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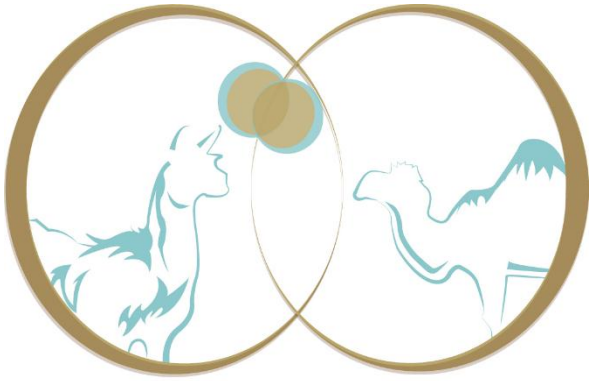

The ICAR 2016 Satellite Meeting on Camelid Reproduction

1st – 3rd July

Tours, France

Eds: J. Juhasz, P. Nagy, J.A. Skidmore, C. Malo

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PROGRAMME AND EXTENDED ABSTRACTS

ICAR 2016 SATELLITE MEETING ON CAMELID REPRODUCTION

Organized by

Emirates Industry for Camel Milk and Products (Camelicious)

Dubai, United Arab Emirates

and

Camel Reproduction Centre

Dubai, United Arab Emirates

at

Universite Francois-Rabelais, Faculty of Science and Technology

Tours, France

ICAR 2016 SATELLITE MEETING ON CAMELID REPRODUCTION

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Preface

Following the success of the 1st and the 2nd ICAR Camelid Satellite Meetings held in Budapest and Vancouver, it was unambiguous for us that this excellent tradition should be continued. As the 18th International Conference on Animal Reproduction (ICAR) is being held in Tours, France this summer, we also embarked on the challenging task to organize the 3rd ICAR Camelid Satellite Meeting. Our aims have not changed over the years, we would like to facilitate communication between scientists and professionals working on Old and New World Camelids, promote the development of Camelid research and help the transfer of knowledge from science to practice. In addition, we hope to provide another platform for young scientists, students engaged on this field to present their data in oral presentation to an appreciative audience.

However, the outcome of our project lies on three important pillars. First, we needed enthusiastic and motivated colleagues who were ready to volunteer the uneasy job of the Organizing Committee. As chairman, I would like to thank all members of the committee for the hard work and support in the entire process. Second, we have been delighted that so many authors responded favorably to the call for papers and invitations. Without your scientific contribution and efforts, the satellite meeting would have no meaning. Thanks to your papers, we were able to put together a solid scientific programme covering not only topics on reproductive physiology, theriogenology, assisted reproduction but also on breeding and genetics, interaction between nutrition and reproduction and on camel milk.

But all these endeavors would have been at risk without strong financial support. Here, I particularly would like to express our gratitude to our GOLD (Advanced Scientific Group and Emirates Industry for Camel Milk and Products), SILVER (Minitube and Lallemand Animal Nutrition) and BRONZE (IMV, Vetoquinol and Prime Medical Technology) level sponsors for their financial contribution that helped putting this idea into reality.

The conference would not be complete without a relevant social program. Similarly to the 2012 meeting, we organize a full day field trip where we can enjoy together the atmosphere, the history and the scenic view of the Loire Valley. Hope this day will be as memorable as the conference itself.

Looking forward to welcoming you to Tours, France.

Dr. Peter Nagy
Chairman of the Organizing Committee

Scientific Programme

FRIDAY 1st July 2016

8.00 – 9.00 Registration

9.00 – 9.15 WELCOME Dr. Peter Nagy

SESSION 1	**Female Reproduction**	
Time	Presenting author	Title
9.15 – 9.35	B.M. Manjunatha	Pattern of follicular development and its manipulation in dromedary camels: present knowledge and future research
9.35 – 9.55	M. Silva	An update on the mechanism of action and luteotrophic effects of ovulation-inducing factor (OIF/NGF) in llama seminal plasma
9.55 - 10.10	M. Silva	Effect of natural mating, intrauterine infusion of raw seminal plasma or seminal plasma purified β-NGF on ovulation and CL development and function in llamas
10.10 – 10.25	J.M. Morrell	Apparent seasonality in the willingness of alpacas to mate in the Peruvian Andes
10.25 – 10.40	H. Chhaibi	Pharmacokinetics of a long-acting progesterone formulation in female camels
10.40 – 10.55	A.J. Campbell	Histological evaluation of the endometrium in pregnant and non-pregnant alpacas
10.55 – 11.15	General discussion	
11.15 - 11.45	**TEA/ COFFEE**	
SESSION 2	**Nutrition and Reproduction**	
11.45 – 12.05	P.W. Bravo	The effect of nutrition on reproduction of domesticated South American camelids
12.05 – 12.25	B. Faye	Selenium supplementation and its effects on reproductive performance in dromedary camels
12.25 – 12.45	J.L. Vaughan	Selenium supplementation in alpacas
12.45 – 13.00	P. Nagy	Effect of live yeast and Se-yeast on milk production and calf health in dromedaries
13.00 -13.15	General discussion	
13.15 – 14.15	**LUNCH**	

SESSION 3	**Embryo transfer and IVF**	
Time	Presenting author	Title
14.15– 14.35	J.L. Vaughan	Selection of alpaca females as recipients in embryo transfer programmes
14.35 – 14. 55	J.A. Skidmore	Synchronization protocols and management of recipients in dromedary camel embryo transfer programmes
14.55 – 15.10	B.M. Manjunatha	Superovulation of dromedary camels with two injections of FSH dissolved in hyaluronan solution
15.10 – 15.45	**TEA/ COFFEE**	
15.45 – 16.05	M. Herrid	Current status and future direction of cryopreservation of hatched blastocysts from the dromedary camel (*Camelus dromedarius*)
16.05 – 16.25	C. Malo	In vitro sperm-oocyte interactions: assessment of dromedary camel sperm quality
16.25 – 16.40	B.P. Mulligan	The early development of camel oocytes, collected from slaughterhouse ovaries, matured *in vitro* and inseminated with frozen thawed semen
16.40 – 17.00	General discussion	
19.00 -	**COCKTAIL PARTY**	**OCEANIA L' UNIVERS HOTEL, TOURS**

SATURDAY 2nd JULY

SESSION 4	**Male Reproduction**	
Time	Presenting author	Title
9.00 – 9.15	S. Al-Bulushi	Characteristics of semen collected from dromedary bulls
9.15 – 9.30	P.W. Bravo	Semen quality and fertility of male alpaca
9.30 – 9.45	V.H. Medina	Reduction of thread formation and its effect on llama sperm cells morphology
9.45 – 10.00	S. Desantis	Effect of bromelain and papain treatments on the glycan pattern of cryopreserved dromedary camel spermatozoa

Time	Presenting author	Title
10.00 – 10.15	C. Stuart	Effect of diluent type, cryoprotectant concentration, storage method, freeze/thaw rates and seminal plasma addition on the post-thaw quality of cryopreserved, papain-treated alpaca spermatozoa
10.15 – 10.30	P. Durand	Effect of seminal plasma added at post-thawing on spermatozoa obtained from Alpaca vas deferens
10.30 – 10.45	General discussion	
10.45 – 11.15	**TEA /COFFEE**	
SESSION 5	**Reproductive Efficiency**	
11.15 – 11.35	A. Tibary	Pregnancy and pregnancy loss in camelids
11.35 – 11.55	J. Juhasz	Management of pregnancy and the neonatal period in dromedaries
11.55 – 12.10	J.B. Sumar	Fertilization failure or early embryonic death in alpacas and llamas?
12.10 – 12.25	A. Tibary	Clinical incidence of various reproductive disorders in male alpacas (*V. pacos*): a retrospective study
12.25 – 12.40	C. Stelletta	Long term retrospective analysis (2004-2015) of reproductive and health management of South American camelids breed in Italy
12.40 – 13.00	General discussion	
13.00 -14.00	**LUNCH**	
SESSION 6	**Breeding and genetics**	
14.00- 14.15	I. Gunsser	Alpaca and Llama breeding in Europe - dreams and reality
14.15 – 14.30	A. Jemmett	Population restoration of the critically endangered Wild Camel in Mongolia
14.30 –14.50	P. Burger	Genetic background of reproductive problems in camelids and other livestock: a mini-review
14.50 – 15.10	E. Ciani	Bridging the gap between the genotype and the phenotype: the role of *omics* technologies

Time	Presenting author	Title
15.10 – 15.25	E. Ciani	First insight on the genetic structure of *Camelus dromedarius* populations through genome-wide SNP markers
15.25 – 15.45	General discussion	
15.45 – 16.15	**TEA/ COFFEE**	
SESSION 7	**Camel Milk**	
16.15 – 16.30	A. Bakheit Sallam	Effect of management system on camel milk production in Western Sudan
16.30 – 16.45	A. Ryskaliyeva	Proteomic analysis of *Camelus* milk from Kazakhstan
16.45 – 17.00	M.E. Babar	High polymorphic sites in 5´flanking region of beta-casein gene in Pakistani dromedary camel
17.00- 17.15	**General discussion and summing up**	

SUNDAY 3rd JULY (8.30 am – after 6 pm)

Field trip to Argenton Alpacas in Le Breuil sous Argenton, www.argentonalpacas.eu
to Saumur, le Chateau de Saumur http://www.chateau-saumur.fr
and if time permits to Chateau and Garden de Villandry, http://loire-chateaux.co.uk/en-gb/chateaux/villandry/chateau-and-gardens-villandry

The organizers reserve the right to change order and content of this programme.

Table of Content

Pattern of follicular development and its manipulation in dromedary camels: present knowledge and future research

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Introduction

The fertility rate is low for camels when bred naturally based on external oestrus signs using conventional breeding practices. This occurs because oestrous signs do not always correlate with ovarian follicular status (Skidmore et al., 1996). Transrectal ultrasonography has been one of the most important advancements and hence widely used technique in the field of animal reproduction for efficient breeding. This technique has been used to monitor follicular growth and to determine the optimum breeding time. Over the last several decades, precise regulation of follicular development for timed breeding (TB) programs has been the focus of extensive research in several species. Several hormonal protocols for synchronisation of ovulation have been developed to breed farm animals at a predetermined time as an alternative to using the detection of oestrus. Recently some hormonal protocols have been developed for the synchronisation of ovulation (Skidmore et al., 2009) and synchronisation of follicular wave for timed breeding (TB) programs in dromedary camels (Nagy and Juhasz, 2012; Manjunatha et al., 2015; Swelum and Alowaimer, 2015). The aim of this review paper is to provide recent advances in the pattern of follicular development and its regulation in dromedary camels.

Follicular development

Ovarian follicular development in dromedary camels occurs in a wave like pattern (Skidmore et al., 1996; Manjunatha et al., 2012a). The growth of small follicles (3-4 mm in diameter) in each wave was characterized by a common-growth phase subsequent to wave emergence, which was followed by a follicle deviation, i.e., a change in the growth rate between the dominant follicle (DF) and the largest subordinate follicle (Manjunatha et al., 2014). Follicle deviation occurs 2.4 days after wave emergence at a DF diameter of 7.4 mm. After follicle deviation, DF continues to grow, whereas subordinate follicles cease to grow and, eventually, decrease in size. The growing DF acquires ovulatory capacity at 9 mm in diameter

and when it reaches 11 mm in diameter, it is capable to ovulate a competent oocyte (Manjunatha et al., 2015b).

Characteristics of follicular wave in dromedary camels are presented in Table 1. The number of follicles recruited in each wave between animals varied whereas within individual animals the number of follicles recruited during different follicular waves was highly repeatable (Manjunatha et al., 2012a). The DF reaches a static phase, few days before the end of mature phase in some of the waves. However, in most cases the growth of the DF towards the end of the mature phase was erratic or inconsistent among the individual animals or waves. The DF after losing its dominance allows the emergence of a new wave, but continues its growth and develops into an oversized follicle in dromedary camels. Oversized follicles develop in 73.3% of the follicular waves and the inter-wave interval (IWI) is repeatable within an individual but varied between the animals. Codominant follicles also observed in 45% of follicular waves in dromedary camel (Manjunatha et al., 2012a).

Table 1: Characteristics of ovarian follicular dynamics (Manjunatha et al., 2012a).

Traits	**Mean** ± SEM	**Range**
Follicular wave		
Number of follicles recruited per wave	12.77 ± 0.93	8-34
Duration of inter-wave interval (days)	16.36 ± 0.37	11-21
Duration of follicular wave (days)	47.11 ± 2.94	21-63
Dominant follicle		
Duration of growth phase (days)	6.10 ± 0.15	4-8
Duration of mature phase (days)	10.20 ± 0.47	4-16
Growth rate between day 0 to 10 (mm/day)	1.17 ± 0.02	
Maximum diameter (mm)	27.30 ± 0.78	19-40
Duration of regression phase (days)	24.71 ± 3.79	12-39
Oversized follicle		
Duration of Growth phase (days)	10.64 ± 1.53	3-25
Maximum diameter (mm)	38.43 ± 1.41	26-48
Duration of regression phase (days)	18.50 ± 2.23	9-32

The characteristics of the DF in dromedary camels appears to be unaffected by the progesterone secreted from the induced CL (Manjunatha et al., 2012b). However, progesterone secretion from induced CL resulting from ovulation of a large follicle (≥18 mm in diameter) or double ovulations affects the growth of DF (unpublished data).

Controlling the follicular wave

Several therapies have been proposed to control the follicular development for timed mating and to facilitate the use of modern reproductive technologies in dromedary camels for the last two decades.

Transvaginal ultrasound-guided aspiration of all follicles ≥ 5 mm in diameter causes the emergence of new follicular wave at 2.3 days after treatment (Skidmore et al., 2009). This technique is very effective in synchronizing follicular development; however, it is not practical to use in the field.

Administration of 100–150 mg progesterone-in-oil (i.m.) daily over a range of 10–14 days (Skidmore et al., 1992; McKinnon et al., 1994) suppress the growth of large follicles, however, progesterone treatment alone did not effectively synchronize wave emergence in camels as follicular waves continued to emerge during the period of treatment (Skidmore, 1994). The use of the progesterone-releasing intravaginal device (PRID) for 7 days has also yielded unsatisfactory results (Cooper et al., 1992; Skidmore et al., 1992). The combination of oestradiol benzoate (5 mg) and progesterone (100 mg) was also not effective in follicular wave synchronisation (Skidmore et al., 2009). In a recent study, controlled internal drug release (CIDR) application for 14 days synchronised the follicular wave for TB at 2 to 4 days after CIDR withdrawal in 68.7 and 70% camels, respectively (Swelum and Alowaimer, 2015).

GnRH is widely used in the synchronisation of ovulation or follicular wave protocols to induce a new wave emergence. Fixed-time mating 14 days after GnRH treatment at random stages of follicular development results ovulation in 73.9% camels. However, GnRH (100 µg, Cystorelin) treatment was successful in inducing synchronous new wave emergence only when a DF of ≥ 11 mm in diameter was present (Manjunatha et al., 2015). The new wave emergence failed to occur in camels to GnRH treatment when small (4 to 7 mm) and immature follicles (8 to 10 mm) were present.

Two GnRH injections 14 days apart or two GnRH injections 14 days apart and PG on Day 7 after the first GnRH were effective to synchronise ovulation rate in dromedary camels at a fixed time interval of 14 days after treatment (Skidmore et al., 2009). In another study, follicular wave synchronisation with two GnRH injections at 14 days intervals and TB 14 days after the second GnRH injection resulted ovulation in 90% camels (Nagy and Juhasz, 2012).

We have developed FWsynch protocol (Fig. 1) for synchronization of follicular wave and have been evaluated for TB program at farm and field conditions (Manjunatha et al., 2015a). The effectiveness of each hormonal injection was assessed in the FWsynch protocol initiated at different stages of follicular development using ultrasonography.

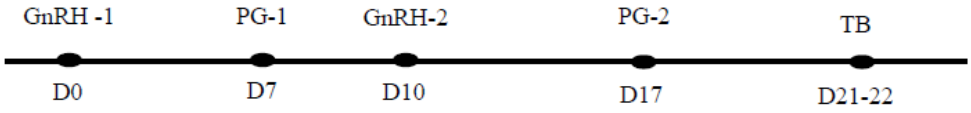

Figure 1: Treatment schedule of hormones in the follicular wave synchronisation (FWsynch) protocol. GnRH (100 µg, i.v., Cystorelin, Ceva Sante Animale, Libourne, France). PG (500 µg of $PGF_{2\alpha}$ analogue, Estrumate, Schering-Plough Animal Health, New South Wales, Australia). TB (timed natural mating).

FWsynch was found to be effective in synchronising the follicular wave for a TB program in a group of animals regardless of the stage of follicular development at the beginning of the protocol. This protocol regulated follicular development and produced an optimal size DF (11 to 17 mm) for TB in 93 to 96 % camels.

Camels diagnosed as non-pregnant after 1st TB program needs to be bred again in a timely manner to maintain reproductive efficiency. We have designed Resynch protocol (Fig.2) to resynchronize the follicular wave for 2nd TB program in camels which failed to become pregnant after 1st TB (unpublished data).

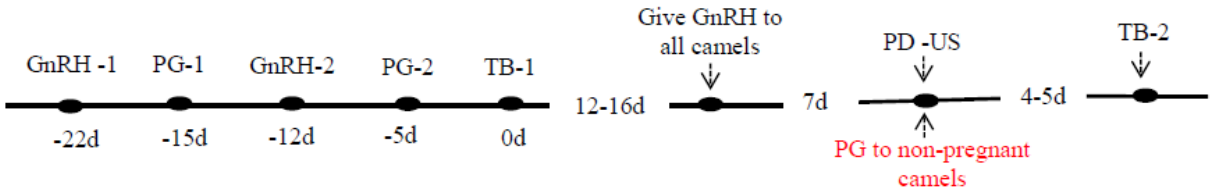

Figure 2: Treatment schedule of hormones for resynchronisation of the follicular wave consists of GnRH injection (100 µg, i.v., Cystorelin) at 12-16 days after breeding, irrespective of pregnancy status, and a $PGF_{2\alpha}$ injection (500 µg, i.m., Estrumate) 7 days later in non-pregnant camels. US (ultrasound scanning), PD (pregnancy diagnosis).

Resynch protocol initiated on Day 12-16 after 1st TB, with unknown pregnancy status, was effective in resynchronising the follicular wave without having any effect on the pre-established pregnancy in dromedary camels.

In conclusion, the knowledge on follicular dynamics and its regulation helps in adopting modern reproductive technologies and improves the fertility in dromedary camels. At present, GnRH based protocols are found to be effective in controlling follicular development to produce an optimal sized DF or synchronise follicular wave emergence at a known time. Further research is needed to assess: 1) endogenous hormonal regulation of follicular dynamics. 2) the relationship between DF size at the time of breeding and the success of pregnancy. 3) the efficiency of GnRH based TB protocols at different conditions and in different breeds of dromedary camels.

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An update on the mechanism of action and luteotrophic effects of ovulation-inducing factor (OIF/NGF) in llama seminal plasma

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Introduction

Llamas ovulate in response to a copulation-induced surge in circulating concentrations of LH. The ovulatory response is a function of the degree of absorption of a seminal factor from the genital mucosa into circulation, and not a response to physical stimulation of the tubular genitalia itself. This seminal factor has been recently isolated and identified as beta nerve growth factor (β-NGF; Ratto et al., 2012).

Mechanism of action

Although it is clear that the ovulatory effect of OIF/NGF is mediated through a surge release of LH into circulation, there is no consensus about whether the tissue target is solely at the level of the hypothalamus or also involves the pituitary gland. Pre-treatment of llamas with a GnRH antagonist (cetrorelix) ablated the effects of OIF (i.e., blocked LH release and ovulation), suggesting a direct or indirect effect of OIF on GnRH neurons in the hypothalamus (Silva et al., 2011). Additionally, the modulatory effect that ovarian steroids exert on the hypothalamic neurons to influence GnRH pulse secretion (Caraty et al., 1998) was evaluated in ovariectomized llamas (Silva et al., 2012a). The LH response to OIF/NGF treatment was muted in ovariectomized llamas and was partially restored by pretreatment with estradiol, consistent with the hypothesis that the pathway of OIF/NGF involves the hypothalamus. Conversely, results of *in vitro* studies document that OIF/NGF also has a direct effect on pituitary gonadotrophs. Treatment of primary cultures of llama and bovine anterior pituitary cells induced LH secretion, in a dose-dependent manner (Bogle et al., 2012), consistent with earlier studies in which the addition of purified OIF or seminal plasma from Bactrian camels

or alpacas to a primary culture of rat pituitary cells induced secretion of LH (Zhao et al., 2001; Paolicchi et al., 1999).

The neural pathways involved in the activation of GnRH neurons in induced ovulators are poorly understood, and no studies have been undertaken in camelids. Recent reports of the pattern of distribution of GnRH neurons in the hypothalamus of llamas revealed that over 60% of GnRH neurons were located in the anterior and medio-basal hypothalamus on the lateral aspects of the third ventricle, but were scattered widely rather than in focal accumulations or nuclei (Carrasco, 2016). The proximity between the cerebral ventricle and GnRH neurons suggest a potential route for OIF/NGF to stimulate the preovulatory secretion of GnRH/LH; i.e., via the cerebrospinal fluid. However, this route implies that circulating OIF/NGF crosses the blood-brain-barrier, a matter of some controversy given the size of the OIF/NGF molecule (i.e., >100 amino-acids and 26kDa; Frieden et al., 1993; Banks, 2009). In an initial attempt to bio-track exogenous OIF/NGF, we were able to detect the biotinylated molecule in the cerebrospinal fluid in the rabbit model (Berland et al., 2013), but could not replicate these results in llamas.

Studies are needed in induced ovulators to determine if this seminal protein, that is absorbed into circulation from the uterine lumen, passes through the blood-brain-barrier and if its receptors are co-localized with GnRH neurons in the hypothalamus. It is interesting to note that among studies, the LH surge elicited by exogenous OIF/NGF begins and peaks 1 to 2 hours after that elicited by exogenous GnRH. The relatively delayed response with OIF/NGF may reflect an intermediate step (i.e., a different cell type) in the pathway required to elicit GnRH/LH release. It is also notable that magnitude of the LH surge was similarly dose-dependent subsequent to treatment with both peptides, OIF/NGF (Tanco et al., 2011) and GnRH (Silva et al., 2012b).

Other possible pathways include: a) a direct action on GnRH neuron terminals outside the blood-brain-barrier, b) an indirect action through tanycytes, or c) a direct action on the pituitary gonadotrophs themselves. The first hypothesis, involves the organum vasculosum of the lamina terminalis (OVLT) whose complex arrangement of GnRH neuron dendrites in the rostral preoptic area are putatively outside of the blood-brain barrier, and therefore able to directly sense molecules travelling in systemic blood (Herde et al., 2011; Rodriguez et al., 2010). Regarding the second hypothesis, some suggest that β1 tanycytes participate in the pulsatile release of GnRH into the portal blood (Rodriguez et al., 2005). Most GnRH nerve fibers and

their endings are concentrated in the lateral regions of the median eminence and are separated from the perivascular space by a continuous cuff formed by tanycytes, a unique cell type lining the floor of the third ventricle (Rodriguez et al., 1979). Perhaps OIF/NGF within the median eminence induces the secretion of molecules that control the transient and cyclic release at the GnRH terminals (Prevot, 2002). The third hypothesis is supported by the presence of NGF in 75% of LH-containing gonadotroph cells, 44% of which expressed the high affinity NGF receptor trkA in rat anterior pituitary cells (Patterson & Childs 1994), suggesting a functional link.

Luteotrophic effect

Just as surprising as the ovulation-inducing effect of seminal plasma was the apparent positive effect it had on the ensuing CL (Adams et al., 2005). Female llamas treated intramuscularly with a conservative dose of homologous seminal plasma developed a CL that tended to be larger, regressed later, and produced more than twice as much progesterone than CL resulting from GnRH-induced ovulation. Apparently, the luteotrophic effect was the result of a more prolonged LH secretion pattern (Adams et al., 2005). The sustained LH-release and luteotrophic effects of seminal plasma have been confirmed in several subsequent studies using OIF/NGF purified from the seminal plasma of llamas (Fernandez et al., 2014; Ratto et al., 2011; Silva et al., 2011b; 2014; Tanco et al., 2011; Ulloa et al., 2014). A similar relationship between LH secretion and luteogenesis has been described in primates and laboratory species (Ishikawa, 1992; Chandrasekher et al., 1994).

In a recent study designed to determine if the luteotrophic mechanism is related to vascular perfusion of the developing CL in llamas, blood flow was assessed by power-flow Doppler ultrasonography every 4 hours after treatment with OIF/NGF or GnRH. Compared to GnRH treatment, llamas treated with OIF/NGF had greater vascular flow to the preovulatory follicle 4 hours after treatment (Ulloa-Leal et al., 2014), and greater vascular flow to the CL and greater plasma progesterone concentration 6 days after treatment (Ulloa-Leal et al., 2014; Fernández et al., 2014). In addition to a dose-dependent effect (Tanco et al., 2011), the luteotrophic effect of OIF/NGF was influenced by the timing of treatment. Two doses of OIF/NGF, given before and at the time of ovulation, induced the development of larger CL with greater vascularization and that produced more progesterone than CL induced by a single pre-ovulatory dose (Fernandez et al., 2014). Additionally, OIF/NGF exerted its luteotrophic effect independently of the preovulatory follicle diameter (Silva et al., 2014).

However, the role of OIF/NGF directly at the level of the ovary remains an open question. NGF stimulated progesterone secretion from mid-luteal stage bovine CL *in vitro* cell culture (Miyamoto et al., 1992). In this regard, both camelid and bovine seminal plasma were found to be luteotrophic in cattle (a spontaneous ovulator) despite that no measurable increase in plasma LH concentrations was detected (Tanco et al., 2012; Tribulo et al., 2015). The most recent data in cattle suggest that the luteotrophic effect of OIF/NGF is mediated, in whole or in part, directly at the level of the ovary through interaction with trkA receptors on granulosa and theca cells of the dominant follicle and developing CL (Carrasco et al., 2016).

Conclusions

The effects of seminal plasma (OIF/NGF) on ovarian function strongly support the idea of an endocrine mode of action (i.e., systemic distribution with distant target tissues). The ovulatory response to seminal OIF/NGF in llamas is brought about by a surge in circulating concentrations of LH, and is a function of the degree of absorption of this seminal factor from the genital mucosa into circulation (i.e., systemic dose), and not a response to physical stimulation of the female reproductive tract during copulation. The mechanism and sites of action of OIF/NGF in the hypothalamo-pituitary-gonadal axis are not fully understood, but may well involve tissue receptors at all three levels. The luteotrophic effect of OIF/NGF exemplifies a multi-layer mechanism since the effect has been associated both with the magnitude of LH release (central mechanism) as well as temporal changes in the expression of specific receptors in ovarian follicles (local mechanism).

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Effect of natural mating, intrauterine infusion of raw seminal plasma or seminal plasma purified β-NGF on ovulation and luteal function in llamas

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Introduction

In llamas and alpacas ovulation is induced by a protein present in the semen rather than by the mechanical stimulation of penis during copulation (Adams et al., 2005; Ratto et al., 2005; Silva et al., 2011; Kershaw-Young et al., 2012). The molecule identified as β-NGF (Ratto et al., 2012; Kershaw-Young et al., 2012) has potent luteotrophic properties when given intramuscularly to female llamas (Silva et al., 2011, Tanco et al., 2011; Ulloa-Leal et al., 2014), through the induction of a more sustained and prolonged increase in LH plasma concentration, resulting in a greater progesterone secretion from the CL, when compared to GnRH-treated females. It is suggested that this effect on LH secretion generates a greater vascularization of both pre-ovulatory follicle and early Corpus Luteum (Ulloa-Leal et al., 2014; Fernández et al., 2014). Route of administration (i.e. intramuscular, intravenous and intrauterine) of seminal plasma (Adams et al., 2005; Ratto et al., 2005) or β-NGF (Silva et al., 2015) significantly affect pituitary LH release and the ovulatory response in female llamas. However, it is unknown if the intrauterine administration of a physiological dose (i.e. average amount found in an ejaculate) of β-NGF exerts a luteotrophic action. The aim of this study was to compare the effects of the natural mating, intrauterine infusion of llama seminal plasma or purified β-NGF on ovulation rate and CL development and function in llamas.

Material and Methods

Adult non-pregnant, non-lactating llamas (*n*=20) were examined once daily by B mode transrectal ultrasonography (Aloka SSD-500 scanner with a 7.5 MHz linear-array probe). Females with a growing follicle ≥8 mm in diameter were randomly assigned to one of the following groups: a) Single mating with an intact adult male (mating; *n*=6); b) Intrauterine

infusion of 4 ml of raw llama seminal plasma (PS; *n*= 4); c) Intrauterine infusion of 10 mg of β-NGF contained in 4 ml of Phosphate Buffer Saline (β-NGF; *n*=6) or d) Intrauterine infusion of 4 ml of PBS plus an intramuscular administration of 50µg of gonadoreline (GnRH; positive control; *n*=4). Selected doses of seminal plasma and β-NGF for intrauterine infusion resembled physiological conditions after a single natural mating. After treatment ovaries were examined by ultrasonography every 12 hours until Day 2 (Day of treatment = Day 0) to determine ovulation. Afterwards, ultrasonographic examinations were performed on alternate days until Day 8 to evaluate CL diameter and vascularization area (VA) by B-mode and Color-Power Doppler ultrasonography (MyLab 5, Esoate) respectively as described (Ulloa-Leal et al., 2014; Fernandez et al., 2014). VA was evaluated on the maximum cross-sectional area of the CL and was assessed by off-line measurements of the number of colored pixels as an indicator of blood flow area (the average of 3 still images was used). Images were edited and analyzed using the ImageJ software (NIH open access, USA). Additionally, blood samples were taken on Day 0, 2, 4, 6 and 8 (Day of treatment = Day 0), by jugular venipuncture, for the measurement of plasma progesterone concentration, using a commercial, double-antibody radioimmunoassay kit (Coat-a-Count total progesterone, DPC). Single point data were compared among groups using one-way analysis of variance (ANOVA). Proportional data (ovulation rate) was analyzed by Fisher's test. Serial data (CL diameter, vascularization and progesterone concentration) were analyzed by one way analysis of variance for repeated measure by the MIXED procedure using SAS. All values are expressed as mean ± SEM, and significance was declared at $P \leq 0.05$.

Results

The pre-ovulatory follicle diameter at the time of treatment did not differ among groups (P=0.8; Table 1). Similarly, ovulation rate, interval from treatment to ovulation and CL diameter at Day 8 did not differ among groups (P≥ 0.7; Table 1). There was not effect of route of administration (P= 0.4) nor interaction (P= 0.4) on CL diameter. Although, plasma progesterone concentration was numerically higher in both natural mating and β-NGF treated groups there was not effect of treatment (P= 0.9) nor interaction (P= 0.7) on this parameter among groups. Only a day effect (P<0.001) was found on CL diameter and vascularization area and plasma progesterone concentration which significantly (P<0.05) increase by Day 6. Natural mating tended (P=0.08) to increase CL vascularization area when compared to seminal plasma administration.

Table 1: Mean (± SEM) follicle diameter at treatment, ovulatory response and CL diameter on Day 8 in llamas given different ovulatory treatments (Day 0 = Day of treatment).

	Groups			
	Mating (n=6)	**S. Plasma** (n=4)	**β-NGF** (n=6)	**GnRH** (n=4)
Follicle diameter (mm)	12.0 ± 1.0	10.2 ± 0.8	10.7 ± 0.7	9.9 ± 1.5
Ovulation rate (%)	83 (5/6)	100 (4/4)	83 (5/6)	100 (4/4)
Interval to ovulation (h)	29.5 ± 0.8	30.2 ± 0.9	29.7 ± 0.7	30.1 ± 1.1
CL diameter Day8 (mm)	13.2 ± 0.8	14.5 ± 0.4	13.6 ± 0.7	12.8 ± 1.0

Discussion

Present results support the notion that, contrary to the luteoptrophic effect observed after i.m. treatment of β-NGF, intrauterine administration of seminal plasma or β-NGF, at volume and concentration to mimic that present in an ejaculate, does not affect CL size and function when compared to a standard intramuscular administration of GnRH. Our results do not support previous studies on which the administration of purified β-NGF intramuscularly increased CL vascularization area and progesterone secretion (Ulloa-Leal et al., 2014, Silva et al., 2014). An early study (Ratto et al., 2006) described similar luteal development and progesterone secretion in llamas induced to ovulate by natural mating or an ovulatory dose of Cystorelin. Interestingly, llamas subjected to a second copula, within 24 h, did not displayed an increased in plasma progesterone concentration (Bravo et al., 1992), reinforcing the notion that when administered by intrauterine route, independent of dose, β-NGF does not improve CL function compared to GnRH treated females. It is concluded that the intrauterine administration of seminal plasma or β-NGF, to mimic the volume and the molecule concentration present in an ejaculate, does not affect CL size and function when compared to a standard i.m. administration of GnRH.

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Apparent seasonality in the willingness of alpacas to mate in the Peruvian Andes

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Abstract

Background: South American camelids are induced ovulators, and are considered to breed all year round, as determined by receptivity to the male. An embryo transfer experiment was conducted in the Peruvian Andes (4000 m above sea level) in April and September 2014. The alpacas were not superovulated. Many alpacas refused to mate in September whereas they had permitted mating by the same males in April. Llama females kept under the same conditions, accepted mating in September. A retrospective analysis of their mating efficiency was performed using Wilson score confidence interval for binomial parameters with continuity correction (wolframalfa.com). Assumed confidence interval was set at 95%. **Results:** In April 2014, 44 out of 48 mating attempts in alpacas were successful; twentytwo embryos were obtained. In September, only eight out of 20 of the same females permitted mating. Seven of the mated females were flushed, two embryos were recovered. For llamas 13 matings occurred out of 20 attempts in September; seven embryos were obtained. The difference in sexual receptivity in alpacas between April and September is significant ($P < 0.05$); the difference between alpacas and llamas in September is also significant ($P<0.01$). The number of matings resulting in embryos was similar between alpacas in April and llamas in September. Nutrition may have affected the willingness if the animals to mate. **To conlcude,** alpacas kept in the High Andes of Peru appear to show seasonal differences in their willingness to mate: such differences are not seen in other countries where alpacas are kept under less harsh environmental conditions. Such possible seasonality should be considered when planning breeding experiments.

Introduction

Alpacas *(Vicugna pacos)* are native to South American but have been exported to many other countries. All South American camelids are induced ovulators, requiring a stimulus from copulation to trigger a surge in luteinizing hormone to initiate ovulation. Females show waves of follicular growth which sometimes overlap (Vaughan et al., 2003); they will mate if there is a suitably-sized follicle (usually >7 mm). After ovulation, or in the absence of a suitable follicle, they refuse to mate by remaining standing and spitting at the male ("spit-off"). They are considered to breed all year round.

Embryo transfer has been caried out successfully in this species, usually in well-eqipped research facilities or on commerical premises. An embryo transfer study was carried out at a field station in the Peruvian Andes in 2014, to see if embryo transfer could be made under field conditions. The study was in two parts, in April and September with non-superovulated females. Although most of the female alpacas permitted mating in April, they refused to mate in September, despite using the same males. Llama females kept under the same conditions, permitted mating in September. Therefore a retrospective analysis of the mating efficiency was performed.

Materials and Methods

Animals and facilities

Alpacas (10 female, 10 male) and llamas (20 female and 10 male) came from a mixed herd of 320 animals in Abra la Raya, University of San Marcos, Peru (4300 m above sea level). Anthelmintics were administered twice a year. Only animals considered to be in good condition were used in this study. Males and females were kept in separate paddocks except for mating attempts, when they were transferred to individual mating pens. A field laboratory was situated in close proximity to the pasture. The facilities do not have running water or a fixed electrical supply, the electricity needed for heating, light and the microscope was provided by a portable generator run with gasoline whereas water for basic hygiene was obtained from a nearby stream.

Embryo flushing

The donors were restrained on a table with a rope around the abdomen and hind legs, and a towel covering the face. An epidural injection of 1.5 ml lidocaine (Lidocaina, hydrochloric lidocaine 2%, Laboratorios Unidos, S.A) was administered. The tail was wrapped in a cloth and tied dorsally to the rope encircling the abdomen. The perineal area was washed with soap

and water after the rectum was emptied. A Foley catheter (size 14) was introduced into the uterus under transrectal guidance. The uterus was flushed two or three times with warm flushing medium (100 mL each time), consisting of physiological saline solution with serum from the same herd (1 mL per 1000 mL), and antibiotics (Penduostrep; Penicillin procaine 200 000IU+ dihydrostreptocillin sulfate 250000 IU, Agrovet Market). After the procedure, prostaglandins (Lutaprost, Sodium Cloprostenol 0.263 mg/ml Agrovet market) were administered to induce luteolysis. The lavage fluid was passed through an Agrotech filter; the last 5-10 mL being collected and transferred to a petri dish. The filter was rinsed with 5 mL of medium and both the rinsing fluid and the filtrate were examined for the presence of an embryo. The procedure was performed rapidly with the petri dish on a heated surface to protect embryos from the cold.

Statistics

The data were compared using wolframalfa.com's Wilson score confidence interval for binomial parameters with continuity correction. Assumed confidence interval was set at 95%.

Results

The outcome of the mating attempts and the number of embryos obtained are shown in Table 1.

Table 1: outcome of mating attempts in alpacas and number of embryos flushed in April and September, and also in llamas in September.

Species	Alpacas, April	Alpacas, September	Llamas, September
Successful mating attempts	44/48[a]	8/20[ab]	13/20[b]
Number of embryos flushed	22	2	7

Note: same superscript denotes statistical significance: a = $P<0.05$, b= $P<0.01$.

The number of matings resulting in embryos was similar between alpacas in April and llamas in September. There is no information available concerning the mating performance of the llamas in April since this was not a part of the original study.

Discussion

The retrospective analysis of mating attempts and number of embryos indicated a difference in alpacas between April and September, and between alpacas and llamas in September. Even though similar in body condition score to the alpacas, the llamas showed no unwillingness to mate: of the 20 llamas introduced to a male, 13 mated and 7 produced an embryo.

Seasonal dependency or reproduction has been reported previously by some authors e.g. in New Zealand (Pollard, et al., 1994) and Australia (Vaughan & Tibary 2006). In contrast, an extensive study involving laparoscopic inspection of alpaca ovaries indicated that they were likely to have one or more follicles above 7 mm all year round, independently of rainfall, photoperiod or temperature (Bravo & Sumar, 1989). Another study found no difference in sexual receptivity, ovulation or fertilization rate year round (Fernandez-Baca, et al., 1972).

The herd at La Raya is held in the treeless zone in the Altiplano. The rainfall is 250-500 mm annually, falling mainly between November and April, and affects the nutritional value of the pasture. Growth of the newborn is associated with the nutritional status of the dam (Burton et al., 2003). Thus it would be natural for alpacas to adapt their reproduction to variability in pasture availability, so that the birth of young coincides with the availability of grazing. The dry season, when the pasture is meagre (consisting mainly of the harsh Peruvian feather grass called "Ichu"), ends in September. No supplementary feed was supplied. It is possible that the willingness of the animals to mate was affected by nutritional state since the succulent forage preferred by alpacas was not available after the dry conditions. This speculation requires further research.

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Pharmacokinetics of a long-acting progesterone formulation in female camels

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Introduction

Camelids are induced ovulators. Progesterone does not display a cyclic pattern unless the female is mated and ovulation occurs. In absence of fertilization, diestrus (life span of the corpus luteum) is very short and luteolysis occurs approximately 10 days following mating. Ovarian progesterone is required throughout pregnancy in these species. Therefore, any attempt to manipulate the follicular wave or help maintain pregnancy requires frequent administration on progesterone. Intramuscular (IM) progesterone administration has been used extensively in the dromedary camel for synchronization of follicular development in embryo transfer programs (McKinnon et al., 1994). Most protocols used to achieve follicular wave control require IM administration of 100 to 150 mg of progesterone daily for 14 days. Daily administration of progesterone has also been used for preparation of non-ovulating, ovariectomized or asynchronous recipients in embryo transfer (Skidmore et al., 2011, Tibary and Anouassi 2001).

Administration of progesterone at a dose of 1 mg/kg intravenously (IV) resulted in a sharp rise in serum progesterone with a half-life of 26±2 minutes (Al-Busadah and Homeida, 2004). There are no studies on the pharmacokinetics of IM administration of commercial preparation of progesterone in oil. Daily administration is often the suggested method in order to maintain luteal levels of progesterone. Daily IM injections in a large group of animals present several difficulties associated with frequent animal handling and compliance with timing and dose of each injection. In addition, frequent injections may render some animals less tractable. Other techniques for extended delivery of progesterone such as CIDRs are generally not tolerated by camels, may be lost, and are associated with the development of vaginitis. Compounded long-acting (Biorelease) progesterone formulations are available for horses and have been shown to be effective in mares for maintenance of pregnancy (Burns et al 2008, Vanderwall et al 2007). The present experiment was designed to evaluate progesterone pharmacodynamics following

a single standard dose administration of compounded proprietary long-acting progesterone that was formulated for mares.

Materials and Methods

Fourteen (n=14) nulliparous, non-pregnant female camels aged 3-4 years and weighing 250-400 kg were used in the study. Females were selected on the basis of normal health and cyclicity. All females were examined by transrectal ultrasonography and females with a visible corpus luteum (CL) on one of the ovaries were given an IM injection of cloprostenol (375 µg) and were included in the study if they did not have high progesterone on the day of progesterone administration. Each female was given an IM injection of 5 mL of a proprietary progesterone formulation (BioRelease P4 LA300, 300 mg of progesterone per mL BETPharm, Lexington KY). Blood samples were collected daily starting one day prior to treatment and continuing for 14 days after injection. Serum was harvested and stored at -20°C until assayed for progesterone using radioimmunoassay. Changes in daily serum progesterone concentrations following treatment were examined by repeated measurement ANOVA using a statistical software (Statistix v. 10, Analytical software, FL, USA).

Results

Two females were removed from the pharmacokinetic analysis. One because she had elevated progesterone levels on the day of treatment. The other female was an outlier with respect to progesterone profile. Serum progesterone concentration (mean ± SEM) for the remaining 12 females is presented in Figure 1. All females showed a significant increase in serum progesterone concentration within 24 hours of treatment (36.76 ± 3.8 ng/mL, $P<0.01$). Serum progesterone concentrations remained above 2 ng/mL in all animals for 10 days. By 12 days after injection only 50% of the females had serum progesterone concentrations below 2 ng/ml. By 14 days after treatment, 5 females (36%) had serum progesterone concentrations between 1 and 2 ng/mL while all of the other females had concentrations less than 1 ng/ml.

Discussion

Administration of 5 ml of a long-acting formulation of progesterone in camels produced a significant increase in serum concentration of progesterone. Serum progesterone was consistently above 2 ng/mL in all animals for 10 days. These results are similar to those obtained in mares following a similar treatment (Burns et al 2008). However, absorption and bioavailability of progesterone following treatment appears to be faster in camels as the peak

progesterone concentration was reached within 24 hours after injection while in mares peak progesterone is observed at 48 hours.

In the present experiment, one female (discarded from the analysis) did not show the same kinetics of serum progesterone following injection. This may have been due to an error in injection as she was the only camel with noticeable injection site reaction (swelling and pain).

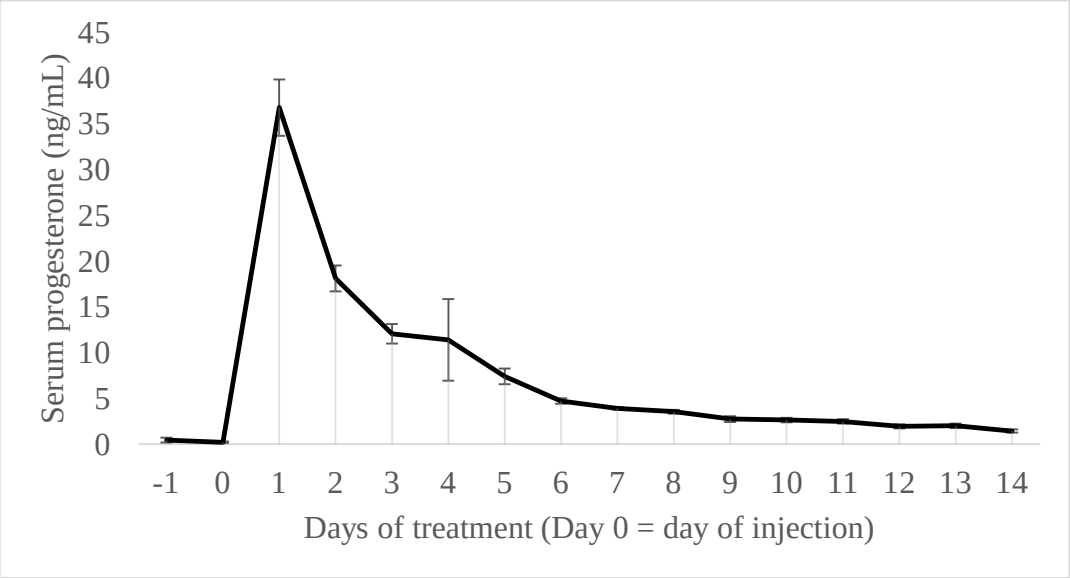

Figure 1: Mean (± SEM) serum progesterone concentration in female camels (n=12) following intramuscular injection of 5 mL of BioRelease P 4LA 300 containing 300 mg or progesterone /ml on day 0.

Conclusion

This study demonstrated that administration of 5 ml of BioRelease P4 LA300 to female camels provides elevated serum progesterone concentrations comparable to those expected during the luteal phase with normal luteal function for at least 10 days. This treatment may be useful to eliminate the need for repeated daily administration of progesterone. Studies are underway to determine the effect of this compounded long-acting progesterone on ovarian function. Sources of variation in individual response need further examination.

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Histological evaluation of the endometrium in pregnant and non-pregnant alpacas

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Introduction

Development and maintenance of pregnancy requires a uterine environment that is healthy and adaptive to the needs of the growing conceptus. The conceptus must provide a timely biochemical signal, as part of the biological process of maternal recognition of pregnancy (MRP), to prevent luteolysis of the corpus luteum (CL) (Tibary et al., 2015). Following MRP, endometrial histoarchitecture changes occur to facilitate endocrine and secretory functions necessary for the establishment and maintenance of pregnancy.

Camelids are induced ovulators, with ovulation occurring equally from both the right and left ovary. Almost all pregnancies are carried in the left uterine horn, regardless of the site of ovulation, suggesting that embryo migration to the left uterine horn is important to prevent luteolysis. This is attributed to a difference in $PGF_{2\alpha}$ release between the two uterine horns with release from the right uterine horn being local and release from the left uterine horn being systemic. While the exact mechanism of embryo migration is not known, gross examination of the uterus from fetal, pre-pubertal, and non-pregnant female camelids has demonstrated that the left uterine horn is consistently larger when compared to the right uterine horn (Tibary et al., 2015).

Early endometrial histology during pregnancy has been studied in several species. However, this information in alpacas is lacking. The objective of the present study was to compare uterine histology in the left versus right uterine horn in non-pregnant and early pregnant alpacas.

Materials and methods

Reproductively sound adult female alpacas (n=20) that were scheduled for uterine and ovarian sampling as part of a larger experiment on MRP were included in the study. Group I

and II females were scheduled for postmortem collection of the reproductive tract and embryos at either Day 9 (n=5) or Day 14 (n=5) post-mating respectively. Group III and IV females were scheduled for postmortem collection of the reproductive tract at either Day 9 (n=5) or Day 14 (n=5) following induction of ovulation with 50 µg GnRH (Cystorelin®, Merial, Duluth, GA, USA) respectively. All females were euthanized using an overdose of barbiturates on Day 9 or Day 14. The uteri were collected *en bloc* immediately after euthanasia. Uterine tissue was cut in 5µm sections and stained with hematoxylin eosin. Histological sections were assessed for differences in glandular density and tortuosity, vascularity, and infiltration of inflammatory cells between the left and right uterine horns in pregnant and non-pregnant animals. The endometrium and myometrium were measured for the left and right uterine horn of each female. All parameters were evaluated using ANOVA with pregnancy status and uterine horn side as the main factors.

Results

Results of the histological evaluation are reported in Tables 1 and 2. Histological evaluation did not show any difference in glandular density and tortuosity, vascularity, or infiltration of inflammatory cells between the left and right uterine horn for females in each experimental group. There were differences in glandular tortuosity and infiltration of inflammatory cells between 14-day pregnant sections (group II) and 14-day open sections (group IV). The endometrial glands were more tortuous for 14-day pregnant sections (group II) when compared to 14-day open sections (group IV). There was a marked infiltration of inflammatory cells for 14-day open sections (group IV) compared to 14-day pregnant sections (group II).

The epithelium was similar in the 9-day open (group III) and 9-day pregnant sections (group II), but was noticeably proliferated in the 14-day pregnant sections (group IV). The luminal epithelium in the 14-day pregnant sections (group IV) exhibited development of microvilli. Evaluation of the 14-day open sections (group IV) revealed a reduction in size of the glandular epithelial cells and sloughing of some areas of luminal epithelial cells. While there did not appear to be a difference in glandular numbers or glandular content between any of the groups, increased tortuosity with greater numbers of longitudinal sections and increased luminal diameter was visualized in the 14-day pregnant sections (group IV). Vascularity within the endometrium and myometrium appeared similar within and between groups. Mean thickness of the endometrium and myometrium in the uterine horns is presented in Table 1 and the ratio of endometrium to myometrium was calculated for each section and is presented in Table 2.

Table 1: Mean (Mean±SE) of the endometrial and myometrial diameter of the left (LH) and right (RH) uterine horn in 10 pregnant and 10 non-pregnant alpacas.

Diameter (mm)	Pregnant Day 9	Open Day 9	Pregnant Day 14	Open Day 14
RH Endometrium	2.91±0.20[a]	2.91±0.25[a]	3.06±0.17[a]	2.17±0.19[b]
LH Endometrium	3.46±0.38[a]	3.27±0.37[a]	3.13±0.44[a]	2.53±0.13[b]
RH Myometrium	3.36±0.41[a]	2.58±0.37[a]	3.76±0.44[a]	2.75±0.19[b]
LH Myometrium	3.44±0.34[a]	2.78±0.17[a]	4.11±0.46[a]	2.58±0.40[b]

[a,b] Significance was set at $P < 0.05$

Table 2: Mean (Mean±SE) of the endometrial and myometrial diameter of the left (LH) and right (RH) uterine horn in 10 pregnant and 10 non-pregnant alpacas.

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[a,b] Significance was set at $P < 0.05$

Day 14 open females (group IV) had significantly ($P<0.05$) thinner endometrium and myometrium than the other groups (group I, II, and III).

There were numerous lymphocytes and neutrophilic granulocytes among the luminal and glandular epithelial cells within the 14-day open sections (group IV). Lymphocytes, neutrophilic granulocytes, and macrophages were observed in the lamina propria. This was consistent among all females within this group. Fewer lymphocytes and neutrophilic granulocytes were seen scattered among the luminal epithelial cells and lamina propria for females in group I, II, and III.

Discussion

Although the vast majority of camelid pregnancies attach within the left uterine horn, histologic structure of the uterus in bilateral horns was similar. These results agree with previously reported observations in camels (Chen et al., 2003). There are no glandular differences associated with embryo migration to the left uterine horn in alpacas. Glandular

tortuosity and increased luminal diameter was observed between Day 9 and Day 14 of pregnancy, and is likely due to luteal function and increased histotroph production.
The endometrium and myometrium were significantly thinner at 14 days post-induction of ovulation in non-pregnant females. This can be attributed to luteolysis and resumption of a new follicular dominance phase.

Findings of increased inflammation at 14-days open were consistent with previous findings in the camel (Chen et al., 2003), and suggest that the mucosal immunity of the female reproductive tract in alpacas is very active. It has been shown that ovulating non-pregnant female camelids return to receptivity and develop a dominant follicle 14 days after mating. Semen is deposited deep into the uterine horns throughout the mating period, which lasts up to 30 minutes, and mechanical irritation to the endometrium is an important component for induction of ovulation. The large influx of inflammatory cells observed in the sections from the 14-day control females (group IV) could be initiated in preparation for the process of mating.

Although no histological differences were observed between the left and right uterine horns in the present study, it is possible that significant changes may be occurring at the molecular level. Genomic and proteomic studies are necessary for further understanding the mechanism of MRP in this species. Pregnancy loss is most common during early pregnancy. This is especially true in females with age-related or pathologic changes to the endometrium. Although a grading system for histological evaluation has been proposed in camelids (Powers et al., 1990), it is not widely used in a clinical setting. Further evaluation and understanding of normal histological changes associated with the development of early pregnancy will improve our understanding of pathologic conditions of the reproductive tract that may lead to infertility or early embryonic loss.

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The effect of nutrition on reproduction of domesticated South American camelids

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Introduction

Domesticated South American camelids have been raised for thousands of years on the highlands of Peru, Bolivia, Argentina, Chile, and Ecuador. They have been subjected to detailed screening on many aspects of their reproductive physiology and success of their breeding has been instrumental for their export to other countries and latitudes. Lately, a drive on the application of reproductive technologies is underway. Techniques of artificial insemination, embryo transfer, in-vitro fertilization have been used with relative but promising results. However, most of llamas and alpacas live under a seasonal rainfall and management of reproduction has been according to pasture availability, and season of the year. Animals are subjected to a spring and summer rainfall for approximately four months of the year, and eight months of dry fall and winter. Parturition occurs during the spring and summer to guarantee survival of the crias (offspring) after 11.5 months of pregnancy. This scenario of seasonal pastures is reflected on the body condition and weight of animals, consequently, nutritional status of animals are also seasonal. This paper is a summary of the author research and his associates on the area of nutrition related to reproduction. Quality of semen, embryonic mortality, and the lactation period are covered.

Quality of semen and early pregnancy

Recently, semen quality was assessed during breeding. Males receiving nutritional supplement increased significantly on motility and sperm concentration. Motility in control males and without supplement was 24% in contrast to 50% in males receiving intravenous amino acids, vitamin E and zinc supplement. Sperm concentration increase was dramatic, in control males was 60 million spermatozoa/mL, and 192 million in males receiving nutritional supplement. There was no difference in normal (80%) and live spermatozoa (70%) between the three groups. Altogether, this information is promising. Males may have a boost on semen production and quality which will affect positively their fertility.

In this regard, the same males bred different groups of females, and the pregnancy rate at 30 days following a single breeding is shown in Table 1.

Table 1: Fertility of males and females receiving nutritional supplement.

Animal group	Pregnancy rate, %
Males preñatec®	81.4*
Males control	75.6
Males catosal®	50.0
Females preñatec	88.0*
Females control	83.6
Females catosal	72.6
Interactions	
Male preñatec x female preñatec	90.0*
Male preñatec x female catosal	73.9
Male preñatec x female control	82.9
Male catosal x female preñatec	59.1
Male catosal x female catosal	60.0
Male catosal x female control	83.8
Male control x female preñatec	84.9
Male control x female catosal	62.7
Male control x female control	83.9

®Preñatec, TQC química, Catosal, Bayer; *Indicates significant difference, P<0.05 within groups of animals

Embryonic mortality

The first report on embryo mortality in alpacas indicates that 50% of embryos are lost during the first month of pregnancy; however, a recent report following pregnancy by ultrasonography from 12 through 45 days of pregnancy, indicates that embryo lost is less than previously reported and is affected by reproductive experience of the female. Indeed, maiden, lactating, and barren females had 28.9, 29.6, and 47.7% of embryo reduction.

A recent work comparing females maintained on native pastures, like most animals are raised in South America, and improved pastures (rye grass and white clover) indicate that embryo loss was 11% higher in females maintained in native pastures, than 4% in females maintained in improved pastures, P<0.05; see Figure 1. This less embryo loss in South America is similar with 5% reduction of pregnancy on the same period in alpacas maintained in North America,

wherein, animals are maintained on improved pastures and on a better nutrition. Interestingly, concentrations of blood urea nitrogen is 40 mg/dL on alpacas maintained on improved pastures, and 17 mg/dL on alpacas maintained on native pastures. This represents a difference of more than half of values between the two types of pastures. High blood urea nitrogen also means that urea is being recycled more in alpacas maintained in improved pastures. Concentrations of beta hydroxy butyrate on the same groups of animals were similar and around 3 mg/dL. These values may represent that alpacas are converting efficiently butyric acid into beta hydroxy butyrate and may have extra source of energy.

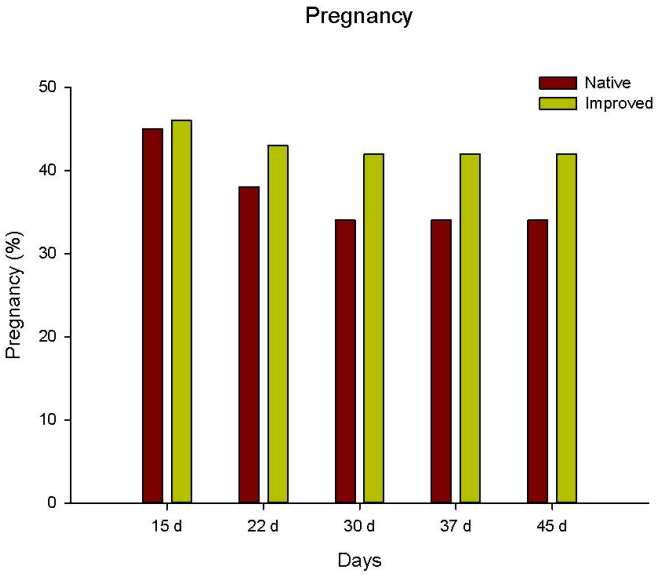

Figure 1: Early pregnancy of alpacas maintained on improved and native pastures from days 12 through 45 of pregnancy.

Lactation period

Female camelids may have a cria to her side and lactating for a period of 6 to 8 months and coincides with lush pastures at the beginning from February to March, and then dry pastures from April through September. During this period females are lactating, may be pregnant and also they have to produce fiber. Work on alpaca reveals that total protein al time of parturition is 6 g/dL and then declines slightly to 5 g/dL at time of weaning of the cria. Blood urea nitrogen is about 25 mg/dL at parturition and declines to 10 mg/dL at time of weaning of the cria, Figure 2.

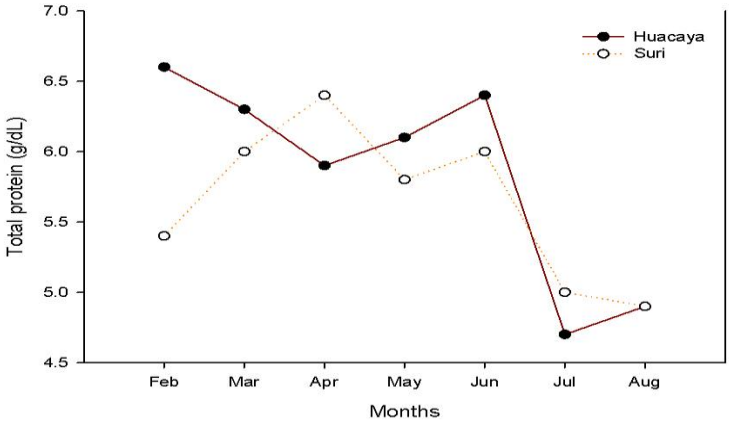

Figure 2: Concentrations of total protein in lactating female Huacaya and Suri alpacas since birth through weaning time.

Glucose is around 100 mg/dL at parturition, declines slightly for the next two months to 80 mg/dL, and then increases back to 100 mg/dL at weaning time. Altogether all three metabolites decline by the presence of the cria, and pregnancy on most of females. A detailed work about the effect of dry pastures during the last three months of pregnancy is underway; however, body weight of the dam shows the effect of pasture availability. Llama and alpaca weight show the same trend during the year, females loss weight twice a year. The first loss occurs by April, and the second loss in October. Llamas Kcara (non wooly) are heavier through the year than llamas Chaku (wooly). Differences in alpaca weight between alpacas Huacaya and Suri thorough the year is comparatively similar; see Figure 3.

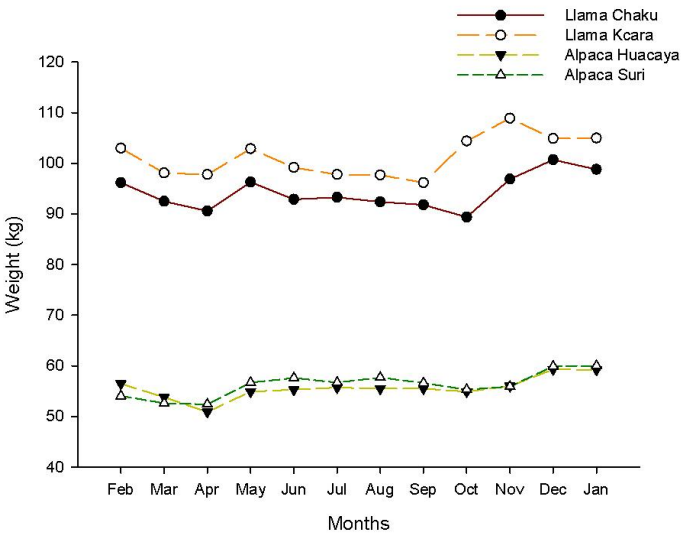

Figure 3: Body weight of female llamas, and alpacas throughout the year. Animals were maintained on native pastures of South America.

Conclusion

Little is known about the effect of nutrition of the reproductive process of domesticated South American camelids. Nutritional supplement definitively improves semen quality in males and females, which is translated in also an improved fertility of the female and male. Embryo reduction is also affected by type of pastures available to animals. Maintaining females on improved pastures results in smaller reduction of embryos compared to females on native pastures. The lactation period is also affected, total protein and blood urea nitrogen decreases during the dry and winter months in the Southern hemisphere; however, glucose concentrations are maintained relatively constant during this period. Altogether, initial work on the effect of nutrition on reproduction is present. Further work is needed to unravel other metabolites in blood of pregnant females during the last three months of pregnancy.

Selenium supplementation and its effects on reproductive performance in dromedary camels

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Introduction

The minerals are a part of the feeding resources essential for the animal life. The deficiency can occur even in the desert where the minerals are a dominant element in the landscape. So, the camel can be affected by mineral deficiency or in some occasions by mineral toxicity. The requirements in trace-elements are in very low quantity (copper, zinc, manganese, iron, iodine, cobalt and so on). Selenium is one of these elements. It enters in the composition of glutathione-peroxidase, an enzyme playing a central action in the cell protection by anti-oxidative activity. Many studies in domestic animals have shown that selenium (Se) supply is linked to a better immune system by the protection of the cells involving in immunity process (white blood cells). Selenium is also involved in reproduction performance and in muscle metabolism. A lack of selenium can lead to infertility, muscle degenerative and heart failure (Hidiroglou et al., 1987).

Selenium in animal reproduction

Many experiments were done to assess the impact of Se distribution to farm animals on their reproduction performances. It has been proved that Se, especially under organic form has a positive effect on several reproduction parameters like embryo development (Fortier et al., 2012), growth of the young (Stewart et al. 2012), spermatogenesis in male (Ahsan et al., 2014) or fertility in female (Gabryszuk and Klewiec, 2002). The Se supplementation could play a role in the prevention of placental retention and mastitis in dairy cows (Julien et al., 1976). Selenium is generally used in complementation with vitamin E for stimulating reproductive performances. However, the study of Coe et al (1993) did not show any significant effect of Se supplementation on somatic cell count, intercalving interval and total milk production.

Selenium status in camel

For long time, the same references than for cattle where used for camel. The National Research Council recommended a daily supplementation of 0.3 ppm in the diet while INRA reported 0.1 ppm only for dairy cows (Combs and Combs, 1986). Only recently, Se requirements and metabolism were studied in this species (Seboussi et al., 2008 and 2009a). A review regarding Se requirements, deficiency and toxicity is available in Faye and Seboussi, (2009a). Selenium deficiency is common in camel especially in places with Selenodeficient soils and plants. Such situation is generally common in arid areas like Saudi Arabia and United Arab Emirates, where Se deficiency in camel is regularly observed. An important part of the camel calf mortality cases could be attributed to heart muscle dystrophy which is the main symptom of Se deficiency in young animals (El-Khouly et al., 2001). The muscle degenerative due to lack of Se can have high consequence on muscle activity and especially on race animals where this activity is strongly requested but also in young growing animals.

However, there is little evidence to date of clinical Se deficiencies in camel. Only few results on plasma or blood values in field conditions in different areas from Morocco, China, Saudi Arabia or in some zoological parks were available in the literature. The recent data collected in Emirates and the results of the studies of Seboussi et al (2008, 2009a and b) confirmed that the normal selenium blood level in camel without selenium supplementation is around 100ng/100ml. In supplemented animals, the serum values could increase up to 200 ng/ml even more. A deficient situation could be considered when values are below 50 ng/ml.

In milk, the Se concentration is comparable to blood concentration, i.e around 100 ng/ml but it is decreasing after one month lactation. The protection of the young could be done easily by maternal transfer at the end of pregnancy. Indeed, the correlation between Se in colostrum and Se in mother blood before calving is very high. Camel seems to present an apparent good efficiency of Se transfer in milk, higher than in other ruminants (Faye et al., 2011).

Selenium supplementation in camel

Few papers related the impact of Se supplementation on the mineral status of camel. To our knowledge, the first trial achieved to assess the effect of dietary Se on camel was reported by Bengoumi et al. (1998) These authors compared the Se status of camels with that of cattle with similar weight and receiving daily 2 mg Se per os in sodium selenite form for two months. In this study, sharper increase of plasma selenium occurred in camels (10 times the plasma level before supplementation) compared to cows (twice the starting level) (Figure 1).

As the magnitude of the decrease of plasma selenium concentration after stopping supplementation was similar to the previous increase, it was supposed that plasma (or serum) selenium concentration in camel was an extremely sensitive indicator of selenium intake. The fast selenium depletion at the end of the supplementation period seemed also to indicate a better efficiency of selenium absorption and excretion in camel compared to cow.

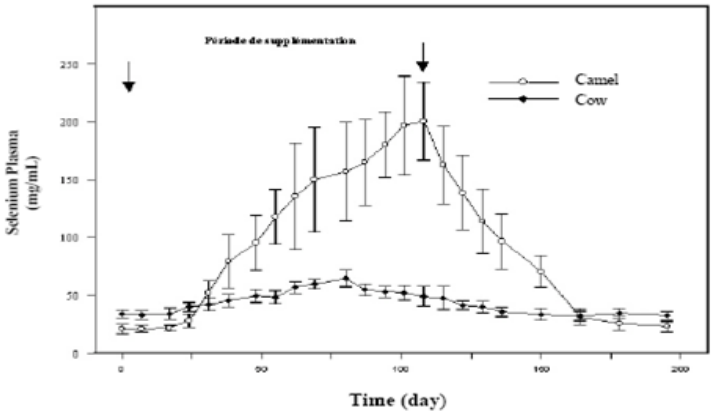

Figure 1: Comparative change in plasma selenium concentration in cow (●) and dromedary camel (o) receiving 2 mg/day selenium under sodium selenite form (from Bengoumi et al., 1998).

In selenium-deficient camels with muscular dystrophy, Al-Qarawi et al. (2001) gave oral treatment involving selenium/vitamin E by IM injection at a dose rate of 0.5 mg/kg body weight for two consecutive days. Following treatment, mean Se concentration rose from 2.3 ng/mL up to 23.7 ng/mL, i.e., with a similar trend to that observed by Bengoumi et al. (1998).

To determine the right requirements in Se for camel, several experiments were achieved in Emirates and according to the meta-analysis performed (figure 2), it has been suggested to limit Se supplementation in camel at 0.01-0.02 mg/kg LW, i.e. approximately 4-8 mg per day for adult animals or 0.5-1 ppm in the diet (Faye and Seboussi, 2009).

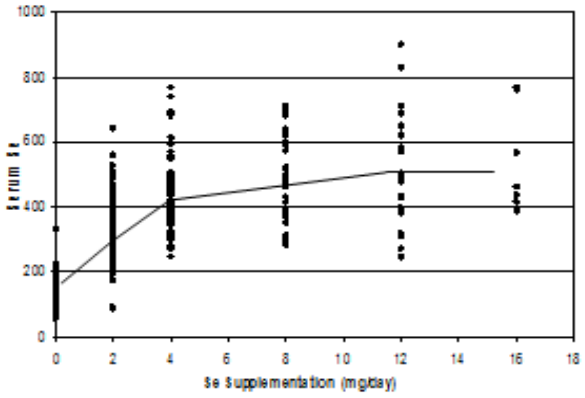

Figure 2: Change in camel serum selenium according to the level of oral supplementation.

It has been demonstrated also that diet including barley (1 kg/day) is favorable for a better Se status during pregnancy (Althamma et al., 2012). A recent study achieved in Saudi Arabia has

underlined also the advantage of organic Se compared to inorganic one for Se status of the camels (Faye et al., 2013). According to dietary Se supply and mean weight of the animal, selenosis (Se intoxication) appeared with 0.05 mg/kg LW Se supply only showing a higher sensitivity of camel than other animals to Se toxicity. Severe intoxication occurred with 16 mg Se supplementation, i.e. 0.10 mg/kg LW. These values were 5 times lower than those for sheep and cattle (Seboussi et al., 2009b).

Conclusion

The camel appeared more sensitive to Se supplementation and the effect of Se on reproduction performances and udder health is not still clearly investigated. No clear effect on Somatic cell count has been reported but the assessment of impact on reproduction performances requires taking in account the right recommendations.

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Selenium supplementation in alpacas

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Selenium is a constituent of glutathione peroxidase, an intracellular enzyme that protects cells from oxidative damage during normal cellular metabolism and is an essential trace element required by grazing livestock (SCARM 1990). Selenium works with other antioxidants such as vitamin E and superoxide dismutase to maintain cell integrity by protecting polyunsaturated fatty acids found in cell membranes and to ensure normal immune and reproductive function (McDonald et al. 2002, Suttle 2010).

Selenium requirements and optimal method of supplementation in South American camelids are yet to be resolved. The currently recommended daily intake of oral selenium is between 0.74 and 1 mg/day/animal (Van Saun 2016). This may be eaten in selenium-adequate pasture or hay, but in areas where soil, pasture and hay are deficient, selenium may be supplemented to alpacas either in a loose mineral lick or using an injectable supplement. In extensive grazing situations where the primary source of nutrition is from pasture and animals are not being supplementary fed on a daily basis, injectable methods of supplementation may be preferred to simplify management.

Sodium selenate and sodium selenite are found in some injectable selenium supplementation products. The selenium in this format is rapidly absorbed and available to the animal. Subcutaneously administered sodium selenate has caused acute liver failure (death in < 24 hours) in at least one alpaca in Australia (Vaughan et al., submitted for publication) therefore it seems prudent to avoid using 5-in-1 vaccines and injectable anthelmintics containing selenium in rapidly available forms.

A study on injectable selenium supplementation in alpacas is currently underway in Australia. The study thus far indicates that alpacas may be injected subcutaneously with barium selenate, a depot product designed to release selenium slowly from the injection site over a period of 12 months. Alpacas that were tested deficient in blood selenium and glutathione peroxidase were supplemented with barium selenate subcutaneously in June 2015. One month after injection,

blood levels had risen to adequate concentrations, and 9 months into the study, alpacas still have adequate blood selenium concentrations. The findings will be published when final samples have been tested in June 2016.

Selenium is an essential trace element required for normal cell, immune and reproductive function in camelids. Ensure animals are blood tested prior to supplementation to detect if a deficiency is present, and ensure supplement calculations are correct to avoid over-dosing. Too much selenium is toxic and it has and will kill camelids.

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Effect of live yeast and Se-yeast on milk production and calf health in dromedaries

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Introduction

Though, camels are not true ruminants, they ruminate and their digestion is essentially foregut fermentation based on microbial activity. The digestive microflora of dromedaries also contains bacteria, protozoa and fungus, but the digestion of the species is adapted to arid climate, scarce and lignified forage (in nature, their main diet is made of trees and bushes). The use of live yeast as a nutritional tool to optimize digestibility of the diet in order to improve milk production and feed efficiency have been extensively documented on ruminants (De Ondarza et al. 2010). Live yeast strains such as the one used in our study (*Saccharomyces cerevisiae* I-1077) have been specifically selected for their ability to optimize rumen function and nutrients utilization. Live yeast improves NDF degradation in the rumen, which is particularly interesting in the case of camels, typical fed a fiber-rich diet. This is due to the ability of *S. cerevisiae* I-1077 (1) to consume oxygen in the rumen (O_2 is toxic to fibrolytic bacteria); (2) to stabilize and raise pH, which is favorable for the growth of fibrolytic bacteria and (3) to stimulate growth and activity of fungi resulting in a greater breakdown of lignocellulosic tissues. Live yeast also improves nitrogen utilization by stimulating ammonia uptake by rumen microorganisms (Chaucheyras-Durand et al. 2008). Under intensive management, there is a constant need to optimize and increase milk production of machine milked dromedaries using various method including nutrition (Nagy and Juhasz, 2016). In addition, selenium imbalance and deficiency related problems such as poor performance, low reproductive efficiency in adults, sudden death and white muscle disease (WMD) in camel calves have been frequently observed under our conditions (Faye et al. 2014). The aim of this paper is to demonstrate through two different examples how oral feed supplementation may affect the performance and disease status of dromedary camels.

Materials and Methods

In the first experimental study, from June to October 2010, 90 dromedaries in mid lactation were randomly assigned to one of two treatment groups. Control animals (Control group) were fed a basal diet consisting of 4.0 kg wheat bran and 6.0 kg alfalfa hay per day, divided into 2 daily portions. In LSC group camels, the basal diet was supplemented with 0.5 g/ animal/day of live yeast ($1x10^{10}$ CFU; *S. cerevisiae* CNCM I-1077; Levucell SC 20, Lallemand, France). Camels were milked twice daily and milk yield was measured using an ICAR approved milk meter. Feed intake was monitored on a daily basis. Camels were weighed and their body condition was recorded at monthly interval. Milk gross chemical composition was analyzed 5 times at monthly interval with FT120 milk analyzer (Foss, Denmark). Udder health parameters (TVC and SCC) were also evaluated each month. Blood samples for hematology and biochemistry parameters were collected every two months. Statistical analyses were performed through a mixed model (SPSS 17.0) with treatment and DIM as fixed factors and weeks for repeated measures. For blood parameters, only treatment was used as fixed factor.

In the second observational study, we have monitored and compared the occurrence of WMD and sudden death in calves in association with selenium supplementation of dams during pregnancy. During the 2009-2010 breeding season, pregnant animals were given a basic diet consisting of 1.0 kg of wheat bran and 5.0 kg of Rhodes hay (*Chloris gayana*) per day, divided into 2 portions. During the subsequent breeding seasons, the diet of pregnant camels was supplemented with 1 g/animal/day of selenized yeast (Alkosel, Lallemand, France) starting from the last 3 months of gestation. This dose provided 2 mg of organic selenium intake per animal daily. During all seasons, calf mortality was closely monitored, all cases were sent for post-mortem examination and the incidence of white muscle disease was determined.

Results and Discussion

The live yeast supplementation significantly improved average milk production by 10% (0.7 kg extra milk per day): 7.26 ± 0.14 vs 7.99 ± 0.17 kg/day (Table 1). The increase in milk yield started 5 weeks after the beginning of supplementation. This increase in daily milk yield was not accompanied by dilution of milk solids and nutrients (Table 1). On the contrary, milk fat content and composition tended to increase in yeast-fed animals but the difference was not significant. Protein yield was significantly increased in LSC group compared to controls. We found no difference in udder health parameters such as TVC, SCC and in the incidence of

clinical mastitis. Blood hematology profile also remained within normal limits and showed no difference between groups. In contrast, important differences were noted in liver and renal function parameters as it is shown in Table 2. In yeast supplemented animals, creatine kinase, lactate dehydrogenase, aspartate aminotransferase, blood urea nitrogen and creatinine were lower, while albumin was higher compared to controls. This blood biochemistry profile indicates improved overall metabolism supporting higher milk production in LSC camels. Body weight in both groups increased significantly towards the end of the study in parallel with decreasing daily milk yield. In conclusion, live yeast in dairy camels helped optimizing their diet utilization and provided a 10 % increase in milk yield resulting in an excellent cost-return ratio (1:35).

Table 1. Body weight, milk yield and milk composition after live yeast supplementation in lactating dromedaries.

	Control group (±SEM)	**LSC group (±SEM)**
Body weight, kg	554.1 ± 4.9	557.3 ± 4.8
Milk per day, kg	7.26 ± 0.13^{b}	7.98 ± 0.16^{a}
Milk Fat, %	2.44 ± 0.05	2.48 ± 0.05
Milk Protein, %	2.82 ± 0.03	2.81 ± 0.03
Fat yield, g	176.3 ± 4.8	186.9 ± 4.8
Protein yield, g	199.4 ± 4.5^{a}	212.5 ± 4.5^{b}
Lactose, %	4.32 ± 0.02^{a}	4.24 ± 0.02^{b}

Different letters within the same row indicate significant difference at $P<0.001$.

During the 2009-2010 breeding season, we had 498 deliveries on the farm. Sudden death and confirmed WMD were observed in 51 of these calves (9.8 %). The disease was manifested always above 30 days of age, in healthy, fast growing and mainly male calves. Organic selenium supplementation started in the 2010-2011 breeding season and has been continued since then. As a result of oral Se supplementation of dams during late pregnancy, the occurrence of sudden death and WMD in calves decreased significantly. We recorded 2 (0.6 %) and 3 (0.5 %) of such case out of 323 and 559 deliveries in 2012-2013 and 2013-2014 breeding seasons, respectively. Occasionally, we observe chronic WMD, but at later age after the cessation of Se supplementation. Therefore, we also started selenized yeast supplementation to growing animals after weaning. In conclusion, oral organic selenium to dams provide an efficient protection to newborn calves against sudden death and WMD.

Table 2. Blood biochemistry parameters in live yeast supplemented and control dromedary camels.

	Control group (±SEM)	LSC group (±SEM)
CK (U/l)	160.3 ± 5.76^{a}	125.4 ± 5.76^{b}
LDH (U/l)	336.6 ± 6.30^{a}	316.0 ± 6.30^{b}
AST (U/l)	80.2 ± 2.04^{a}	71.8 ± 2.04^{b}
TP (g/l)	69.2 ± 0.59	70.7 ± 0.59
ALB (g/l)	41.0 ± 0.41^{a}	43.0 ± 0.41^{b}
BUN (mmol/l)	11.3 ± 0.20	10.7 ± 0.20
Creatinine (μmol/l)	195.0 ± 2.41^{a}	188.5 ± 2.42^{b}

Different letters within the same row indicate significant difference at $P<0.01$.

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Selection of alpaca females as recipients in embryo transfer programmes

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Introduction

Three to five days after a female alpaca is mated, and 2-4 days after ovulation, a corpus luteum (CL) develops on the ovary at the site of ovulation and plasma progesterone levels rise 4-6 days after mating (Sumar et al. 1986, Aba et al. 1995).

In a female that fails to conceive the CL reaches a maximum size of 10-15 mm and maximum progesterone output 8-9 days after mating. The progesterone output of the CL starts decreasing about 9-11 days after mating and the CL has halved its diameter 12 days after mating (Fernandez-Baca et al. 1970, Adams et al. 1989, Adams et al. 1990, Sumar et al. 1991). The lifespan of the CL is therefore 8-9 days. Females will be sexually receptive again approximately 12-14 days after mating if conception fails.

In pregnant females, ovulation, CL growth and progesterone production occur as in ovulatory but non-pregnant camelids for the first 8 days after joining. There is a temporary decline in blood progesterone from Day 8 to 12 after mating during the period of maternal recognition of pregnancy (Fernandez-Baca et al. 1970, Sumar et al. 1993, Adams et al. 1991, Aba et al. 1995). The embryonic signal for maternal recognition of pregnancy must be transmitted as early as Day 10 after mating in order to rescue the CL of pregnancy (Aba et al. 1997). Thereafter, progesterone levels increase and the diameter of the CL reaches a maximum of 10-19 mm on about Day 21 after mating (Adams et al. 1991, Bravo et al. 1993). Plasma progesterone levels remain elevated above 2 ng/mL but fluctuate throughout gestation (Bravo et al. 1996). Progesterone declines between 14 and 1 day pre-partum, markedly in the last 24 hours, and is basal by the day of parturition (Leon et al. 1990, Aba et al. 1998). The CL is the major source of progesterone throughout pregnancy and its presence is required to maintain pregnancy (Sumar 1988, Skidmore et al. 1996).

The relationships among CL size, plasma progesterone concentration, and ability to conceive in alpaca embryo transfer recipients have not been reported. This paper aims to identify recipient alpaca females, using ultrasonography on the day of transfer, that are more likely to conceive following transfer of an embryo into their uterus.

Materials and Methods

Forty-two female alpacas were prepared as recipients in two separate commercial embryo transfer programmes in north-eastern Victoria. Embryos were flushed from donors and transferred within 30 minutes into synchronised recipients according to Vaughan et al. (2013). On the day of embryo transfer, a transrectal ultrasound was performed on each recipient female to visualise and record digital images of the ovaries using a SonoSite Vet M-Turbo ultrasound machine (Fujifilm SonoSite Inc., USA) equipped with a 10 MHz linear array transducer. The maximum height and width of each corpus luteum and the maximum height and width of any non-echogenic fluid filled central cavity, or lacuna, were measured from stored images and a mean diameter calculated for each.

A 10 mL blood sample was collected from each recipient female via jugular venipuncture prior to embryo transfer. Blood was allowed to clot at room temperature for at least 1 hour, then centrifuged at 3000 rpm for 15 minutes. Serum was harvested and stored at -20°C for future analysis.

Transrectal ultrasound was performed in recipient females approximately 60 days after embryo transfer to confirm pregnancy status.

Plasma progesterone concentrations were determined using the Coat-A-Count Progesterone® kit (Diagnostic Products Corporation, Los Angeles, USA), previously validated for use in alpacas (Sumar et al. 1988).

Logistic regression was used to analyse various parameters in recipient females that did and did not conceive.

Results

There were no significant differences between females that did and did not conceive with respect to parameters listed in Table 1 and Figure 1.

Eight of 23 non-pregnant females and 9 of 19 pregnant females exhibited a lacuna inside their corpus luteum, ranging from 2-13 mm and 2-7 mm in mean diameter respectively.

Table 1: Mean (± SEM) of various parameters in recipient alpaca females that did and did not conceive following embryo transfer. There were no significant differences between groups (P > 0.05).

		Females that failed to conceive (n=23)		Females that conceived (n=19)	
	Unit	**Mean**	**SEM**	**Mean**	**SEM**
Recipient age	**years**	5.9	0.5	5.0	0.5
CL age	**days**	6.9	0.1	6.8	0.1
[Progesterone]	**ng/mL**	7.2	1.0	7.4	0.5
Body condition score	**1-5 scale**	2.3	0.0	2.4	0.1
Embryo diameter	**mm**	1.8	0.2	1.8	0.2
Embryo quality (1=good, 2=ok, 3=poor)		1.6	0.2	1.6	0.1
Mean CL diameter	**mm**	13.2	0.4	12.1	0.4
Mean lacuna diameter	**mm**	1.8	0.6	2.2	0.7
(CL-lacuna diameter)	**mm**	11.4	0.4	9.9	0.5

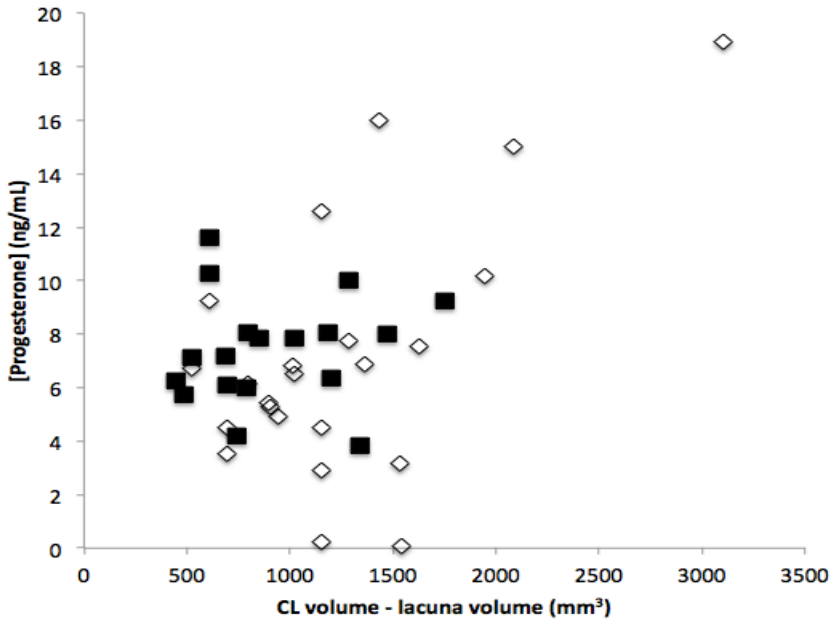

Figure 1: Relationship between plasma progesterone concentration (ng/mL) and [corpus luteum volume minus lacuna volume (mm^3)] in recipient alpaca females that did (n=19; solid squares) and did not (n=23; open diamonds) conceive.

Discussion

Neither plasma progesterone concentration nor corpus luteum or lacuna diameter or volume had any effect on ability of recipient alpaca females to conceive in this study despite the observation in a previous study that progesterone is higher in pregnant than non-pregnant females 8 days after mating (Sumar et al. 1993).

In this study, 17 of 42 females had a hollow cavity ranging from 2-13 mm diameter within their corpus luteum 7 days after induction of ovulation, in accordance with a previous study in llamas where 5 out of 36 llama CLs had a non-echogenic fluid filled central cavity ranging from 3-8 mm diameter (Adams et al. 1991).

The multifactorial nature of conception suggests that it is not possible to use ultrasound on the day of embryo transfer to select synchronised recipient alpaca females that are more likely to conceive following transfer of an embryo into their uterus.

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Synchronization protocols and management of recipients in dromedary camel (*Camelus dromedarius*) embryo transfer programmes

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Introduction

The use of assisted reproductive technologies, such as embryo transfer and artificial insemination, are becoming increasing popular in both racing and milking camels as they provide the opportunity to produce more of the genetically superior animals. One of the essential prerequisites of embryo transfer is to be able to synchronize ovarian follicular waves in the donors and recipients. This can pose particular problems in dromedary camels as they are induced ovulators, and therefore lack the cyclical corpus luteum of spontaneous ovulators such as the horse and cow. This means that the more conventional methods of synchronizing cattle by giving two injections of prostaglandin PGF2α 11 days apart (Cooper *et al*, 1976) are inappropriate for the camel. This paper reviews various protocols for synchronization of follicular waves in donor and recipient camels.

Synchronization of follicular waves

Various studies have been conducted to compare the efficacy of different treatments intended to synchronize follicular development in camels. Skidmore *et al*. (2009) randomly assigned camels to one of five groups (n=15 per group) and treated them with either (i) 5mg oestradiol benzoate (i.m.) and 100mg progesterone (E/P), (ii) 20µg GnRH analogue, buserelin (GnRH), (iii) 20µg buserelin on Day 0 (T = 0) and 500µg prostaglandin on Day T + 7 (GnRH/PG) (iv) transvaginal ultrasound-guided follicle ablation of all follicles ≥0.5cm (ABL) and (v) 5ml of saline (controls). All camels were subsequently injected with 20µg buserelin 14 days after the first treatment and follicular development and ovulation monitored by ultrasonography. A total of 11/15 camels in both the GnRH and GnRH/PG groups had dominant follicles between 1.3 and 1.9 cm 14 days post treatment, and 21/22 follicles ovulated after GnRH injection on T + 14. The ABL, E/P and control groups however, showed greater variability in follicle size with fewer camels having dominant follicles between 1.3 and 1.9 cm and more in the ≥2.0cm or follicle regressing groups, therefore fewer follicles ovulated

(ABL n=7; E/P n=9; Control n=6) after GnRH injection on Day *T* + 14. They concluded that two GnRH injections 14 days apart, with or without PGF2α on Day 7 after the first GnRH, were the most effective methods to synchronise ovulation rate in dromedary camels at a fixed time interval of 14 days after treatment.

Other studies by Nikjou *et al,* (2008) compared two different protocols for synchronizing follicular wave emergence prior to superovulation in Bactrian camels. Group 1 camels received two injections of GnRH 14 days apart (injected on Days -18 and -4 when initiation of superovulation = Day 0) and Group 2 camels received two consecutive progestogen treatments (three norgestomet implants and 200mg of progesterone) 7 days apart (Day -14 and Day -8) with removal of all implants 14 days after the first progestogen treatment. Results indicated that whereas the majority of follicles in the GnRH group ovulated and at the initiation of superovulation the diameters of the largest follicle was 0.7 ± 0.59 cm, in the progestogen treated group the growing follicles either became atretic or persistant and the average diameter of the largest follicle was 2.0 ± 2.26cm at the initiation of superovulation. They concluded that two GnRH injections was a better method to synchronize follicle wave emergence in Bactrian camels prior to superovulation.

Subsequent studies investigated the pregnancy rate obtained after using the synchronization protocol of two GnRH injections 14 days apart, with or without PGF2α, in a fixed-timed breeding programme. Nagy and Juhasz (2012) obtained pregnancy rates of 53.7% in their treatment group of camels that were injected with GnRH on days 0 and 14 and were mated at a fixed-time interval on day 28. This was an improvement on the pregnancy rates of 28.3% achieved in the control camels that were randomly mated.

Manjunatha *et al,* (2015) investigated the use of a hormone protocol (FWsynch) for the synchronization of follicle waves and timed breeding. The FWsynch protocol, which consisted of GnRH on Day 0, PGF2α on Day 7, GnRH on Day 10 and PGF2α on Day 17, was initiated at random stages of follicular development and animals were bred at a fixed time interval on Day 22. They concluded that the FWsynch protocol synchronized the follicular wave in 92.8% (91/98) of animals, ovulation occurred in 89.9% (88/98) and they obtained a pregnancy rate of 68.4%. Thus it was concluded that the FWsynch protocol was effective in synchronizing the follicular wave for timed breeding irrespective of the stage of follicular development at the start of the protocol.

Synchronization of donors and recipients

Synchronization of follicular waves is not only important for fixed - time breeding but also for synchronizing donor and recipient animals in embryo transfer programmes. Pregnancy rates of 65 – 75% are now routinely achieved in camels after transfer of fresh Day 7 embryos into synchronized recipients that have ovulated 24 – 48 h after the donor was mated (McKinnon *et al*, 1994; Skidmore *et al*, 2002).

Synchronization of ovulation between donor and recipient camels can be achieved by selection of recipients from a random group of camels. Recipients are examined 24 and 48 h after the donor is mated and those with a follicle 1.3 – 1.7cm in diameter in their ovaries are injected with GnRH to induce ovulation (Skidmore *et al*., 2002). This method is labour intensive and only works if large numbers of recipients are available. Alternatively recipients can be treated with daily injections (i.m.) of progesterone-in-oil (100 mg/day) for 10–15 days, terminating on the day that the gonadotrophin was first given to the donor, and followed by administration of 1500 – 2000 i.u. eCG. The eCG treatment is planned for the day after the donor receives eCG and guarantees the presence of mature follicles in the recipient 24 – 48 h after the donor has ovulated. Results demonstrated that 90% of recipients treated with eCG after progesterone withdrawal showed normal follicular development and ovulated in response to GnRH 24 – 48 h after the donor was mated (McKinnon *et al*, 1994). However this method is also labour intensive as it requires daily handling and injections. More recently following the promising results from the studies of Nikjou, *et al*., (2008), Skidmore *et al*., (2009), Nagy and Juhasz, (2012) and Manjunatha *et al*. (2015), recipient camels have been treated with two injections of GnRH (14 days apart) and PGF2α 7 days after the first GnRH. The second GnRH was scheduled for the day after the donor was mated and results indicated that approximately 85% of the recipients ovulated after the second GnRH (unpublished data).

Management of asynchronous recipients

Pregnancy rates have been shown to be dramatically reduced to <10% if recipients are +1 day ahead or as much as 3 days behind the donor (McKinnon *et al*, 1994; Skidmore *et al*, 2002). Studies have therefore been carried out to determine ways to make use of asynchronous recipients. Recipients that had ovulated as much as 3 or 4 days behind the donor were injected daily with 75mg progesterone from 2 days before transfer to 6 days after ovulation, thereafter progesterone doses were reduced to 50mg, 25mg and 25mg on day 7, 8 and 9 respectively.

Nine of 16 recipients became pregnant (56%, ov+3, n=4; ov+4 n=5) compared with none of the non-progesterone treated controls (Skidmore *et al*, 2011).

This review shows that synchronizing follicular waves in donor and recipient camels is possible using either two injections of GnRH 14 days apart with or without PGF2α on day 7 or a combination of progesterone and eCG injections. However if recipients ovulate as much as 3 days behind the donor then acceptable pregnancy rates can be achieved by using exogenous progesterone from 2 days before to 9 days after transfer.

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Superovulation of dromedary camels with two injections of FSH dissolved in hyaluronan solution

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Introduction

Multiple ovulation and embryo transfer (MOET) technology has been applied in dromedary camels for last two decades. Several hormonal protocols have been used to induce multiple ovulations with variable success. The gonadotropins used for superovulation treatment in dromedary camels were equine chorionic gonadotropin (eCG) and follicle stimulating hormone (FSH) of porcine, ovine and camel origin (reviewed by Anouassi and Tibary, 2013). The most widely used methods of inducing superstimulation in dromedary camels are by twice daily intramuscular (IM) administration of porcine FSH in decreasing or constant doses over a period of 3 to 7 days (Mckinnon et al., 1994; Tibary and Anouassi, 1997; Nowshari and Ali, 2005) or a combination eCG and twice daily IM injections of FSH (Skidmore et al., 2002; Nowshari and Ali, 2005). Recently several approaches have been attempted to simplify superstimulation protocols by reducing the number of doses of FSH in cattle. A single dose or two doses (48 h apart) of FSH diluted in slow release formulation (SRF) compound, hyaluronan preparation resulted in similar superovulatory response, as the conventional twice daily IM administration of FSH (Tribulo et al., 2011, 2012). These simplified protocols reduce stress and effort of donor handling. Hence, this study was conducted to examine the effect of superovulation treatment involving two doses of FSH diluted in hyaluronan solution, on ovarian response and embryo production in dromedary camels.

Materials and Methods

This study was carried out at the Animal Research Centre Farm, Royal Court Affairs, Muscat, Sultanate of Oman, during the peak breeding season, from December to March (2015-16). Female camels were housed in pens isolated from males, were fed fresh green grass/dry

fodder, and had free access to water. They were also fed a diet of mixed concentrates once daily.

Follicular development of thirty two adult dromedary camels (aged 8-14 years) was synchronized by FWsynch protocol as described previously (Manjunatha et al., 2015) using i.v., injections of GnRH (100 µg of GnRH, Cystorelin, Ceva Sante Animale, Libourne, France) on days -22 and -12 and i.m., injections of $PGF_{2\alpha}$ (500 µg of $PGF_{2\alpha}$ analogue, Estrumate, Schering-Plough Animal Health, New South Wales, Australia) on days -15 and -5. On Day 0, animals were examined by transrectal ultrasonography to record the dominant follicle (DF) size on the ovaries and were treated with a further injection of GnRH to induce ovulation. Animals were scanned on Day 2 to examine ovulation and only camels that had ovulated were randomly divided into two groups.

On Day 4, camels in group I (n =11) received 400 mg NIH-FSH-P1 of Folltropin-V (Bioniche Animal Health, Belleville, Ontario, Canada) diluted in saline (20 mg/mL, recommended by manufacturer) in decreasing doses (60-60, 50-50, 40-40, 30-30, 20-20 mg), twice daily IM schedule for 5 days. Camels in group II (n = 19) received 200 mg of Folltropin-V diluted in a 5 ml solution of 5mg/mL hyaluronan (MAP-5, Bioniche Animal Health, Inc.) in two IM injections at 48 h intervals (first dose on Day 4: 120 mg, second dose on Day 6: 80 mg). PGF2α was administered on Day 7 and animals were bred by natural mating randomly with one of the 5 fertile bulls on Day 11. The ovaries were examined by ultrasonography on Days 11 and 13 to record the number of ovulatory sized follicles of ≥ 9 mm and ovulations. Animals were flushed 8 days after mating (Day 19) as described previously (Skidmore et al., 2002). Embryos were graded based on gross morphological appearance. Grade A (Excellent): Symmetrical and spherical in shape with smooth surface and uniform color. Size corresponds with its expected stage of development. Grade B (Good): Same as grade A with mild irregularities in overall shape and color with few protruded cells. Grade C (Fair): Collapsed or irregular shaped or small embryo with few dark patches and some protruded cells. Grade D (Poor): Non-transferable, collapsed or small embryos with very dark or size and stage of embryos does not corresponds with its expected stage of development. All statistical analysis was carried out using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). Student's t test was used to find significance between the groups.

Results

In response to GnRH treatment on Day 0, ovulation occurred in 30 out 32 animals. The mean number of ovulatory sized follicles was higher (P = 0.01) in group I than group II animals, but there was no difference in the number of ovulations between groups (Table 1). The number of anovulated follicles and grade C embryos were higher (P < 0.001) in group I than group II animals.

Table 1: Ovarian response and embryo yield in dromedary camels treated with 400 mg Folltropin-V given by twice daily IM injections over 5 days (Group I) or 200 mg Folltropin -V diluted in hyaluronan solution given by two IM injections at 48 h apart (mean ±SD).

	Group I (n = 11)	Group II (n = 19)	*P values*
Ovulatory sized follicles	23.1 ± 6.9	17.2 ± 5.0	*0.01*
Ovulations	19.3 ± 5.8	15.9 ± 4.3	*0.08*
Anovulated follicles	3.8 ± 1.9	1.3 ± 1.2	*<0.001*
Embryos	6.2 ± 3.5	6.3 ± 3.0	*0.93*
Embryo grade			
Grade A	3.7 ± 2.3	5.3 ± 2.6	*0.09*
Grade B	1.5 ± 1.4	0.8 ± 1.1	*0.12*
Grade C	0.9 ± 0.8	0.1 ± 0.3	*<0.001*
Grade D	0.2 ± 0.4	0.1 ± 0.3	*0.63*
Unfertilized ova	0.3 ± 0.8	0.2 ± 0.7	*0.65*

Discussion

The present study showed that a superovulation treatment protocol involving IM administration of FSH diluted in hyaluronan solution in two doses (48 h apart) was effective for in vivo embryo production in dromedary camels. New follicular wave emergence occurred at 2.94 days after GnRH treatment in ovulated animals and follicle deviation occurred at 2.4 days after wave emergence in dromedary camels (Manjunatha et al., 2014). Therefore, in the present study FSH treatment was initiated on Day 4 (i.e., 1 day after wave emergence) in the presence of actively growing small follicles of 3 to 6 mm in diameter. Based on the ovarian response and embryo yield to different doses of Folltropin-V in hyaluronan, a dose of 200 mg Folltropin-V is considered to be the optimal dose for dromedary camels at our centre (unpublished data).

Ovarian response in terms of number of ovulations and embryo yield in group II animals treated with two doses of FSH in hyaluronan was similar to that in group I animals treated with conventional twice daily IM administrations of FSH for 5 days. Similarly, two doses (48 h apart) of hyaluronan diluted FSH resulted in a superovulatory response that was comparable to the conventional twice daily protocol in cattle (Tribulo et al., 2012). The number of anovulatory follicles and grade C embryos were lower in group II when compared to group I animals. These findings suggest that two doses of FSH (48 h apart) diluted in hyaluronan provides sufficient FSH to support follicle growth through to an ovulatory size. Hyaluronan (5 mg/mL) solution was viscous but there were no problems in mixing of Folltropin-V or extraction of the mixture from the vial into the injection syringe. In summary, these results indicate that superovulation treatment with two doses of FSH in hyaluronan is an efficient and simple protocol for in vivo embryo production in dromedary camels.

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Current status and future direction of cryopreservation of hatched blastocysts from the Dromedary camel (*Camelus dromedarius*)

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Introduction

Cryopreservation of embryos is an integral part of assisted reproduction technologies (ART) in humans and animals (Vajta G., 2013). Two methods of cryopreservation, slow freezing and vitrification, are widely used to maintain functional capacity of biological materials during a cooling/warming process. Although ART has been successfully applied in human medicine and in some animal breeding programs, the cryopreservation of camel embryos is in its infancy, with a focus on the modification of established protocols commonly used for other animal species. Possible reasons for the slow development of an effective cryopreservation protocol for camels include: 1) the permeability of cryoprotective reagents (CAPs) during the cooling/warming processes might be influenced by the lack of zonae pellucidae in hatched embryos (Edger *et al.*, 2012). 2) a much larger variation in embryo size (150-500 µm from Day 6, 7 and 8 donors) in camels appears to influence the survival rate of hatched embryos during cryopreservation, and the development of protocols to fit different sized embryos has proven difficult (Herrid *et al.*, 2016). 3) there is no reliable evaluation system for embryo quality. The morphological integrity of cryopreserved embryos does not always correlate to developmental potential, and thus it is insufficient to assess cryopreservation outcome and to predict ET success.

Thus there is a need to compare and summarize recent progress in the field to provide researchers with new insight into designing experiments which will lead to development of effective cryopreservation protocols that can be used in ET programs to facilitate a more widespread application.

Slow Freezing

In the initial phase of developing cryopreservation protocols, slow freezing methods have been applied to test the toxicity of standard embryo CPAs to camel embryos. Briefly, the results of those works have shown that camel embryos are sensitive to propandiol (PROH), dimethylsulfoxide (DMSO) and glycerol, but tolerant to ethylene glycol (EG) (Skidmore et al., 2004). Subsequent studies were performed to determine the minimum exposure time to 1.5M EG required to achieve cryoprotection and to compare different methods of rehydration with or without sucrose. The best pregnancy rate (37%) was achieved when the embryos were exposed to EG for 10 min, cooled slowly at a rate of -0.5ºC/ min to -33ºC before plunging into liquid nitrogen and then thawed and rehydrated in 0.2M sucrose in holding media (HM) for 5 min (Skidmore *et al.,* 2004). The relatively higher pregnancy rate from slow frozen embryos may be due to more intact cytoskeleton integrity (Skidmore *et al.,* 2009). Cell death resulting from slow freezing was comparable to the unfrozen control, but freezing caused widespread disruption of the actin cytoskeleton, indicating that levels of cells death in an embryo may not be as critical as cytoskeleton integrity for embryo survival and implantation.

Vitrification

Vitrification is currently widely used for cryopreservation of human and bovine oocytes/ embryos because of its simplicity and efficacy. A combination of higher concentration of CPAs and an increased cooling/warming rate reduces ice crystal formation and thus improves the survival of biological material. Although the current vitrification methods differ considerably in technical detail between laboratories or clinics, two basic components are similar in all disciplines: 1) the commonly used equilibration and vitrification media respectively are 7.5% (v/v) ethylene glycol (EG) and dimethylsulfoxide (DMSO), and 15 - 16% (v/v) EG and DMSO plus sucrose; 2) room temperature (22-27°C) or mammalian body temperature (37°C) are used for equilibration/vitrification/warming.

In camels, Nowshari *et al,* (2005) vitrified 20 embryos using a high concentration of ethylene glycol (7.0 mol/L) with sucrose (0.5 mol/L) which resulted in the birth of one live calf [6]. In our study, although the rate of survival judged by the morphological appearance of the embryos after warming and in culture was high (91%) (Herrid *et al.,* 2016), the transfer of 18 vitrified embryos (Day 7 or 8) into 6 recipients during the breeding season resulted in no pregnancy (unpublished data). In addition, after warming the morphological appearance of larger embryos (250–500 mm) appear to be better than smaller ones (<250 mm) (Herrid *et al.,* 2016). However, in the longer term smaller embryos seem to be more tolerant to cryo-injuries than larger

embryos, since a 38% pregnancy rate was obtained from Day 6 vitrified embryos (after exposure to vitrification solution 20% glycerol +20% EG + 0.3M sucrose + 0.375M glucose + 3% polyethylene glycol in 3 steps) whilst no pregnancy was detected with Day 7 embryos (Skidmore *et al.*, 2005).

Future direction

Recent achievements with *in vitro* survivals and *in vivo* developments after vitrification are promising, however, more work is required to clarify the reasons of different requirements for *in vitro* and *in vivo* development. To optimize further parameters (applied cryoprotectants, concentrations, equilibration times and temperatures) that support both, cytoskeleton stabilizing agents may also be added to equilibration media. In addition development of modified carrier tools adapting better to the larger size and special structure of zona-free camel embryos may improve results. Finally, inconsistencies related to the manual handling of samples and differences between individual embryologists can only be eliminated by full automation of the established procedure - an unavoidable task of the next decade applicable to cryopreservation of all species including humans, and all procedures in assisted reproduction.

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In vitro sperm-oocyte interactions: assessment of dromedary camel sperm quality

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Abstract

This article reviews methods used for the evaluation of dromedary camel sperm, with particular emphasis on sperm-oocyte interaction *in vitro*. Dromedary camel sperm assessment has mostly relied on standard light microscopic analysis of parameters such as motility, morphology and acrosome status. Recently, *in vitro* fertilization and an *in vitro* test of sperm-oocyte interaction using the zona pellucida-free oocytes of prepubertal goats have been used to assess camel sperm function. This offers a more direct test of sperm function in this species.

Introduction

The most valuable test of male fertility and sperm fertilizing ability is to obtain viable pregnancies and normal offspring following natural mating or artificial insemination (Tsakmakidis et al. 2010). Meanwhile, a variety of methods are available to measure sperm viability, motility and organelle integrity These parameters, while important to know as a measure of initial sperm quality and processing procedures, are of limited value in predicting fertility outcomes. A third option can be the measurement of sperm function *in vitro* as it relates to interaction with female reproductive tract tissue, or even oocytes wherein binding, penetration, decondensation, cleavage and even blastocyst development can result (Rodriguez-Martinez et al. 2006). However, the latter most often can only be achieved with homologous eggs which are not always ready available. In this review, we have summarized the current state of *in vitro* fertilization (IVF) using homologous and heterologous oocytes in the camel.

IVF with camel sperm

Among the *in vitro* assays, an IVF test is the most suitable for assessment of overall sperm function. This assay definitely demonstrates that sperm can capacitate, acrosome react, bind to an oocyte, penetrate and fertilize the oocyte under the conditions tested. Typically, *in*

vitro conditions are more limiting than *in vivo* conditions. In the camel, several attempts to *in vitro* mature oocytes have been reported (Nowshari et al. 2005; Wani and Nowshari 2005; Kathir et al. 2009; Wani and Wernery 2010; Abdoon et al. 2011). Kathir et al. (2007) demonstrated the importance of oocyte maturation in the developmental competence of camel blastocysts. IVF has been accomplised in camels but the data are limited. Kathir et al. (2004) fertilized *in vitro* matured oocytes with fresh semen and co-cultured the embryos with oviductal or granulosa cells. Blastocysts resulted only from oocytes co-cultured with oviductal cells (10%). Later these researchers reported that hatched embryos obtained by culture in a semi-defined medium (mKSMaa) had better *in vivo* developmental ability after 60 days of pregnancy (33%) than those co-cultured with oviductal cells (0%; Kathir et al. 2005). The first camel offspring from IVF was obtained with this system (Kathir and Anouassi, 2006). Wani (2009) used epididymal spermatozoa stored 2, 4, 6 or 8 days at 5ºC in Tris-Tes and Tris-lactose egg yolk extenders to fertilize *in vitro* matured camel oocytes and obtained blastocysts. More recently, Fathy et al (2014) demonstrated a positive effect of 10 mM caffeine supplementation during *in vitro* maturation of camel oocytes on the frequency of embryo development and increased blastocyst formation (27.7%) compared to controls (11.7%).

Although IVF still offers the best method to assess overall sperm functions *in vitro*, the limited availabilty of biological material from female camels is a major impediment to the routine use of this assay. However, ZP- free oocytes from a heterologous species offer an attractive way to evaluate the fertilizing capacity in species with a limited oocyte source because it does not require the use of valuable homologous gametes and can offer vast numbers of oocytes for experimentation.

Zona-free oocyte penetration test with camel sperm

The heterologous *in vitro* sperm penetration test was introduced by Yanagimachi et al (1976) and involved the use of oocytes from hamsters after removing the ZP to allow interspecies interactions. It evaluated sperm competence in distinct biological processes required for fertilization: capacitation, acrosome reaction, spontaneous recognition and fusion with the vitelline membrane and finally chromatin decondensation. Positive penetration was confirmed by the presence of swollen sperm heads or a male pronucleus. However, this test is unable to examine other aspects of fertilization such as penetration of the ZP and embryo formation. Heterologous *in vitro* penetration tests have been reported between wild and domestic bovids (McHugh and Rutledge 1998), felids (Herrick et al. 2010), and goats (Lopez-Saucedo et al. 2015).

We have developed the first heterologous zona pellucida-free oocyte penetration test for the assessment of camel sperm function (Malo et al. 2015). Using *in vitro* matured, zona-free goat oocytes, we have demonstrated the ability of camel sperm to fuse with the oolema (>79%), penétrate, decondense and form pronuclei (>67%). Subsequently, we have successfully used this test to analyze sperm function resulting from a number of research projects (Malo and Skidmore et al. 2016, Crichton et al., manuscript submitted). Future research in our lab will attempt to correlate this *in vitro* test with conception rates in order to better understand the utility of this test to accurately predict *in vivo* fertilizing ability of camel sperm.

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The early development of camel oocytes, collected from slaughterhouse ovaries, matured *in vitro* and inseminated with frozen thawed semen

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Introduction

To date very few studies have reported the successful production of dromedary camel embryos with the use of frozen thawed semen (El-Sayed et al., 2012; El-Sayed et al., 2015). In the present study a small cohort of slaughterhouse derived oocytes were matured and subjected to *in vitro* fertilization (IVF) with frozen thawed semen derived from a single bull of proven fertility. IVF embryos were successfully produced using two systems which utilized either intact, expanded, cumulus oocyte complexes (COCs) or cumulus cell free oocytes (CCFOs). In tact COCs (n 69) were subject to a longer (18 hr) sperm co-incubation period, whilst CCFOs (n 96) were given a shorter co-incubation time period (5 hrs).

Cumulus cells appear to play a vital role during IVF and protect the oocyte from oxidative stress whilst helping to regulate normal fertilization. However, denuding the oocyte prior to IVF may reduce gamete co-incubation times, and has resulted in the production of embryos using frozen thawed semen in other species (Romar et al. 2003). Our results demonstrate that camel embryos can be derived from frozen thawed semen and that this may potentially be achieved with or without cumulus cell attachment. Modest rates of blastocyst formation were achieved with and without cumulus cells, with increased blastocyst formation rates recorded with the use of COCs (11.6 vs 4.2 %). A cumulus cell free IVF system whilst compromising normal embryo development may facilitate successful IVF in cases of poor sperm parameters typically observed in frozen thawed dromedary camel semen.

Materials and Methods

Recovery of oocytes and in vitro maturation

Ovaries were collected from a local slaughterhouse in normal saline solution (NSS) and transported to the lab within 4 hours of collection at room temperature. Aspirated COCs were examined under a stereo microscope and good quality, compact COCs were selected for maturation *in vitro* using tissue culture medium 199, supplemented with 10 IU PMSG, 10 IU HCG, 1 µg FSH, 1 µg Estrogen, 15 ng EGF and antibiotics for 36-40 hrs at 38.5 °C in an atmosphere of 5 % CO_2 in air.

In vitro fertilization

Several ejaculates of fresh semen were collected from a single bull of proven fertility and stored in liquid nitrogen for use in all experiments. Prior to use, several 0.5 ml straws of frozen semen were thawed at 30 °C for 60 seconds and then diluted with Dulbecco's PBS supplemented with 0.1% bovine serum albumen (BSA). The diluted sample was then subjected to a percoll density gradient separation (90 % over 45 %) and centrifuged twice (1400 rpm). The sperm pellet was re-suspended in fertilization medium (TALP, supplemented with 4 mM caffeine). Matured COCs were split into two groups and then either subjected to the longer term IVF period of 18 hrs, or denuded and used as CCFOs for the shorter 5 hr duration of IVF in 50 µl droplets of fertilization medium overlaid with mineral oil. Sperm were added to the drop at a final concentration of 1.5 x 10^6. Following IVF, presumptive zygotes were pipetted repeatedly to remove bound sperm and remaining cumulus cells and then cultured in modified KSOM with amino acids (mKSOMaa).

In vitro culture and assessment of development

Embryos were cultured in mKSOMaa for 48 hr at 38.5°C in an atmosphere of 5 % CO_2 in air and then cleavage rates were recorder in either system. All cleaved embryos were cultured for a further 5 days in mKSOMaa supplemented with 10% Fetal Bovine Serum (FBS). Morula formation was recorded on day 4 post IVF and blastocyst formation rates were recorded on Day 6. Embryos reaching the blastocyst stage were subjected to staining with Hoechst 33258 solution in order to assess total cell numbers.

Results and Discussion

The aim of this study was to assess the suitability of two *in vitro* fertilization systems for producing camel embryos derived from *in vitro* matured oocytes and frozen thawed semen. The proportion of oocytes that underwent cleavage (CCFOs 29.1 % vs COCs 30.4 %) and development to the morula stage (CCFOs 20.8 % vs COCS 23.2 %) was similar in either system (Table 1). However, development to the blastocyst stage was greater for longer term

incubation, cumulus cell intact oocytes (11.6 % vs 4.2 %). Very few blastocysts in either group underwent cavitation and expansion and none of them hatched (data not shown). Average cell numbers in resulting blastocysts were also low but similar amongst both groups (36 vs 38). This poor development at the blastocyst stage may have been because of the culture conditions and high oxygen tension used in this experiment (5 % CO_2 in air). Lowered oxygen tensions (5 % CO_2 5 % O_2 90 % N_2) during the *in vitro* culture of camel embryos has resulted in the birth of live dromedary camel offspring with fresh semen (Khatir H, Anouassi A, 2006). Further investigations will decide whether decreased oxygen tension during embryo culture might facilitate the complete development of camel IVF embryos derived from frozen thawed semen.

Table 1: The *in vitro* development of cumulus cell intact and denuded dromedary camel oocytes following IVF.*****

Group	No.Oocytes	No. (%) cleaved	No. (%) morula	No. (%) blastocysts	No. total cells in blastocysts
COCs	69	21 (30.4)	16 (23.2)	8 (11.6)	36
CCFOs	96	28 (29.1)	20 (20.8)	4 (4.2)	38

*The number of replicates was 5

In conclusion both cumulus cell intact and cumulus cell free camel oocytes fertilized *in vitro* with frozen thawed semen have the potential to develop to the blastocyst stage when inseminated with frozen thawed semen. The removal of cumulus cells appears to allow for a shorter co-incubation period but may compromise later stages of embryo development. Further study is required to confirm these results and assess any underlying causes for these findings.

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Characteristics of semen collected from dromedary bulls

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Introduction

Artificial insemination (AI) is not widely applied in dromedary camels due to difficulties in the collection of quality semen and a lack standard of semen freezing techniques (Tibary & Anouassi, 1997). Semen collection using an artificial vagina (AV) is the most common approach used in dromedary camels (Deen *et al.*, 2003, Skidmore & Billah, 2006, Medan *et al.*, 2008, Morton *et al.*, 2011, Ziapour *et al.*, 2014) although little is known about the effect of repeated semen collection on ejaculate characteristics or inter-male variation. Therefore, this study was carried out to define the semen characteristics of dromedary bulls during a six-week period of weekly collections.

Material and Methods

Six dromedary bulls aged between 14-17 years, with a history of normal fertility in natural breeding programs were selected for this study. These bulls were trained to donate semen by AV. A bovine AV (30 cm length and 5 cm internal diameter) was used in this study. Inside the AV, a smooth latex liner was mounted and fixed at both ends of the AV and a transparent graduated glass water-jacketed semen collection vessel was attached to the apex of the internal latex liner. The inner chamber of the AV was filled with water (45 to 48°C) in order to maintain an internal AV temperature of 41–42°C during semen collection. The water–jacketed semen collection vessel was then filled with warm water (37°C) and the inner surface of the AV was lubricated with KY jelly. Semen was collected at weekly intervals for 6 consecutive weeks during the peak breeding season (December to January). Bulls were exposed to the sexually receptive female for a period of 10 min before semen collection. After teasing, the bull was allowed to approach and mount a female camel restrained in sternal recumbency. The semen collector positioned on the left side of the female then grasped the male's prepuce, cleaned the preputial orifice and directed the erect penis into the AV for copulation. During

copulation, the tight feeling of the cervix was imitated manually by holding the latex liner between the AV and semen collection vessel. The bull was allowed to copulate into the AV up to 6 min during each collection.

Ejaculate volume and colour were recorded immediately after collection and transferred to a 35°C water bath. Gross activity (oscillatory activity of spermatozoa, Scale 1-4) was examined by placing a drop of neat semen on a pre-warmed slide and examined under a phase contrast microscopy. Viscosity (Scale 1-4) was assessed by measuring the strand formed between a glass slide and a pipette. To examine sperm morphology, semen smears were made on glass slides, air-dried and stained with Farrelly stain according to manufacturer's instructions (Minitube, Germany). At least 200 spermatozoa were examined with a phase contrast microscope under oil immersion. Spermatozoa with protoplasmic droplets, abnormal head, mid-piece and tail were categorized as abnormal spermatozoa. Ejaculates were diluted 1:1 with pre-warmed Optixcell (IMV, France) extender, kept in a water bath (35°C) and evaluated for liquefaction by pipetting at 5 min intervals. After complete liquefaction, sperm concentration was recorded using a Makler Counting Chamber (Sefi-Medical Instruments, Israel). Motion characteristics of spermatozoa were evaluated using CASA (CEROS, Version12, Hamilton Thorne Biosciences, USA) pre-adjusted for camel sperm analysis. Three microliters of semen (50×10^6 spermatozoa/ml) were placed in a 20 µm standard count analysis chamber (Leja, Nieuw-Vennep, The Netherlands). Five randomly selected microscopic fields were scanned five times each and approximately 500 spermatozoa counted. The total motility (TM), progressive motility (PM), path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) of spermatozoa were analyzed. Sperm viability and acrosome integrity was evaluated by using FITC-PNA/PI as described previously (Kershaw-Young & Maxwell, 2011) . Statistical analysis was performed using mixed model regression in GENSTAT (version 17, VSN International, Hemel Hempstead, UK) and $P > 0.05$ was considered non-significant.

Results

Thirty-six ejaculates were collected from six bulls (6 ejaculates per bull) and the colour of each ejaculate was creamy white. There was no difference between bulls in the volume of ejaculate, gross activity, sperm concentration, percentage of abnormal spermatozoa and viable spermatozoa with damaged acrosome (Table 1). Viscosity and percentage of viable intact-acrosome, dead intact and non-acrosome intact spermatozoa varied ($P < 0.05$) among bulls.

Motion characteristics of spermatozoa (TM, PM, VAP, VSL and VCL) varied ($P < 0.05$) among bulls (Table 2).

Table 1: Mean (±SEM) volume of ejaculate, gross activity, viscosity, sperm morphology, sperm concentration, viability and acrosome integrity of dromedary bulls.

	Bull Number						Overall
	1	2	3	4	5	6	
Volume (mL)	4.0±0.4	3.7±0.6	3.7±0.7	3.6±0.6	2.3±0.3	3.2±0.4	3.4±0.3
Gross activity (Scale 1-4)	2.9±0.2	2.7±0.3	2.2±0.2	2.6±0.3	2.0±0.4	2.6±0.2	2.5±0.3
Viscosity (Scale 1-4)	0.5±0.2 [a]	3.0±0.2 [b]	3.0±0.4 [b]	2.4±0.3 [bc]	2.5±0.5 [bc]	2.0±0.1 [c]	2.2±0.4
Abnormal spermatozoa (%)	11.9±0.7	8.9±1.3	11.7±1.8	11.6±0.4	12.1±1.7	14.5±0.8	11.8±1.6
Sperm Concentration (10^6/mL)	472±45	499±83	403±47	416±74	361±64	429±50	430±60
Sperm viability and acrosome integrity							
Viable intact-acrosome (%)	63±6.6 [a]	55±2.8 [b]	46±5.6 [c]	56±2.2 [b]	40±2.3 [c]	61±1.4 [a]	53.5±3.6
Viable damaged acrosome (%)	0	0	1±0.3	1±0.2	0	0	0.3±0.2
Dead intact acrosome (%)	20±5.4 [a]	13±2.1 [b]	28±3.1 [c]	21±0.6 [a]	32±2.3 [c]	20±2.2 [a]	22.3±2.7
Dead damaged acrosome (%)	17±3.0 [a]	32±3.3 [b]	25±2.5 [cd]	22±2.1 [c]	28±1.7 [bd]	19±1.0 [ac]	23.8±2.3

Between bulls, values with different superscripts within a column differ significantly.

Table 2: Motion characteristics of spermatozoa in dromedary bulls (mean ±SEM).

Bull No.	TM (%)	PM (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
1	91.0±0.9[a]	28.0±1.6a	103.8±3.7[a]	54.2±2.2[a]	194.1±11.0[ac]	10.1±0.4[ac]	29.5±0.5	51.7±1.1	27.5±0.3
2	80.0±1.1[bd]	20.0±1.6[bd]	112.8±4.8[a]	58.4±2.6[ac]	222.9±13.1[a]	12.6±0.7[b]	29.8±0.4	48.5±1.1	25.6±0.6
3	76.0±7.0[c]	16.0±3.5[b]	66.2±7.8[b]	34.5±5.9[b]	142.7±17.1[b]	8.3±1.0[c]	33.4±2.2	49.3±1.2	26.0±1.1
4	84.0±1.3[d]	26.0±1.9[c]	79.8±10.0[b]	42.2±6.3[bc]	165.4±20.5[b]	9.8±0.7[ac]	30.9±0.6	50.2±2.1	25.4±0.2
5	81.0±4.5[cd]	23.0±1.8[d]	87.1±16.6b[c]	41.9±8.1[c]	176.9±27.1[c]	10.2±0.9[ac]	32.4±1.5	52.0±1.9	25.0±1.1
6	84.0±2.2[d]	22.0±1.5[cd]	105.6±5.4[c]	52.7±3.5[ac]	217.2±11.3[ac]	11.1±0.5[ab]	29.3±0.7	50.0±0.8	25.9±0.5
Overall	83.0±2.1	22.0±1.7	92.5±7.3	47.3±3.7	186.5±12.6	10.3±0.6	30.9±0.7	50.3±0.5	25.9±0.3

Between bulls, values with different superscripts within a row differ significantly.

Discussion

The results of the present study indicate that some semen characteristics (viscosity, percentage of viable intact-acrosomes, dead intact and non-acrosome intact spermatozoa, TM, PM, VAP, VSL and VCL) vary significantly between individual dromedary bulls. This is a similar result to that observed in other species, for example the alpaca (Bravo *et al.*, 1997) and llama (Giuliano *et al.*, 2008) have displayed inter-male variation in various semen characteristics, while cattle have shown variation in sperm motion characteristics between bulls

(Farrell *et al.*, 1998). Inter-male variation in semen parameters has been postulated to result from variations in secretory activities of the sex glands, scrotal circumference, breed, age, body size and body weight (Leon *et al.*, 1991, Sharma *et al.*, 1991). As the animals used in the present study were of the same breed, age size and weight with similar scrotal characteristics (data not shown) we postulate that differences in seminal plasma produced by the accessory sex glands may be responsible.

The average volume of ejaculates in this study was comparable with that reported in previous studies (Deen et al., 2003, Skidmore & Billah, 2006, Morton et al., 2011). However, sperm concentration in this study was higher when compared to previous work (Wani et al., 2008, Morton et al., 2013, Ziapour et al., 2014). Semen viscosity is one of the main factors that delays semen processing and the time required for complete liquefaction of semen is usually reported as 60 to 90 min when incubated at 37ºC (Wani et al., 2008). However, in the present study, complete liquefaction of semen occurred between 5 to 30 min after diluting (1:1) semen with Optixcell extender. The average concentration of the spermatozoa ($x10^6$/mL) in the present study was 430 ± 60 which is higher than 370 (El-Hassanein, 2003), 185 to 350 (Skidmore & Billah, 2006), 230.4 (Wani et al., 2008) and 160.2 (Ziapour et al., 2014) previously reported. The percentage of abnormal spermatozoa in this study was similar to that reported in previous studies (Ziapour et al., 2014)(Wani et al., 2008). Morton et al. (2011) reported higher sperm membrane integrity and acrosome integrity than the present study (61.7% vs 53.8%) and (87% vs 75.8%), respectively. Total motility in the present study (83%) was comparable to previous studies in dromedary camels (Wani et al., 2008, Ziapour et al., 2014), although in previous studies motility was assessed subjectively. The reason for these various differences between our results and those reported in other studies may be due to a variety of factors. While it is possible due to the inherent differences between bulls (as reported herein), it may also be due to different semen collection length, frequency of semen collection or time of collection in relation to the breeding season. Some of these factors may be worth further investigation.

In conclusion, we report inter-male variation in semen characteristics between individual dromedary breeding bulls during the breeding season. The data reported herein could be used as reference values for breeding soundness evaluation of male dromedary camels.

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Semen quality and fertility of the male alpaca

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Semen parameters were determined and then associated to fertility of the male alpaca. Two hundred and fifty-six females and 20 males were used in this study. Alpacas were maintained grazing native pastures at La Raya research center in Cusco, Peru. Animals were separated at random from a group of 120 males and 400 females. Three types of breeding were used according to common practices in South America, being alternate, four breeding per day and single breeding per day were compared. Semen was collected per vaginal aspiration following breeding of the male to sexually receptive females. Animals were marked with number on their side to easily identify them on field conditions. Breeding was done every morning for four consecutive days. Semen characteristics were motility, live, morphology and concentration. Pregnancy was determined by ultrasonography at 21 days after breeding. Chi square test was used to determine differences between types of breeding on pregnant females. Analysis of variance was used to determine differences on semen quality. There was no difference in pregnant females between types of breeding, being 74, 71, and 69% for alternate, four times, and single breeding, respectively; X^2= 0.45. Spermatozoa motility affected differently on pregnancy ($P<0.05$), 75% of females became pregnant when motility was 50 to 90%, than 40 to 45% pregnant when motility was less than 40% ($P<0.05$). Live spermatozoa also affected fertility of the male, being more than 77% when live spermatozoa was 70 to 100%, in contrast to 10% females pregnant when live spermatozoa was 50 to 60%. Normal spermatozoa in the ejaculate also affected the percentage of pregnant females, being 89% when normal sperm was greater than 61%, in comparison to 2 to 35% when 20 to 50% of spermatozoa were normal. Sperm concentration varied from the most, from 9 to 190 sperm/mL, with no clear association ($P>0.05$) to fertility. There was difference ($P<0.05$) in fertility, 74%, when males bred 1 or 2 times per day: however, it was less than 22% when males bred 3 or 4 times per day. Fertility of individual males was also different and varied from 3 to 81%. In conclusion, seminal characteristics and number of breeding per day affected fertility of the male alpaca, which should be considered in breeding programs.

Introduction

The management of breeding in alpacas of South America is different according to number of females per owner. Alternate breeding is practiced when large of females are available, in this case two teams of males take turns in breeding females for a week. A second type of breeding is when two or three males are available for a small number of females, and in this case males bred many times without control or any registry. A third type is when males are kept separated and a controlled breeding is practiced, a registry of breeding exists and the owner knows how many females a male bred (Condorena and Fernandez-Baca, 1972; Condorena and Velasco, 1979). However, under the situations described above, fertility of the male, and semen characteristics after each breeding are unknown. The aims of this study were, first to determine semen characteristics after each breeding, and second, to determine the fertility of the male.

Materials and Methods

Two-hundred and fifty-six females and 20 males were used in this study. Animals were maintained on native pastures at La Raya research center, which is located at 4200 m sea level, and on the highlands of Cusco, Peru. Females were separated at random from a group 400 breeding animals. Likewise, males were also separated at random from a group of 120 breeding animals.

Females were assigned to three types of breeding. First, alternate breeding; second, four breeding per day, and third, a single breeding per day. Overall, the breeding period lasted for a month. Females were bred to assigned males on the morning hours of every day according to breeding schedule. Females were bred a single time and then marked with number to her side and to identify them on the field.

Semen samples were collected after each breeding by vaginal aspiration and following the protocol of Neely and Bravo (1998). Semen characteristics considered were, volume, motility, concentration, live and morphology, and were evaluated following the protocol of Garnica et al. (1993). Semen volume was determined in a 15-mL tube. Motility was assessed using a light microscope and expressed in terms of spermatozoa with movement of the tail. Sperm concentration was determined using the method of Newbauer. Spermatozoa morphology was determined on semen smears stained using Diff quick. Live spermatozoa were assessed on semen smears with Hancock stain.

Early pregnancy was determined by transrectal ultrasonography. A prostatic prove was inserted into a lubricated rectum. The presence of an embryonic vesicle was indicative of pregnancy, and its absence as open.

Statistical analysis: Data on fertility were analyzed using the Chi square test. Data on semen characteristics were analyzed using analysis of variance. Computer software, Statistical number crunching system, NCSS, was used. A P value of 0.05 was used to determine differences, if any.

Results

Proportion of females ovulating and early pregnancy in females of the three types of breeding is presented in Table 1. There was no difference in ovulating and early pregnant females between the three types of breeding, P>0.05.

Table 1: Percentage of early pregnancy in female alpacas subjected to three types of breeding.

Breeding type	Females ovulating	Females pregnant
Alternate	80.6	73.9
Controlled	82.2	71.3
Single breeding	71.4	68.6
Total	81.3	72.3

Semen characteristics

Main semen characteristics considered in this study appear on Table 2. Most semen characteristics varied significantly i.e., time of copulation, motility, live spermatozoa, sperm concentration, abnormal heads, and presence of cytoplasmic droplets (P<0.05).

Table 2: Means for semen characteristics of males subjected in three types of breeding.

Semen characteristics	Alternate breeding	Controlled breeding	Single breeding
Copula time, min sec	12’	12’ 30”	16’ 30”
Motility, percent	52.0^{b}	41.7^{a}	47.1^{b}
Live spz, percent	66.9^{a}	66.2^{a}	86.0^{b}
Conc, million, spz/mL	57^{a}	75^{b}	109^{c}
Normal spz, percent	52.5^{a}	52.6^{a}	57.7^{a}
Abnormal heads, percent	15.5^{b}	15.4^{b}	7.3^{a}
Cytoplasmic droplet, percent	4.6^{a}	6.1^{b}	7.4^{b}
Abnormal tails, percent	24.9^{a}	24.1^{a}	19.9^{a}

Spz= spermatozoa, Conc= sperm concentration. Different superscripts by rows indicate difference, P<0.05.

Semen traits associated to fertility

Relationship between sperm motility, live and normal spermatozoa are depicted in Figures 1, 2, and 3, respectively. More females became pregnant when motility was greater than 50%, when live spermatozoa was greater than 70%, and when normal spermatozoa in the ejaculate was greater than 61%.

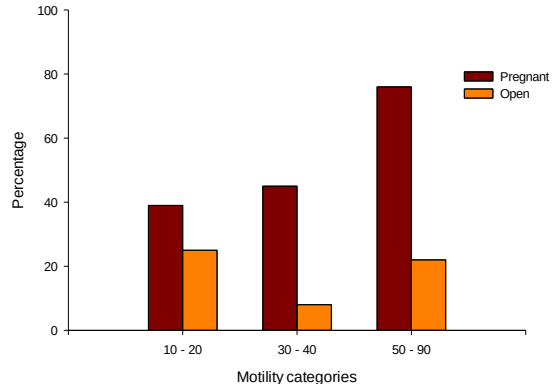

Figure 1: Relationship between sperm motility and pregnancy in the alpaca.

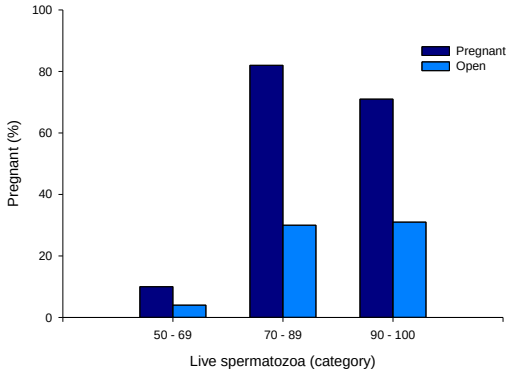

Figure 2: Relationship between categorized live spermatozoa of the ejaculate and fertility of the alpaca.

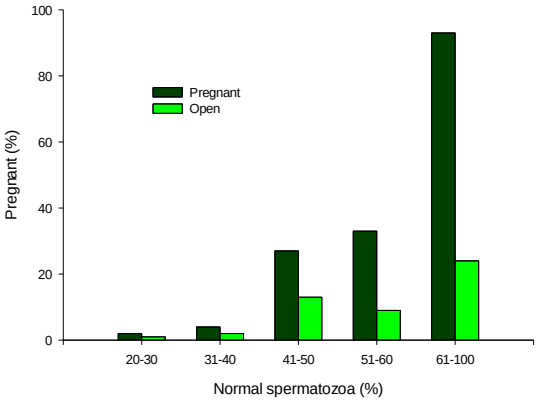

Figure 3: Relationship between categorized normal spermatozoa of the ejaculated and pregnancy in the alpaca.

Discussion

This study associated semen characteristics of the male alpaca to his fertility. Type of breeding, as practiced in South America, did not result in an increased ovulation rate and early pregnancy. As a matter of fact ovulation rate and pregnancy were similar to previous work in the alpaca (Fernandez-Baca and Novoa, 1968; Condorena and Fernandez-Baca, 1972; Bravo et al., 1997).

Semen characteristics found in this study varied between the three different types of breeding. Copulation time was extended when a male bred only one female as compared to four breeding per day and alternate breeding. A single breeding male took his time, was separated from other males, and was not competing with other males as in the other two types of breeding. By contrary the other males were breeding in the same area and could see other males. These results are in agreement with previous results in alpacas (Fernandez-Baca and Novoa, 1968; Bravo et al., 1997a; Bravo et al., 1997b).

Semen characteristics associated to fertility were more difficult to assess. Semen traits, motility, live spermatozoa and normal spermatozoa were positively correlated to fertility of the male. The increase in fertility could be attributed to a greater number of spermatozoa showing motility and consequently more spermatozoa reaching the place of fertilization. Likewise, more live and normal spermatozoa are beneficial for fertilization. Comparable results have been obtained in bulls breeding cows (Hafs et al., 1959; Correa et al., 1997), and rams (Hulet et al., 1965; Allison, 1975).

In summary, type of breeding, and semen characteristics did not affect fertility of the male alpaca. Semen characteristics more related to fertility were motility, live and normal spermatozoa than volume of ejaculate and sperm concentration.

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Reduction of thread formation and its effect on lama sperm cells morphology

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Introduction

Semen extension, cryopreservation and artificial insemination (AI) in llamas are continuously developing technologies. Whilst initial observations suggested that seminal plasma acted as a vehicle for the transport of spermatozoa into and within the female reproductive tract, further investigations highlighted an important role of seminal plasma in sperm function. One of the most important physical characteristics of camelid semen is its high viscosity seminal plasma, which makes it very difficult to handle during laboratory procedures and mixing with extenders, being currently the major impediment to the development of AI technologies in South American Camelids. Thereby, it limits contact between the sperm cell membranes with cryoprotective compounds during cryopreservation. Viscosity of the semen is usually attributed to the presence of mucopolysaccharides from secretions of the bulbourethral glands and the prostate. The physiological role of this characteristic is not clear. The degree of viscosity depends on the individual male and on the proportion of seminal gelatinous fluid The morphological abnormalities should be reported according by type and location. All sperm abnormalities found in other livestock species can be found in camelid semen (Tibary and Anouassi, 1997; Tibary. and Vaughan, 2006). Sperm morphology is an essential part in the study of ejaculates, since poor sperm morphology has been correlated with low fertility (Barth et al., 1992). The aim of this study was to evaluate the use of mechanical techniques to reduce thread formation, avoiding the use of enzymes, evaluating the effect on morphology lama sperm cells.

Materials and Methods

Animals

Two adult lama males were used as semen donors at the Laboratory of Theriogenology "Dr. H. Morello", Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, Río Negro, Argentina (38° 50´44" S and 68° 04´ 03" W). Animals were kept in individual pens and supplemented with balanced diet and water *ad libitum.*

Semen collection and evaluation

Semen samples were collected from December 2015 to March 2016. Ejaculates were obtained by means of a modified ovine artificial vagina, wrapped in an electric warming pad with a temperature controlled device (42°C to 45°C), Samples from males who had copulated for more than 15 min were processed, as this amount of time is required to achieve camelid semen with semen good quality. Ejaculates were placed in a bath at 32°C and were transported to the laboratory for analysis. Macroscopic and microscopic raw semen characteristics evaluated were: volume, motility and sperm morphology. Volume was measured in a graduated tube; sperm motility (percentage of total motile sperm) was evaluated using a warm stage (37°C) in a phase contrast microscope (100x) and sperm morphology was evaluated using eosin-nigrosin supravital stain (aliquots of 10 μL of the sperm and 10 μL eosin-nigrosin were mixed and extended on a slide). A minimum of 200 spermatozoa were evaluated using a phase contrast microscope (400x), considering percentage of normal cells and ten categories of abnormal spermatozoa.

Experimental design

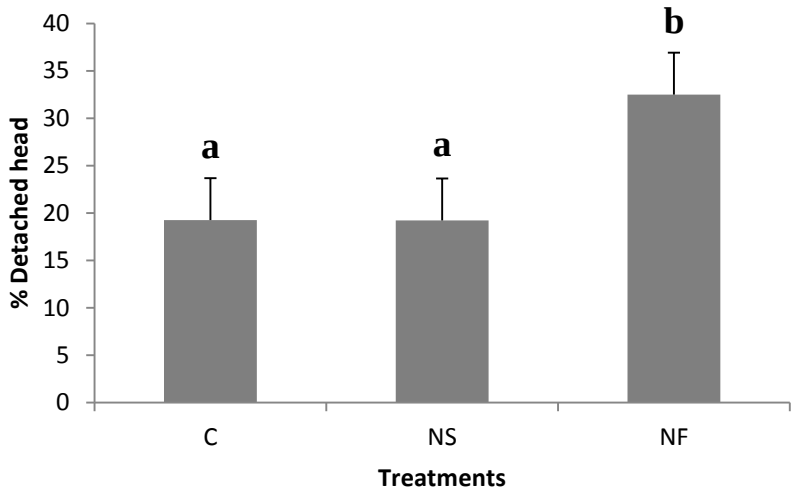
To reduce thread formation, raw semen samples were treated by means of: A) mechanic slow method, with a needle (0.5 mm) attached to a syringe, B) mechanic fast method, using the same syringe and C) control (without treatment). Each parameter was analyzed by ANOVA (main effects), with Fisher–LSD *post hoc* test, using the software StatSoft, Inc. (2007).

Results

After the initial analysis, no interaction between male and treatment factors was found. Needle fast treatment showed a lower percentage of normal sperm cells ($p< 0.05$) than needle slow and control (23.35±3.08%, 35.47±2.99% and 35.18±4.53%, respectively). This difference was indicated especially by increment ($p<0.05$) of cells with detached heads (Figure 1) in needle fast treatment (32.51±4.38%), compared with needle slow (19.21±4.03%) or control

(19.24±3.37%), as well as in coiled tails (38.77±4.38%, 28.45±9.08 % and 16.08±5.40%, respectively).

Figure 1: Detached sperm head in different treatments. Control (C), needle slow (NS) and needle fast (NF).



Data are expressed as mean ± standard error; values over a column with different superscripts are significantly different ($p < 0.05$).

Discussion

Bérgamo et al. (2015) demonstrated that the mechanic method was effective to reduce thread formation. The present study showed that the use of the mechanic slow method have not altered the number of normal sperm morphology. Possibly, the control of the flow rate - by counting no less than 10 seconds in each movement of the plunger of the syringe - determines that the turbulence within the fluid is minimal. This situation allows the preservation of morphological integrity of sperm cell during the process. Medina et al. (2012), studying the spermatozoa of Lama glama, reported abnormalities of the head, mid-piece and tail. The proportion of abnormal spermatozoa (obtained by artificial vagina) was very high (57.8 ± 13 %), being the morphology of the sperm head the most common abnormalities.

In conclusion, the fast method increases detached heads and shows a lower percentage of normal cells, indicating that the mechanic slow method it is better if it chooses to reduce thread formation. This condition would be beneficial allowing subsequent dilution and its use in artificial insemination.

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Effect of bromelain and papain treatments on the glycan pattern of cryopreserved dromedary camel spermatozoa

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Introduction

The liquefaction of dromedary camel ejaculates would enhance the nutritive/protective effects of extenders on post-thaw sperm characteristics and, hence, the application of artificial insemination and *in vitro* fertilization with frozen-thawed semen in this species. Recent studies reported using two proteases, bromelain and papain, to liquefy the dromedary camel ejaculates (Crichton et al., 2015; Kershaw-Young et al., 2016; Monaco et al., 2016).

Sperm glycocalyx consists of many different glycoconjugates that represent the interface between the male gamete and the extracellular environment. Therefore, they play an important role in reproductive biology (Tecle and Gageux, 2015). The evaluation of the sperm surface glycans could facilitate understanding the changes that occur to the spermatozoa in different conditions, including semen liquefaction and processing.

To date no reports addressed the influence of dromedary semen liquefaction on cryopreserved sperm glycocalyx pattern. Therefore, the current pilot investigation aimed to demonstrate the effect of bromelain and papain treatments on the glycan features of frozen-thawed dromedary sperms using the lectin histochemistry technique, which is a useful approach for *in situ* sperm glycocalyx analysis (Desantis et al., 2010).

Materials and Methods

Two ejaculates were collected from two dromedary bulls using an artificial vagina and were divided into three aliquots. The first aliquot served as control (untreated), whereas the other two aliquots were treated either with papain (0.1 mg/ml) or bromelain (5 u/ml) for 5

minutes. Thereafter, the treated specimens were supplemented with the anti-enzyme E 64 to terminate the effects of the two proteases.–All semen aliquots were then diluted with a 4% glycerolated Tris-lactose glucose extender, and were equilibrated at 4°C for 180 min before cryopreservation. After thawing, the spermatozoa of control and treated specimens were fixed in 4% (w/v) neutral formalin, washed, smeared on poly-L-lysine coated glass slides and were air-dried. Afterwards, the dried slides were incubated for 1 h in the dark with appropriate dilutions of 14 fluorescent lectins (Table 1). Slides were subsequently rinsed in the same buffer and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, USA). Controls included: (1) substitution of the substrate medium with buffer lacking lectin; (2) incubation with each lectin in the presence of its hapten sugar (0.2 M in Tris buffer). Both these control experiments gave negative staining. Slides were observed and photographed under a light photomicroscope Eclipse Ni-U (Nikon, Japan) equipped with a digital camera (DS-U3, Nikon, Japan). The images were analyzed by the image-analyzing program NIS Elements BR (Vers. 4.20) (Nikon, JP).

Table 1: Lectin used, their sugar specificities and the inhibitory sugars used in control experiments.

Lectin Abbreviation	Source of lectin	Sugar specificity	Inhibitory sugar
SNA	*Sambucus nigra*	Neu5Acα2,6Gal/GalNAc	NeuNAc
RCA_{120}	*Ricinus communis*	Terminal Galβ1,4GlcNAc	Galactose
Con A	*Canavalia ensiformis*	Terminal/internal αMan>αGlc	Mannose
PHA-E	*Phaseolus vulgaris E*	bisected complex GlcNAc β1-2Man	Mannose
PHA-L	*Phaseolus vulgaris E*	GlcNAc βl,2Man	Mannose
sWGA	*Triticum vulgaris*	Terminal/internal βGlcNAc	GlcNAc
GSA I-B_4	*Griffonia simplicifolia*	Terminal αGal	Galactose
HPA	*Helix pomatia*	GalNAc α1,3GalNAc	GalNAc
DBA	*Dolichos biflorus*	Terminal GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1	GalNAc
SBA	*Glycine max*	Terminal α/βGalNAc	GalNAc
PNA	*Arachis hypogaea*	Terminal Galβ1,3GalNAc	Galactose
GSA II	*Griffonia simplicifolia*	Terminal D-GlcNAc	GlcNAc
LTA	*Lotus tetragonolobus*	Terminal α L-Fuc	Fucose
UEA I	*Ulex europaeus*	Terminal L-Fucα1,2Galβ1,4GlcNAcβ	Fucose

Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, N-acetyl neuraminic (sialic) acid; s, succinylated.

Results

The acrosomal cap from all specimens showed N- and O-linked asialo glycans. N-glycans were high-mannose, biantennary and complex-types (Con A, PHA-E, succinylated (s)WGA affinity) and terminated with lactosamine (RCA_{120} reactivity). O-linked (mucin-type) glycans ended with N-acetylgalactosamine (HPA, SBA binding) and galactoseβ1,3 N-acetylgalactosamine (PNA staining). Rare acrosomal cap expressed few oligosaccharides with galactose (GSA I-B4 binding) in control and bromelain-treated spermatozoa. The latter

expressed also few fucosilated glycans (UEA I reactivity). Further, both bromelain- and papain-treated specimens revealed N-linked glycans (RCA_{120}, Con A affinity) in the equatorial zone of rare sperm heads, which did not bind sWGA in the bromelain-treated sperms. Among the 14 lectins used, sperm tail never reacted with SBA and sWGA. However, the tails of both bromelain- and papain-treated sperms poorly reacted with PNA and UEA I, whereas only bromelain-treated sperms reacted with LTA.

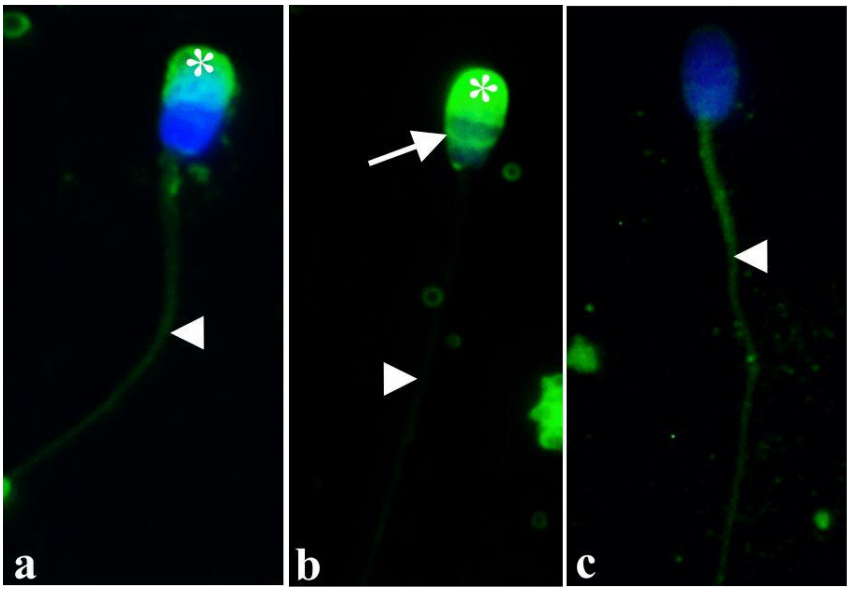

Figure 1: FITC-Con A (a,b) and FITC-UEA I (c) reactivity with dromedary spermatozoa from control (a), papain- (b) and bromelain-treated semens. Asterisk, acrosomal cap; arrow, equatorial region; arrowhead, tail. The nucleus was blue stained by DAPI.

Discussion

The evaluation of the sperm surface glycans is important to understand the sperm conditions because the sperm glycocalyx plays a key role in sperm motility, maturation and fertilization. This pilot study investigated the effect of the bromelain and papain, enzymes used in semen liquefaction, on dromedary spermatozoa glycocalyx. The results indicate that bromelain and papain caused a few changes in glycan pattern of dromedary sperm as revealed by i) GSA I-B_4 and UEA I reactivity for the acrosomal cap, ii) Con A, RCA_{120}, and succinylated WGA affinity for the head equatorial zone, iii) PNA and UEA I for the tail. Since both papain and bromelain are endopeptidases, they efficiently liquefied the dromedary semen due to their actions on seminal plasma proteins, which were speculated to contribute to semen viscosity in this specie (Kershaw-Young et al., 2013; Kershaw-Young et al., 2016). The minimal effect observed on post-thaw sperm glycan pattern in the current investigation is, evidently, due to

the proteolytic actions rather than the deglycosylation activity of both enzymes. Although few were the glycan changes, functional studies are required to evaluate the effects of these proteolytic enzymes on sperm fertilizing ability for their application in dromedary camels assisted reproductive technologies.

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Effect of diluent type, cryoprotectant concentration, storage method, freeze/thaw rates and seminal plasma addition on the post-thaw quality of cryopreserved, papain-treated alpaca spermatozoa

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Introduction

Semen cryopreservation is now a widely used method for long-term storage and transport of male genetic material in many livestock and wildlife species as well as in human medicine. However, the technology is not well developed in alpacas, due to their low sperm quality and viscous seminal plasma. Recently, a method of reducing semen viscosity using the protease papain, which does not adversely affect sperm quality, was developed. This has facilitated renewed attempts to freeze alpaca spermatozoa.

This study aimed to develop a cryopreservation protocol for papain-treated, ejaculated alpaca semen.

Materials and Methods

Four experiments were conducted to determine the optimal egg yolk concentration (5, 10 or 15%), glycerol concentration (2, 5 or 10%), and diluent type (SHOTOR, lactose, skim milk or INRA-96™) for an alpaca sperm cryopreservation medium as well as ideal freeze rates (2, 4 or 8cm above liquid nitrogen), thaw rates (37˚C for 1 min or 42˚C for 20 secs) and storage vessel (pellets, 0.25 mL straws or 0.5 mL straws). Two further experiments investigated the effects of adding 10% homologous seminal plasma back to ejaculated, papain-treated semen both pre-freeze and post-thaw (experiment 5) and adding varying concentrations (0, 10, 25 or 50%; final concentration) of seminal plasma to cryopreserved spermatozoa after thawing (experiment 6).

In all experiments, semen was collected from 3-4 male alpacas using an artificial vagina fitted inside a mannequin as described previously (Morton *et al.* 2008). Unpooled semen was used and 2-5 ejaculates were collected from each male so that the total number of replicates per

experiment was 12. Within 5 min of collection, semen was assessed for volume, sperm motility and concentration. Only samples with a volume > 1 mL, motility ≥ 40 % and sperm concentration ≥ 20 x 10^6 spermatozoa/mL were used.

After collection, semen was diluted 1:1 with fraction A (11% lactose and 3% BSA; Bathgate *et al.* 2006) and treated with 0.1mg/ml papain (final concentration; Sigma-Aldrich, St Louis, MO, USA) for 20 minutes at 37˚C. The effects of papain were then halted by adding 10μM N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64;final concentration; Sigma-Aldrich, St Louis, MO, USA) at 37˚C for 5 minutes, this caused viscosity to be eliminated for all the ejaculates used. The diluted ejaculate was diluted further (2:1) with fraction B (fraction A supplemented with egg yolk), placed in a water jacket and cooled over 1.5 hours to 5˚C. The cooled semen was further diluted (3:1) with fraction C (fraction B plus glycerol and Equex STM®; IMV Technologies, L'Aigle, France), to a final semen dilution rate of 1:3. The spermatozoa were allowed to equilibrate with fraction C at 5˚C for a further 30 minutes before being assessed for pre-freeze motility, acrosome integrity (FITC-PNA fluorescent staining), plasma membrane integrity (PI fluorescent staining) and DNA integrity (TUNEL fluorescent staining), then loaded into straws and frozen above liquid nitrogen vapour. Straws were thawed by agitating in a 37˚C water bath before the semen was further diluted 2:5 with fraction A of the diluent (final dilution rate of original ejaculate 1:9) and incubated for two hours in a 37˚C water bath. Pellets were thawed by placing them in a glass test tube in a 37˚C water bath while agitating the tube until the pellets liquefied. At 0, 30, 60 and 120 minutes post thaw, motility, acrosome integrity and plasma membrane integrity were assessed. DNA integrity was assessed at 0 and 60 mins post-thaw.

Results

Sperm motility was affected by egg yolk concentration (P<0.001), diluent type (P<0.001), freeze rate (P=0.003) and storage vessel (P=0.001), but not by glycerol concentration (P=0.11), straw volume (P=0.47) or thaw rate (P=0.12). Viability was affected by egg yolk concentration (P<0.001), diluent type (P<0.001), storage vessel (P=0.002) and thaw rate (P=0.03) but not by glycerol concentration (P=0.10) or freeze rate (P=0.15). DNA integrity was only affected by diluent type (P=0.001) and not freeze (P=0.71) or thaw rates (P=0.09).

Addition of 10% seminal plasma either pre-freeze or post-thaw did not improve sperm motility (P=0.49), viability (P=0.82) or DNA integrity (P=0.85). Adding seminal plasma at any of the concentrations back to the spermatozoa after thawing did not benefit motility (P=0.08) or DNA

integrity (P=0.12), but adding 50% seminal plasma incurred more acrosome and plasma membrane damage than the other treatments (P=0.03).

Discussion

As demonstrated by methodical examination of different aspects of the cryopreservation protocol, the highest post-thaw motility and viability of papain-treated, ejaculated alpaca spermatozoa was achieved when using an 11% lactose diluent supplemented with final concentrations of 5% egg yolk and 2, 5 or 10% glycerol, without additional seminal plasma, loaded into 0.5 mL straws and frozen 2 cm above liquid nitrogen for 4 minutes, followed by thawing in a 37˚C water bath for 1 minute.

Previous studies in alpaca semen cryopreservation have yielded post-thaw motilities of less than 25% for both ejaculated (Santiani *et al.* 2005, Kershaw-Young and Maxwell 2012) and epididymal (Morton *et al.* 2006, Morton *et al.* 2010) spermatozoa. By taking a systematic approach to the optimisation of a cryopreservation protocol for alpaca spermatozoa, the current study has achieved mean post-thaw motilities of up to 45% (lactose diluent, experiment three). As the mean pre-freeze motilities in the current study ranged between 35 and 65%, the loss of motility from pre-freeze to 0 h post-thaw was less than 50% of the neat motility across the six experiments, a marked improvement on previously reported results (Santiani *et al.* 2005, Banda *et al.* 2010, Morton *et al.* 2006, Morton *et al.* 2007). Despite these promising *in vitro* findings, further studies are required to determine whether alpaca spermatozoa cryopreserved in this manner retain their fertility.

This study also found that despite the papain treatment reducing the seminal plasma proteins in the ejaculate prior to freezing, adding seminal plasma back to the spermatozoa either pre-freeze or post-thaw did not have a beneficial effect. This suggests that alpaca seminal plasma may not play a role in the freezing resilience (or lack thereof) of alpaca spermatozoa as it does in other species, such as sheep (Rickard *et al.* 2015).

Thus, the recommended protocol is to dilute alpaca spermatozoa 1:3 in a lactose-based diluent, supplemented with 5% egg yolk and 5% glycerol. Freezing should be performed in 0.5 mL straws 2 cm above liquid nitrogen for 4 min before thawing at 37˚C for 1 min. Addition of seminal plasma either pre-freeze or post-thaw is not recommended, especially at concentrations ≥50% when a detrimental effect was seen. By utilising these diluent components, freeze and thaw rates and storage methods, post-thaw motilities of 30-40% can be routinely obtained.

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Effect of seminal plasma added at post-thawing on spermatozoa obtained from alpaca vas deferens

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Introduction

Seminal plasma (SP) contains a large protein component which has been implicated in the function, transit and survival of spermatozoa within the female reproductive tract (Druart et al, 2013). It is well known that SP linked to spermatozoa is necessary to achieve fertility and oocyte binding. The addition of SP to sperm following cryopreservation increased post-thawed motility and fertility in ram, enhanced post-thawed sperm function in boar and increased AI fertility in stallion (Kershaw-Young et al, 2012).

In South American Camelids, the highly viscous seminal plasma is one of the most important barriers to the development of artificial insemination technologies. It impedes the homogenous mixing of semen with the extender, thereby reducing the interaction between the sperm cell membrane and the cryoprotective compounds during cooling and freezing. To avoid this problem, several authors decided to develop their experiments with epididymal or *vas deferens*-collected spermatozoa. Pérez et al (2006) demonstrated that it is possible to obtain sperm cells from surgically diverted *vas deferens* in male alpacas and llamas, thus facilitating the evaluation of concentration, motility, abnormalities and subsequent cryopreservation.

The aim of this study was to evaluate the effect of SP on alpaca sperm cells after freezing-thawing-process.

Materials and Methods

Spermatozoa from three alpaca males were obtained by pipette aspiration of surgically diverted *vas deferens*. The samples collected were quickly diluted with a glycerolized Tris-base extender, evaluated and then frozen in straws. At thawing, each dose was divided into two post-thawing conditions: without addition of SP (control) or with 10% SP (with previous threat-forming reduction by means of mechanical method, Bergamo et al., 2015). After 20 min at 37°C, the samples were evaluated *in vitro*. The sperm cells parameters were: total motility percentage (MOT), sperm motility index (% of motility x movement quality, ISM) viability (eosin-nigrosin supravital stain, Eo-Ni), hypoosmotic swelling test (HOST) and sperm cell morphology (including normal cells, primary and secondary abnormalities).

The parameters were analyzed by ANOVA (main effects), with Fisher–LSD *post hoc* test, using the software StatSoft, Inc. (2007).

Results

No interactions between males, dates and replicates were found. The sperm cells parameters at thawing (including some of the abnormalities registered) are showed in Table 1. The presence of SP after freezing-thawing improved the ISM (p=0.017), compared to the absence of SP (78.5±9.0 vs. 40.0±3.2, respectively). No differences were found for Eo-Ni and HOST with respect to the addition of SP. Normal sperm cell morphology was higher (p=0.022) when SP was present at thawing, compared with no SP treatment (60.9±1.4% vs. 54.6±1.4%, respectively). It was observed a decrease of the presence of bent tailed spermatozoa in the SP group with respect to the control group (0.4±0.3% vs. 2.6±0.7%, respectively).

Table 1: Effect of seminal plasma added at post-thawing on sperm cells parameters.

Treatment	MOT*	ISM*	Eo-Ni	HOST	SPERM CELL MORPHOLOGY			
					Normal*	Detached head	Bent tail*	Coiled tail
Without SP	18.3±2.1	40.0±3.2	61.4±2.5	41,3±1.9	54.6±1.4	1.7±0.6	2.9±1.0	1.2±0.6
With SP	25.8±2.42	78.5±9.0	63.4±1.4	47.8±2.5	60.9±1.4	2.6±0.7	0.4±0.3	1.3±0.6
p value	0.035	0.017	0.51	0.12	0.022	0.32	0.04	0.91

SP: seminal plasma; MOT: total motility; ISM: sperm motility index; Eo-Ni: supravital stain; HOST: plasma membrane functionality. Mean ± Standard Errors (SE). *: indicates significant differences.

Discussion

In this work, MOT and sperm morphology related to the tail status were improved when SP was added after thawing. In this case, the recovery of frozen spermatozoa was better, indicating a direct effect on its survival. It was shown that SP improve some aspects of sperm cell physiology. Motility of ejaculated or epididymal spermatozoa obtained from male alpacas were improved when SP (especially at 10%) was added during incubation al 37°C (Kershaw-Young et al, 2011). Others authors confirmed that the presence of viscous mucus in the utero-tubal junction in llamas was involved in the formation of the sperm reservoir (Apichela et al., 2014).

In the same way, it could be observed a tendency to a higher preservation of plasma membrane functionality, showed by HOST, indicating a probable interaction between SP proteins and the surface of the sperm cell. Regarding this finding, it could be connected with the high ISM achieved.

In conclusion, the presence of SP at post-thawing improves ISM and sperm morphology, indicating that this condition would be beneficial to improve fertility ability of the alpaca spermatozoa.

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Pregnancy and pregnancy loss in camelids

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Pregnancy loss is the second most common complaint in camelid theriogenology. Epidemiological data on the incidence of pregnancy loss in camelids is scarce. In our practice, 7 to 12% of alpaca pregnancies are lost due to reabsorption, abortion, or stillbirth. A study in New Zealand reported 25.7% pregnancy loss rate after 30 days of gestation and 9.6 to 16.7% losses occurring after day 120 of gestation. In autumn-bred females, 17.3% of pregnancy losses occurred before 81 days of gestation, whereas spring-bred females had a loss rate of only 2.8% in the same gestational period. In camels, pregnancy loss is high following embryo transfer and can reach alarming levels particularly if nutrition is inadequate or during outbreaks of diseases (Anouassi and Tibary 2013, Tibary et al 2006, Tibary and Pearson 2015).

General approach to diagnosis of pregnancy loss

Pregnancy losses is generally categorized as early embryonic death (before 40 days); early fetal losses (between 40 and 120 days) or late fetal losses (120 days to term). It is important to precisely define cases when working-up a pregnancy loss problem. Important epidemiological data include number of animals affected (herd vs. individual problem), stage of pregnancy, method of pregnancy diagnosis, reason for suspecting pregnancy loss (behavioral vs. observed abortion), and presence of a common history before pregnancy loss (shipping, clinical signs, treatments etc.). Causes of pregnancy loss in camelids are numerous, which often makes diagnosis costly and frustrating for breeders. The best chance of arriving at a diagnosis requires submission of fresh or cooled fetus and placenta. Uterine culture and paired blood samples (acute and convalescent) from the aborting and control female(s) should also be submitted. The fetus should be weighed, its crown-rump length measured, and be examined for congenital abnormalities, skin lesions and appearance of the epidermal membrane. Samples for bacteriological evaluation (culture and PCR) should be taken from the placenta and fetal organs (stomach contents, liver, lung, brain), packaged in individual sterile bags and submitted

refrigerated. Samples for virological investigations (virus isolation, PCR) should include placenta, fetal blood, and fetal tissues (lung, liver, heart) packaged and submitted fresh or frozen at -80°C. Toxicological samples should include dam serum and fetal liver, kidney and ocular fluid. Fetal thoracic and peritoneal fluid should always be taken with a sterile syringe and refrigerated. The chorionic surface of the placenta (avillous areas) may provide information on possible areas of uterine fibrosis or inflammation. Placental insufficiency (presence of avillous areas or areas of mineralization) has been proposed as a possible cause of abortion in alpacas and llamas. Samples should be taken for histopathology from the middle of each uterine horn, body of the uterus, cervical star, and any apparent lesions. Histopathological samples should also be taken from the umbilical cord and amnion. The female(s) experiencing pregnancy loss at any stage of pregnancy should be isolated from the rest of the herd and examined thoroughly for any systemic diseases or nutritional problems.

Infectious causes of pregnancy loss

Although abortions are reported in cases of systemic viral disease such as coronavirus and camel pox, the most commonly reported cause of abortion is Bovine Viral Diarrhea Virus (BVDV). Other viruses that have been associated with abortion in camelids include Equine Herpes Virus-1 and Equine Viral Arteritis Virus (EVAV). However, the role of these viruses in abortion and clinical disease in these species is unknown. Epidemiologic studies have shown variable prevalence of seropositive camelids to BVDV types 1a, 1b, and 2, with 1b being the most common. Experimental inoculation of pregnant llamas demonstrated seroconversion of the dams but no other clinical effects. One experimentally infected llama aborted 5 months after infection but no BVDV was found in the fetus. Diagnosis of BVDV infection in an alpaca or llama is based on virus isolation from fetal blood, fetal tissues (lymph nodes) and placenta. Immunohistochemistry may be performed on formalin-fixed tissues. Polymerase chain reaction (PCR) on whole blood samples is commonly used for screening newborns for persistent infection.

Bacterial causes of abortion are the predominant infectious cause of pregnancy loss in camelids. Brucellosis (*B. abortus* and *B. meletenis*) is a common cause of abortion in camelids in some areas of the world. Organisms were disseminated through the fetal tissues and placenta (placentitis). *B. abortus* was also isolated from the dam's mammary and lymph tissues after euthanasia. *Chlamydophila spp*. have been identified as a cause of abortion and birth of weak crias in llamas. In camels, serological surveys have established the presence of the organism in

several camel populations. *Chlamydophila abortus* has been associated with infertility and ovarian hydrobursitis. Leptospirosis is suspected as a cause of abortion in camelids. Seroprevalence is high in many regions of the world but there is little evidence for the role of leptospiral organisms in abortion. Commercial vaccines have been used off-label in camelids with variable results. *Listeria monocytogenes* infection has been diagnosed in adult and neonatal llamas and alpacas. Only one case of listeria-induced abortion has been reported. Abortion due to *Campylobacter fetus fetus* was reported in alpacas in the UK. Examination of the placentae shows focal necrosis or generalized necrotic placentitis. Organisms were isolated from placental tissue, as well as fetal stomach contents. *Campylobacter spp*. have been isolated from barren camels but the role of this organism in abortion is not clear. To our knowledge there are no confirmed reports of abortions in camelids due to *C. burnetii*. However, several reports have shown high seroprevalence in camels in many areas of the world. There is an increasing concern about the role of the camel as a reservoir and contamination of humans. Several studies have shown an association with seroprevalence in camels and risk of disease (Q-fever) in camel herder and owners. Other bacteria have been isolated from cases of placentitis. Ascending bacterial placentitis has been observed in females with recurrent vaginal prolapse during late pregnancy. Hematogenous placentitis is suspected in cases of fetal loss in which the dam was diagnosed with severe dental disease, gastric ulcers, or metabolic disorders. Fungal placentitis (*Encephalitozoon cuniculi*) has been diagnosed in a primiparous alpaca which aborted at 290 days of gestation.

Natural infection with *Neospora caninum* has been reported in camelids in several countries. *N. caninum* abortions, mostly mid-gestation, have been confirmed in some alpaca herds. Abortion due to *Toxoplasma gondii* has been long suspected. High seroprevalence was demonstrated in all species of camelids throughout the world, though parasitic cysts in tissues have not been found and the effects on pregnancy remain uncharacterized. Experimental infection has been shown to result in abortion in llamas. A case of near-full term abortion due to *T. gondii* was recently described in the USA. Fetal lesions include meningoencephalitis, hydrocephalus and nephritis. The role of sarcocystosis in pregnancy loss and abortion in camelids has not been fully elucidated. *Tritrichomonas foetus* has been isolated from camels with endometritis but there is no evidence of its involvement in camelid abortion.

Non-infectious causes of pregnancy loss

These are often fetal or placental abnormalities (twinning, umbilical cord torsion, severe deformities, chromosomal abnormalities and placental insufficiency), luteal insufficiency (hypoluteoidism), environmental stressors (severe disease process, long stressful trip, heat stress) or iatrogenic causes (administration of $PGF_{2\alpha}$, corticosteroids, 8-way vaccines). On a herd basis, severe losses may be seen with nutritional deficiencies (selenium, vitamin A, iodine deficiency) or toxicosis (copper, iodine). Lactating and very young maiden females may register an increased incidence of pregnancy loss. The administration of corticosteroids in mid to late gestation will result in pregnancy loss. Pregnancy loss due to administration of topical steroid-containing ophthalmic solutions has been observed in 4 pregnant females (5 to 8 months) in our clinic. The use of multivalent clostridium vaccines in pregnant female alpacas and llamas has also been associated with early gestation pregnancy loss. Luteal insufficiency is hypothesized to occur in alpacas and llamas with recurrent pregnancy loss between 2 and 8 months of pregnancy. Most of cases seen in our clinic are obese females. Treatment for maintenance of pregnancy in cases of suspected hypoluteoidism consists of administration of hydroxyprogesterone caproate (250 mg, IM every 3 weeks). Treatment is stopped at 300 days of gestation to allow normal parturition. Long term progestogen supplementation is associated to a high risk of failure of cervical dilation. Pregnancy loss may occur as a result of poor placentation (placental insufficiency). The authors have seen recurrent pregnancy loss in maiden females with a poorly developed uterus, in cases of uterus unicornis, and in multiparous females with degenerative endometrosis or cervical pathology.

The most common embryonic or fetal factor which can result in pregnancy loss is twinning. Abortion may be due to severe fetal malformations (hydrocephalus, conjoined twins, cyclopia). Umbilical cord torsion is a rare cause of abortion in the late term pregnant female. Early pregnancy losses may be due to chromosomal abnormalities in the dam or the embryo has been suspected but remain poorly studied.

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Management of pregnancy and the neonatal period in dromedaries

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Introduction

Profitability of a commercial camel dairy depends on many factors including pre- and postnatal losses, such as pregnant camel loss, abortion, stillbirth, neonatal and calf mortality. In dromedaries a lot of research and clinical work has been directed towards the assisted reproductive technologies, however reliable data on optimum management and problems during pregnancy, parturition and the neonatal period, clinical symptoms and post-mortem finding of camel calf diseases are scarce (Tibary et al., 2005). Due to the strong emotional bound between mother and calf, it is difficult to milk a camel without the presence of her offspring, that's why it's essential to keep calf morbidity and mortality rate at a low level in the herd. On traditional local farms, even with better veterinary care, calf mortality can reach up to 35-40 % (Agab, H., personal communication). The main reasons of calf mortality are diarrhoea, septicaemia, Clostridium perfringens enterotoxaemia, sudden death due to white muscle disease and obstipation caused by meconium fecolith. Emirates Industry for Camel Milk and Products is a large scale, intensive, camel milking farm where an efficient breeding and reproductive programme has been developed to ensure a continuous supply of milk throughout the year.

The aim of the study was to review the data related to pregnancy losses, parturition, neonatal morbidity and mortality during the last ten breeding seasons.

Materials and Methods

In our herd management program the breeding season lasts from September until June. Pregnant camels are kept in spacious, partially shaded paddocks in a well isolated part of the farm. The feed ration and the necessary mineral and vitamin supplements are calculated according to the stage of gestation and body condition, which is monitored regularly. Two to three weeks before the expected delivery, camels are moved to maternity paddocks to be under 24 hour supervision and veterinary assistance. Birth weight and standing of the calf are recorded and the placenta is thoroughly checked after its pass. The new-borns are closely

monitored for colostrum intake and when it is needed they are bottle fed with colostrum every 2-2.5 hours during the first 24 hours. In the maternity paddocks, calves suckle their mothers ad libitum, no milk replacement is used. One month after parturition camels and calves are transferred to the production area and machine milking begins in the milking parlour. Twice a day a veterinarian team monitors the health status of the camels, any sickness, injury or abnormal condition is treated immediately. There is a well-equipped veterinary clinic on the farm which can provide intensive care for the camels if it is needed. Precise records are kept about all animal husbandry and veterinarian activity. In case of abortion, the camel is separated immediately and the fetus, placenta and blood from the dam are sent for further laboratory tests. All dead animals are sent for post-mortem examination.

Results and Discussion

Of a total of 3220 deliveries monitored during 10 breeding seasons, 3134 (97.3%) calves were born alive and 87 (2.7%) were born dead; only a single set of twins was delivered. Gender wise, 1538 (47.7%) were female and 1683 (52.2%) were male. The mean gestation period was 384 (±12.7) days and the average birth weight was 34.6 (±5.8) kg, but with a strong seasonal fluctuations within each year. There were 188 abortions (5.4% of pregnant camels), amongst which 25 (13.3%) were twins. Abortions usually occurred around 260 days of pregnancy. Despite post-mortem and serological examinations, the cause of abortion, except for twins, could not be determined in most cases. To date, neither *Brucella abortus* nor *B.melitensis* have been isolated from any abortus.

Dystocia occurred in 49 (1.5%) of all deliveries. The most frequent problems were posterior presentation with uni- or bilateral hock or hip flexion, ventral or lateral neck flexion, carpus and shoulder flexion, or transverse presentation; uterine torsion has not occurred to date. Most of the dystocia cases were corrected by transvaginal manipulation. However, this needed to be performed promptly to save the calf's life and despite rapid veterinarian assistance, only 25% of the dystocia cases resulted in a live birth. Fetotomy proved to be an efficient way to resolve cases of dystocia and it gave an excellent prognosis for the dam. Only 5 caesarean sections were carried out over the 10 year period. Post-partum complications included uterine prolapse, retained placenta, post-delivery haemorrhage, and tears in the vaginal wall, the most frequent of which was retained placenta (2.3% of all deliveries).

There were a total of 342 calf mortality (≤ 1 year) in the last ten breeding seasons (13.4 %). Calves just like camels do not show too many clinical symptoms when they are sick. Within

one month after birth the most frequent clinical signs are tiredness with or without fever (13% - 51%), diarrhoea (2%-13%), constipation (1%-6%), and neurological signs (0%-45%). As in other species, the first month of the life is the most risky period in a calf's life. In each season there was a different primary cause of neonatal death, besides the commonly occurring septicaemia, clostridiosis and obstipation caused by meconium fecolith.

In 2009-2010, the main cause was selenium and E-vitamin deficiency manifesting in white muscle disease which mostly occurred in calves born from pregnant camels originated from Africa. Treatment of the calves didn't seem to have any effect, it didn't prevent the typical sudden death. The solution was supplementing the pregnant camels with adequate amount of selenium and E-vitamin. The next breeding season there was hardly any white muscle disease.

In 2010-2011, a new disease, a typical neurological disorder occurred in the calves younger than 4 weeks of age. The pathological findings were brain oedema and cerebro-cortical necrosis caused by malnutrition of the pregnant camels before deliveries. In the affected calves multiple clinical signs and pathological findings were detected. Feeding management of the pregnant camels was corrected and since then no more such typical neurological signs occurred, although there were very few brain oedema cases with no inflammation diagnosed by post-mortem examinations. In 2011-2012, a new feeding system, a TMR machine was introduced to distribute the feed for the camels. The TMR chops up the feed ingredients and mixes them up before distributing it to the camels. Shortly after its introduction some calves started to develop bloody faeces, they showed abdominal pain and became lethargic and many of them died. The post-mortem diagnosis was perforated gastric ulcer and peritonitis caused by the mechanical irritation of the chopped hay particles.

Septicaemia, mainly colisepticaemia may manifest in three different clinical forms, besides lethargy and depression. The most common is diarrhoea, but we had cases with arthritis and meningo-encephalitis as well, caused by E. coli. This disease can be maintained in low level by keeping strict hygienic measures during and after delivery, ensuring adequate colostrum and milk intake, monitoring udder health of the dam and intensive IV fluid and antibiotics therapy of the calf when the first symptoms occur. Controlling clostridial enterotoxaemia requires the same approach like septicaemia. Vaccination of the dams in their last trimester of pregnancy can help preventing the disease, but it is considered as off label use and side effects, like abortion might occur.

EICMP was the World first large scale camel dairy. Introducing this species to an intensive system resulted many predictable and non-predictable problems. Our herd health management

system has the efficiency and flexibility to detect newly occurring abnormalities and problems, to make the necessary changes quickly to compensate or prevent further occurrence and to eliminate those in the future. Proper management of pregnancy and parturition ensures a high live calf birth rate and appropriate correction of complications leaves no negative effects on the future reproductive performance of the dam.

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Fertilization failure or early embryo death in alpacas and llamas?

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Introduction

The early or late pregnancy loss occurs in high levels in many species of farm animal, and in the domestic South American camelids (alpaca and llama), occurs during early embryonic stages (Fernández-Baca *et al*, 1970). According to these authors, 80% of the ova recovered 3 days after mating, were in the process of dividing, and only 50% of them, surviving more than 30 days. Later in a study of the reproductive wastage in a group of 95 females alpacas, of different ages, parity and a group surgically removed the left horn, the overall failure recorded was 82, 3 per cent, after a single mating (Bravo *et al, 1987).* The major pregnancy losses occur from breeding to 35 days post-mating. Under natural breeding condition in the south highlands of Perú, those that uses hand breeding system, told me that 35-40% of their female herds need only one service, and the rests 2 to 3 matings, to get a 92% of pregnancy (Señor M. Diaz, personal communication).

The maternal recognition of pregnancy has been calculated at around day 7 to 8 after mating in the alpaca, and 8-9 days in llamas. Embryos coming from the left ovary reached the uterus on day 6 post-ovulation and probably sometime later those coming from the right ovary. By day 9 the embryo started to elongate and by day 10, the embryo occupy the entire uterine horn. Our working hypotesis, is that females that return to be receptive, at 12 days after mating are those that fails to fertilize, showing a very short luteal phase.

Materials and Methods

Thirteen 3-7 years-old, multiparous alpacas (58.5 k), and thirteen 3-8 multiparous llamas (92 k) all with the cria at foot, were selected from alpaca and llama female herds from the Research Station. The post-partum was in all females from 17 – 20 days (recommended 15 days post-partum before mating). All the females were receptive and mated with fertile males. (Day 1 = day of mating)). Vasectomized males were used for sexual receptivity detection on days 12, 24, 40, 60, and 90 after mating. Females having a CL will reject the male and receptive females were found to be open or non-pregnant (Alarcón *et al,* 1990).

Laparoscopy

Laparoscopy was performed at day 3 after mating to document the ovulation, at 8 days to confirm the corpus luteum size and formation (Sumar *et al*, 1984; Sumar and Bravo, 1991). Blood sampling were collected on days 1, 5, daily from 8 to 20, and 25 to 30 after mating, and then at 7 days interval between 31 and 90 days for pregnancy measurement of P_4 for the period of early embryo and fetal death.

Hormone analysis

P_4 concentration was determined by an enhanced luminescence immune assay technique (Amerlite Kodak Clinical Diagnostic Ltd., England). Values are presented in nmol/L. The main plasma metabolite of $PGF_{2\alpha}$ was analyzed by RIA according to Granström and Kindahl (1982). The Oestrone sulphate (E_1SO_4) was analysed by RIA (Kindahl *et al*, 1982).

Results

From the thirteen alpacas, 6 females became pregnant until parturition (46.15%); 3 alpaca females have a very short luteal phase; the plasma progesterone concentrations started to increase at day 5 after mating, and reached maximum concentrations of 12 nmo/L at day 8 in alpacas. The figure was different in the llamas; from thirteen llamas, 6 became pregnant (46.15%), and seven cases (53, 84%) showed basal levels of P_4 by day 10 to 12 after fertile matings, with a very short luteal phase. The luteolysis started at day 9 in alpacas and day 10 in llamas, reaching basal levels at 10 days in both species. The time span of the luteal phase arbitrarily defined as the time period with progesterone concentrations above 1 nmol/L was approximately 3 days in alpacas and 4-5 days in llamas. The very early embryo loss in llamas was higher than in alpacas, under the present research conditions. In alpacas and llamas mated with fertile males, the plasma progesterone concentration on Day 8, post-service, showed a tendency to be higher in pregnant that in non-pregnant females ($P< 0.5$) (Sumar et al., 1993). One alpaca loss the embryo by day 30, and three more at day 50, 64 and 85. In these four females that loss the embryo, the peaks of PGFM started about 15 to 25 days earlier.

Base line of the prostaglandins metabolite of around 360 pmol/L were recorded from alpacas that showed short luteal phases, and double by day 10, and decreasing to base line at Day 13. In llamas at Day 1 showed around 733, decreasing in Days 5, 8, 9 and 10, to increase to 805 on Day 11, to basal levels of 467 pmol/L at Day 13. No fluctuations in E_1SO_4, was observed in alpacas and llamas with very short luteal phases.

Discussion

Table 1 shows the P_4 concentrations of the female alpacas and llamas mated with fertile males, results of the present experiment, and infertile or vasectomized males from a study done some years before (Sumar et al., 1988). Both domestic species, showed very short luteal phases. As we can see, the day to day levels and the concentration of the P_4 in both cases are very similar. The laparoscopic observation is excellent tool for direct follicular and corpus luteum observation. The third day after mating we observe by laparoscopy, the recent formatted corpus luteum, with a very dark red color, of the same size of the previous follicle, and at 8 days, a very well and round corpus luteum of approximately 10-11 mm, pale pink color. As the ultrasound equipment is now a day intensively used to determine ovulation and corpus luteum formation, it is not possible to see the color of the follicle and of the corpus luteum, very important clues to determine the state of the ovarian structures. Since the results of the infertile mattings are very similar to the results in females mated with entire males, resulting also in both cases in a very short luteal phase, we came to the conclusion on the probability that only the same factor is involved in the so called "very embryo loss": failure of fertilization. We believe that the ageing of the oocyte is the main cause of faulty fertilization. We know that fertilization occurs at about day 6 days after ovulation in the ampulla of the oviduct, and the causes of failure may be due to: improper day of mating or insemination, delayed ovulation (> 30 hours, a factor that is not mentioned in the literature) (Sumar et al, 1987), ageing of the sperm and genetically abnormal oocyte. We cannot rule out other factors like, deficiencies in nutrition, hormonal imbalance, heath stress, etc. Failure of fertilization itself is very difficult to distinguish from early death of the zygote, and the distinction is best drawn by microscopic examination of the egg, and this can be done only in controlled experimental studies, slaughtering 3 to 4 days after mating, and the ova flushed from the oviduct, and classified as either fertilized or non-fertilized. In one study of fertilization after Artificial Insemination in alpacas, fertilization did not occurs until 15 hours after mating or LH injection, and most of the fertilized egg were un the range of 27 – 35 hours after the induction (Calderon et al. 1970). Females showing receptivity after day 12 after mating, is the first indication of a presumptive failure of fertilization, due to the regression of the corpus luteum. In cows classified as repeat breeders, observations based on surgical or non-surgical recovery of eggs, for embryo transfer, suggest that failure of fertilization may be the single most important cause of these repeat-breeder conditions (Hunter, 1994). Knowing that ovulation occurs 30 hr after induction, and the sperm arrived to the oviduct at 30 minutes after mating, how long is the lifespan of the sperms? This and another questions need to be studied.

Figure 1: P_4 levels in alpacas and llamas mated with entire and infertile males, that ovulated showing very short luteal phase.

Day of mating	ALPACAS		LLAMAS	
	Fertile matings +	➢ Sterile matings	Fertile mantings +	➢ Sterile matings
1	0.38	0.20	0.45	0.45
5	1.46	1.20	1.38	1.40
8	12.03	10.50	10.90	12.50
9	3.20	4.00	14.10	16.50
10	0.76	0.80	6.90	3.00
11		0.60	2.90	1.90
12		0.30	0.28	0.30

+ Sumar et al; 2016

➢ Sumar et al., 1988.

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Clinical incidence of various reproductive disorders in male alpacas (*V. pacos*): a retrospective study

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Introduction

Breeding soundness examination (BSE) guidelines have been set and are common practice in equine and ruminant species. Although reports of individual cases or case series on infertility are available in camelids, data on results of a standardized BSE are lacking. The largest study on male alpaca reproductive pathology was based on abattoir specimens (Sumar 1983). In North America, breeding management relies primarily on in-hand mating and males are often purchased at very high prices. Our group has been very active in educating clients on the importance of male BSE. We developed over the years a standard alpaca male BSE which was described extensively in other publications (Pearson et al. 2014). The present retrospective study reports on the clinical findings on male BSE, as well as examination of males presenting for specific complaints related to infertility or reproductive emergencies in alpacas.

Material and Methods

Medical records of male alpacas submitted for routine BSE (n=202), infertility (n=71) or reproductive emergencies (n=25) from 2000 to 2012 were examined. All males presenting for routine BSE or infertility were evaluated using a standard protocol as previously described (Pearson et al 2014, Tibary and Vaughan 2006). A physical examination was performed on all males followed by testicular palpation and ultrasonography and semen evaluation. Semen evaluation was performed on samples collected by post-mating aspiration in the majority of cases (100% for routine BSE and 59.2% for males presenting for infertility). Semen collection by electroejaculation was performed under general anesthesia only in males with infertility.
Males presenting to our theriogenology emergency service (n=25) were evaluated clinically and blood samples were submitted for complete blood count and serum biochemistry to determine prognosis for life and fertility.

Results

All males presenting for routine BSE included in this study were at least 3 years of age. All males were healthy and in good body condition at presentation. The most common physical reproductive abnormalities are reported in Table 1. Males with testicular hypoplasia and testicular degeneration had lower semen quality (<50% motility and >50% abnormalities). Semen quality in males with rete testis cysts was variable. Most males with unilateral rete testis cysts met the minimum sperm motility and normal morphology requirements. However, males with large bilateral rete testis cysts had poor sperm morphology and one was completely azoospermic. The percentage of males without physical abnormalities that showed teratozoospermia (>50% abnormal spermatozoa) was 6.43%.

Reproductive abnormalities found in males presenting for infertility are summarized in Table 1. Testicular degeneration and rete testis cysts were the most common abnormalities. Four (n=4) of 6 males presenting with a complaint of erection failure had various degrees of preputial adhesions. No precise diagnosis was reach in the other 2 males.

Semen quality was below the set standards in 60.6% (n=43) of the males examined (teratozoospermia, n=34; poor motility, n=6; azoospermia, n=3). With the exception of 2 cases (one with asthenozoospermia and one with severe teratozoospermia), poor semen quality was associated with a testicular pathology.

Table 1: Abnormalities of the reproductive systems in male alpacas submitted for routine breeding soundness examination (BSE, N=202) or infertility (N=71).

Condition	**Routine BSE**		**Infertile males**	
	N	%	N	%
Rete testis, bilateral	34	16.8	13	18.3
Rete testis, unilateral	20	9.9	7	9.9
Testicular hypoplasia	17	8.4	1	1.4
Testicular degeneration	9	4.5	23	32.4
Persistent penile attachment	6	2.9		
Cryptorchidism	4	1.9	-	-
Orchitis	2	0.9	4	5.6
Epididymal cysts	1	0.5	3	4.2
Preputial adhesions	-	-	4	5.4
Failure of erection	-	-	2	2.8

Reproductive emergencies consisted primarily of severe scrotal swelling (48%), preputial swelling or trauma (28%), and preputial prolapse (20%). Scrotal/testicular swelling was due to orchitis (66.7%), testicular hemorrhage (16.6%), or seminomas (16.6%). All of these abnormalities were suspected on ultrasonography and confirmed by histopathology after biopsy or castration. Preputial swelling with urethral rupture was diagnosed in 5 cases and carried the poorest prognosis for life and fertility. Preputial prolapse was successfully correct surgically in 4 out of the 5 cases.

Discussion

The clinical findings for BSE and in males with infertility parallels finding reported on abattoir specimen (Sumar 1984). Poor testicular size, testicular degeneration and rete testis cysts are common causes of poor reproductive performance. The most intriguing aspect of testicular pathology found in this study is the high incidence of rete testis cysts. In an earlier report, rete testis cysts were found in 18.5% of animals presenting for castration; 40.6% of cases were bilateral. Cysts ranged in size from 4 to45 mm (mean ± SEM; 13.3 ± 1.3) in length and 2 to28 mm (6.5±0.8) in width. Post-castration, cysts were aspirated; 44.4% of cysts contained immature spermatozoa (Tibary et al 2011). All cystic testes had evidence of spermatogenesis; however, disruption was observed in testes with large cysts. The high incidence of testicular hypoplasia in alpacas is worth noting and may have some underlying genetic causes. Testicular degeneration in this study was diagnosed based on ultrasonographic evidence, histopathological characteristics and change in testicular size. Factors suspected to predispose to this abnormalitiy include heat stress, systemic infectious diseases and trance mineral deficiencies. Although semen evaluation was performed mainly on samples taken by post-coital aspiration, the information gained remains valuable for the field practitioner. Many clients decline electroejaculation because of general anesthesia. In our experience this risk is minimal (Pearson et al 2014). As expected the majority of males with poor sperm quality had a clinically identifiable testicular abnormality. However, a few males appeared to have abnormalities or infertility that was not associated with a clinical disorder. Molecular and cytogenetic evaluation is warranted in such cases.

The most common emergencies of the male reproductive system are scrotal/testicular swelling and preputial swelling or prolapse. With exception of rare cases due to systemic infection (Aubry et al 2000), the majority of orchitis cases seen in our clinic are due to local infections

due to trauma. Preputial swelling is often the result of complications of preputial prolapse and carries a poor prognosis due to azotemia particularly when the animal cannot urinate.

In conclusion, the present study stresses the importance of male BSE for the diagnosis of reproductive disorders. Guidelines for BSE of the male alpaca are proposed. The role of rete testis cysts in infertility remains unclear. However, the high incidence found in the present study suggests that testicular ultrasonography and testicular measurements should be performed on all males presented for reproductive evaluation. Males with testicular cysts should be monitored closely particularly if the condition is bilateral.

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Long term retrospective analysis (2004-2015) of reproductive and health management of South American Camelids breed in Italy

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During the ’90s, llamas and alpacas arrived in Italy. Nowadays there are numerous farms distributed from the North to the South of the country, although their current situation is not yet well documented. A monitoring protocol is required, in order to identify critical points influencing the maintenance of a good heath and reproductive conditions. In this work 10 Italian farms South American camelids were considered. The monitoring was based on updating the herd databases including anamnestic records, Body Condition Score, evaluation of the diet, standardized clinical diagnostic examination. All data collected over time was processed and assessed in order to identify the variability of classes of events related to the health and reproductive management. Vitamine D deficiency, parasitic and environmental dermatitis, gastrointestinal parasitosis, abortion and congenital diseases are the main health and reproductive concerns for the monitored farms.

Introduction

In Italy, the recent increase of the number has been observed and this represent a challenge for vets working with farm animals. Health and reproductive conditions of these animals needs knowledge related to neuro-endocrinology and metabolic peculiarities (Firshman AM et al., 2013) other than specie-specific characteristics of the immunological and reproductive systems (Vaughan JL, 2006; Youngquist, R.S., 2007). Management of South American camelid groups is not different from other farm animals in terms of productive performances and needs to develop indices of evaluation of successful rate of health persistence and reproductive maintenance. Recording data systems, Body Condition Scoring (Fowler, 2010), ultrasound examination (Gazitúa, FJ et al, 2001) had been used as diagnostic tools. Aim of this work was to integrate health and reproductive data collected during scheduled on-farm

clinical visits in ten Italian South American herds during the period running from the 2004 to the 2015.

Materials and Method

Animal and data collected

Ten South American Camelid herds were considered for a retrospective study related to health and reproductive parameters collected during scheduled on-farm visits. The data collection included individual identification (species, sex, age, productive phase), anamnestic information on therapy (parasitosis, dermatitis, rickets and other) (Lusat J et al., 2009; Fonster 2007; Parker JE et al 2002; Scott DW et al, 2010), pathological and productive events were recorded. BCS values, physical examination and diagnostic procedures (Raggi et al., Cebra C et al, 2014; Dawson DR et al, 2011) were carried out starting from the 2008.

Analysis of data

Data collected were reported in one single Excel datasheet and integrative classes were calculated for each parameter considered. The total number of animal was classified for each event time depending on: species, age (<30 days, ≥30-≤179 days, ≤6 months-≤1year, ≥1years-<2years, ≥2years-<5years, ≥5years-<10years; ≥10), physiological (Female - F, Male - M, Young -Y, Adult –A) or productive phase (Pregnant–P, Barren–B or Lactating–L). Annual frequencies of health parameters reproductive events as breeding time, abortion gestational age, calving interval, parturition and birth were calculated. Significant correlation indices identification (BCS and Physiological phase) and regression analysis (diagnostic events: *i.e.* US measurement of biparietal distance–BD, Progesteronemia–P4) were tested using the statistical software SigmaStat 2.03.

Results and Discussion

The total number of animals visited was increased during the observational period as reported in Table 1. Among the farms considered, 40% (4/10) are mixed camelid breeders with separated groups of lama and alpaca and the other only alpaca breeders (60%).

Average age values and standard deviation of the population observed was increased during the observational period (2.81±2,36 years in to 2004 up to 5.04±3.56 years in to 2015). It is important indicate the negative correlations among frequencies of health specific and general treatments (decrement of -40%) with diagnostic events (increment +20%-from 2008 35%) during the period of observation.

Within the Health treatments were evident decrements of general (-20%) or single apparatus drugs use. BCS values (N1290 with 3.01±0.48 as score mean value) were significantly lower in lactating female (-1,52 of SD from the mean value) (Figure. 1).

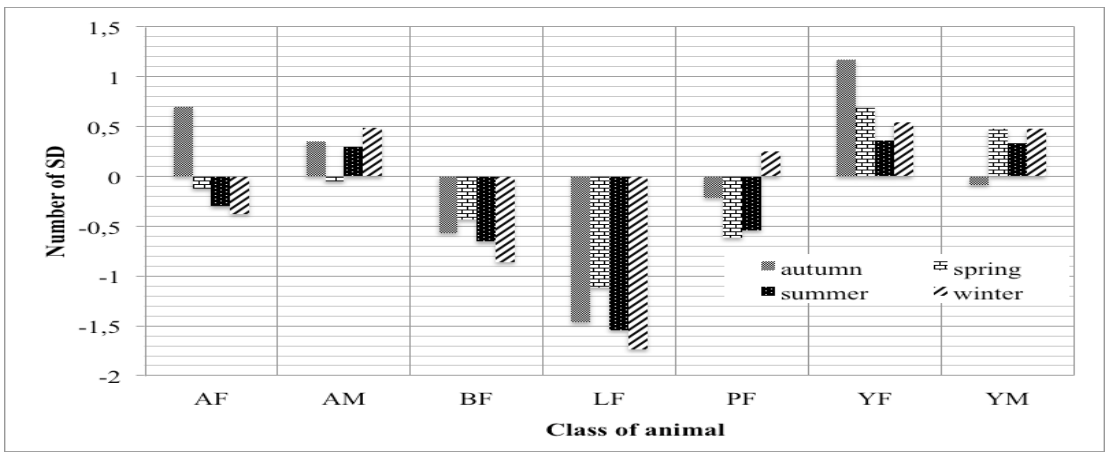
Vitamine D deficiency (282/3881), dermatitis and gastrointestinal parasitosis (1762/3881) and congenital diseases (N 9: 3 cardiac malformations, 3 cystic testis, 1 atresia ani, 1 atresia choane, 1 persistent urachus) are the main concerns.

Table 1: Number of South American Camelids monitored.

	Alpaca Female	Alpaca Male	Lama Female	Lama Male	Total number
2005	29	28	0	0	57
2006	37	31	0	0	68
2007	39	35	0	0	74
2008	36	39	0	0	75
2009	40	45	0	0	85
2010	37	39	0	0	76
2011	37	39	0	0	76
2012	55	45	0	0	100
2013	94	55	7	7	163
2014	121	58	19	12	210
2015	91	26	11	10	138
Total	616	440	37	29	1122

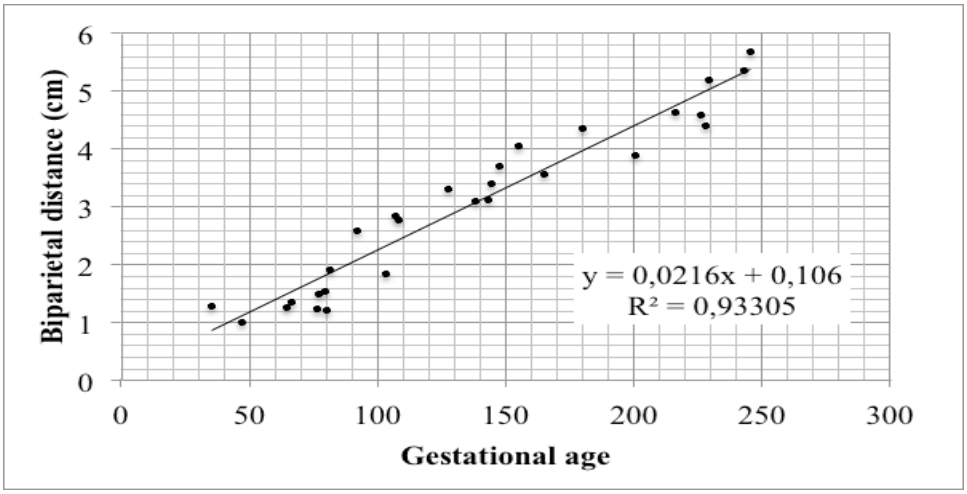
Within the reproductive event class, calving interval increment seems to be linked to the breeding selection of the females justified by the increased aging of the adult female population (75%) ranging from 3.78±1.71 years up to 9.88±2.42 years of age, barren females are represented by older animals (8.01±2.99 years) and pregnant females are represented by ≈6 year old (6,38±2.99 years) animals over the period of observation.

Figure 1: Distance (number of Standard Deviation) from the BCS average value in classified animals.



There are not significant difference among season and class of age in terms of P4 levels (N 123) and vary significantly among trimesters of pregnancy with 5.96±2.77, 5.67±2.82 and 3.89±0.90 ng/ml for the 1st (N28), 2nd (N 14) and 3rd (N 9) trimester respectively. US estimation of the gestational age (N63) (Fig. 2) was performed with a precision of 0.17 ± 16.97 (N 33) days from referred breeding date. Estimated gestational age of the abortion (N 56) reports 199.6 ± 46.34 days as mean value with a variability ranging from 77 days up to 229 days of pregnancy.

Figure 2: Estimation of gestational age using the biparietal distance measurement.



Conclusion

Integration of clinical and diagnostic parameters could increase the health and reproduction management accuracy decreasing the number of treatments and helping to identify risk thresholds. US, P4 as well as BCS are very useful tools to evaluate critical conditions.

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Alpaca and Llama breeding in Europe - dreams and reality

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During the last 23 years the interest in keeping and breeding llamas and alpacas is steadily growing in Europe. In 1993 most owners were looking for classic llamas or, because they were still quite rare in Europe at that time, for llama-guanaco crossbreeds. There was also some interest in alpacas which were acquired, like the llamas, mostly from zoos. In the following years, breeding activities and importations (mainly from South America, e.g. Chile), produced South American Camelids (SAC) with different fiber types: The new llama types are called "woolly llamas", and alpacas with the fiber type "huacaya" were also called "chile type". In recent times, some owners are breeding an additional fiber type in llamas and alpacas, called "suri type". Also the huacaya type has changed: The structure of huacaya fiber shows crimp as found in the wool of Merino sheep, while the suri fiber is straight. The reason for the rising

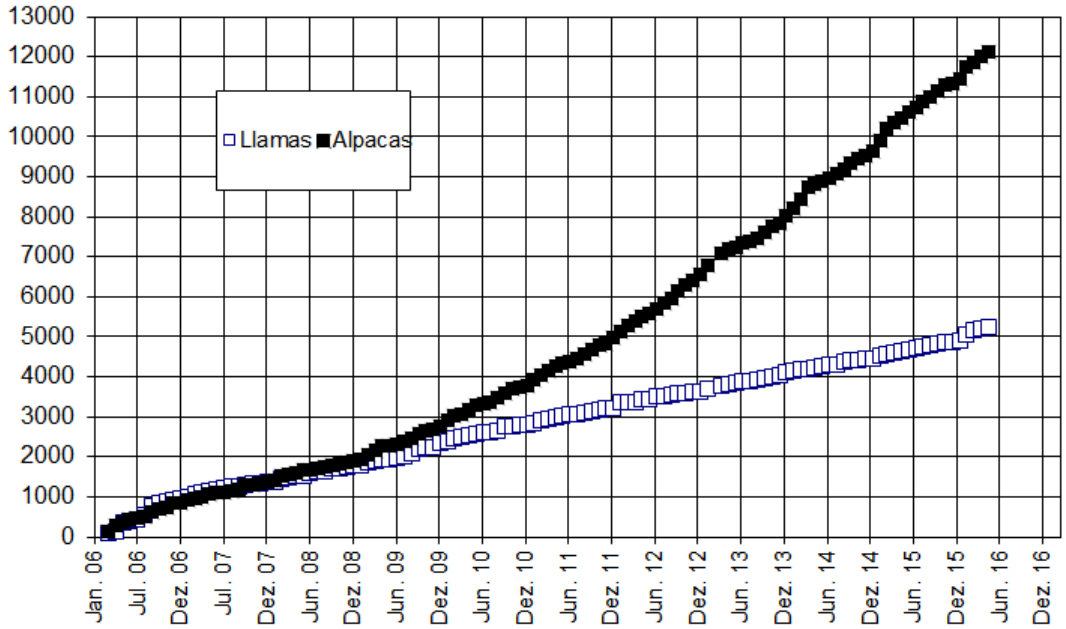

Figure 1: Registration activity as function of time for the European Llama and Alpaca Registry LAREU, separated into llamas and alpacas.

interest in these South American Camelids is for one their nice look but also the appealing character of these animals.

Another reason was also the improving marketing strategy by some animal dealers / breeders, promoting these animals as easy to handle and with good prospects for profit by selling wool and offspring at high prices. The promoters of alpacas worked harder than the promoters of llamas: In 2008 the European Registry LAREU indicates for the first time more alpacas being registered than llamas (see fig. 1). In the first quarter of 2016 the statistics of LAREU show over 17000 animals (12000 alpacas, 5000 llamas) and 2200 breeders. Generally, more female than male alpacas and llamas are registered with LAREU (see Table 1). Presumably not all geldings are being registered. The statistics also show that most of the registered breeders have small herds (5-8 animals).

	Total number	Percentage
Alpacas		
males	4853	40.0
females	6799	56.0
gelding	481	4.0
Llamas		
males	1964	37.4
females	2842	54.2
geldings	439	8.4

Table 1: Distribution of sexes for alpacas and llamas, registered with LAREU, as of April 2016.

Most of the camelid breeders are beginners and do not have any experience with neither keeping agricultural animals nor with the selection of breeding animals. Since alpacas are advertised as fiber animals, the ranking in shows is mainly based on the fiber quality: As a consequence, fiber and fiber color are considered as the most important selection criteria for the breeding program. Many of these “fiber-monsters” are imported to Europe, and the alpaca owners in Europe therefore do not have any certified information about the genetic qualities or potential problems of the animals they are using for breeding. The results of breeding for better fiber are obvious, but the neglect of correct conformation and physiology has consequences, too. Most small breeders are using the service of herd sires from other owners, and in most

cases they have no possibility to see and inspect all the offspring of these sires to get some information about the genetic quality of the male in question.

Some frequently encountered problems

Alpacas and llamas are growing in a rather slow pace, they need 4-5 years to become adults. For that reason some of the problems concerning incorrect conformation, malformation or physiology become visible only quite late. In addition, most owners start breeding their animals as early as possible, usually with 1.5 year old females and 2 year old males. At that age the "parents" still have their milk teeth.

The result of "breeding without information on the genetics" can be observed as incorrect conformation in the region of the legs, mainly described as "x-legs", luxation of the patella, down in pastern, and polydactyly, just to name a few examples. In the region of the spine, problems may result from malformation of the vertebra corpus or the articulation of the vertebra, mostly seen as "kinky tails". But also the head may be affected: Incorrect position of the incisors makes the intake of food difficult, since the SACs transport the food into their mouth with the lips. Malformation of the ear or of the auditory canal can be observed, or deafness, especially for some white animals with blue eyes. The latter will inevitably result in increased stress for these flight animals. Other problems are wry face, insufficient milk production for the offspring, or skin injury after irritating mite allergy. Nearly all SACs have mites, but some of these animals never show allergic reactions.

Classical treatment of the problems

Up to now many of these problems are ignored, or "transferred" to a new ignorant owner by selling him the animal. Some owners try to treat incorrect conformation with vitamin D injections, sometimes in toxic doses. An incorrect position of the incisors is generally corrected by cutting the teeth regularly. Incisors of adult alpacas are very long and during the animal's lifetime the incisors push step by step into the direction of the dental pad. In contrast to general believe they do not "grow" permanently. When cut too frequently, the animals will have too short incisors at older age, which they might even lose after some time.

Another problem is mite allergies. These are treated regularly by medication, but the result does not persist over extended times. As a final example, female alpacas or llamas should be mentioned with insufficient milk production for their own offspring. In such cases the cria will receive the additional milk by the owner using a baby bottle. Wrong imprinting and an entailing berserk syndrome will be the inevitable result. When several crias on a farm need to be bottle-

fed at the same time, special constructions like those to feed lambs are even proposed. Quite rarely, unfortunately, the owners will take such animals out of the breeding program. Some of the “big breeders” use a simple correction of problematic breeding results: animals with faults are being slaughtered to become dog-food. But this solution is, of course, unethical and against the law of animal welfare.

Proposals for future treatments and further developments

The breeders should receive information and education about which animal should be kept in the breeding program and which not. This recommendation is, of course, difficult to accept for owners of small herds. Some breeders will follow the recommendation, but some will ignore the reasoning or listen to other “experts”.

Breeding animals and their offspring should be controlled repeatedly by a qualified authority, e.g. a camelid association. This makes sense only, however, when all offspring are screened. This technique is not very successful in Europe because of the different competing camelid associations and the distribution of the offspring into different associations and countries.

True progress could be made by working on DNA techniques in order to identify the genetic capacity of alpacas and llamas, not only for fiber quality but also for avoiding problems in conformation and physiology.

Maybe one day it will become reality for the breeder to have access to meaningful genetic tests, giving him clear recommendation for a successful breeding program without propagating genetic defects.

Population restoration of the critically endangered Wild Camel in Mongolia

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The Wild Camel Protection Foundation (WCPF – UK charity 1068706) was established in 1997 with the sole aim of protecting the critically endangered wild camel *Camelus ferus* and its habitat from destruction and extinction.

By 2002 WCPF had achieved its first goal of gaining protected status for the wild camel in Mongolia and in China with the establishment of the Lop Nur Wild Camel National Nature Reserve. The estimated numbers of wild camels in 2004 were 600 in China and 350 in Mongolia (IUCN, Hare 2004). Even with protection, pressure on these numbers was downwards. And all attempts by various organisations to estimate the population of wild camel numbers then or since have been unsuccessful. Options including embryo transfer were considered by WCPF for increasing the numbers of the wild camel population. However with generous funding in 2004 the Hunter Hall Wild Camel Breeding Centre in the buffer zone of the Great Gobi Special Protected Area 'A'(GGSPA'A') Zakhyn Us was established with the full support of both the Mongolian Ministry of Nature and Environment (MNE) and the Academy of Sciences and Adiya Yasmusden, a zoologist and wild camel expert, Chairman of the Mongolian Wild Camel NGO. As the wild camel is an IUCN red listed species, WCPF notified them in advance of the planned releases of these wild camels into translocations sites within the GGSPA'A'.

From its very beginning the breeding centre has been successful in breeding wild camels. Managed by the Director of the GGSPA'A' and local herdsmen, under the overall joint management of WCPF and the MNE the aim was to establish a bank of pure wild camels for the eventual release, of some of these captive wild camels into the wild for population reinforcement. A stud book with a record of all the captive wild camels including annual births has been kept since the beginning and is monitored strictly to ensure purity of the breeding centre animals. The centre started with 12 individuals and had grown to 27 in 2015. More bull calves being born than females calves. As the young bulls grew this led to problems caused by aggression amongst the bulls during the annual winter rut. Because of protected status of wild

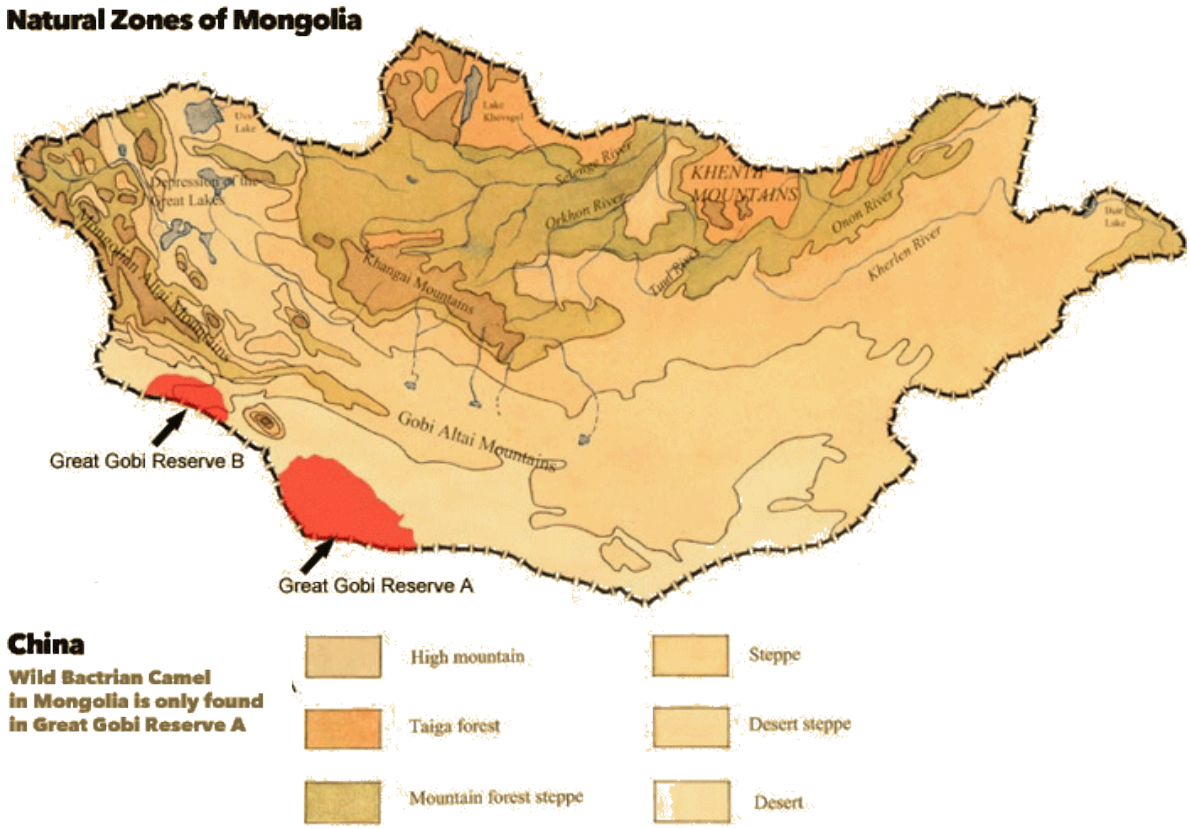
camels in Mongolia, these bulls can be neither castrated nor used in zoological exhibitions. So to reduce competition at the breeding centre, both for the protection of the wild camels there and the staff, the decision was made by the MNE, WCPF and the GGSPA'A' Director to identify a number of young bulls to release. The result was the first of two successful releases, with two bulls in 2013 and a further six bulls in 2015.

The first release in 2013 of two mature adult bull wild camels was to a translocation site, Bogts Tsagaan, a freshwater spring in the Great Gobi Specially Protected Area "A" (GGSPA "A"). Both were fitted with satellite collars for remote sensing given by Professor Liu Shaochuang from the Chinese Academy of Sciences. The collars allowed the movement of the two bulls to be monitored from the release point until the end of their journey within the protected area, which was eventually and surprisingly was back to the breeding centre.

The second release in 2015 of six bull wild camels, into the GGSPA "A", was also a success. Using two translocation sites at springs in the special protected area the six of the captive bred male wild camels aged between three years old and six years old were released. Four camels were fitted with satellite tags to monitor post release movement. To date none of these wild bulls have returned to the breeding centre.

These successful translocations of captive bred wild camels were done only after in-depth discussions with MNE and translocation expert Dr Mark Stanley Price and following the IUCN 2013 Guidelines for Reintroductions and other Conservation Translocations . WCPF considers this strategy has worked albeit, with very limited funding and there is sufficient information now to enable WCPF and Mongolian scientists to develop this strategic approach further, so all future translocation sites will be identified after a comprehensive field study of potential sites within the GGSPA'A' and a full risk evaluation. WCPF is applying for funding to undertake a five year scientific research study into the behaviour of wild camels once released into a translocation site within the GGSPA'A', data collection on available food and water resources in the GGSPA "A", possible predators, impact on other species and the health of its desert habitat. All this is critical to ensure the future of the wild camel.

The first calf born at Zakhyn Us 2016.



Genetic background of reproductive problems in camelids and other livestock: a mini-review

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Introduction

All camelids (Camelini and Lamini) are seasonal breeders, the time and length of their sexual activity depending on local environmental factors such as temperature, rainfall and nutrition. The breeding seasons in Old World camelids have been reported for dromedaries (*Camelus dromedarius*) to take place between November to April in most of the Arabian countries, or even shorter between December and March in Pakistan, and March to August in Sudan; whereas for the two-humped camels (*Camelus bactrianus*, *Camelus ferus*) in China and Mongolia breeding occurs between January and April (rev. in Skidmore 2011, Al-Eknah 2000). There are conflicting reports on the breeding seasons of alpacas (*Vicugna pacos*) and llamas (*Lama glama*) accounting for different management systems, whether sexes are kept in mixed or separate herds, and ranging from November to April with a peak in breeding during January to April (rev. in Brown 2000).

Given the extraordinary importance of camelids for sustainable milk, meat and wool production in marginal eco-agricultural mountain and desert regions, there is a need to increase the reproduction success in these livestock species. This mini-review will give an overview about the reproduction physiology and related reproductive problems in Old and New World camelids. Due to a general lack of literature about genetic factors associated with reproductive problems in camelids, other livestock genome-wide association, gene expression and epigenetic studies will be presented here, which report genetic variants influencing fertility traits. In fact, this points out a large knowledge gap about potential genetic factors connected to infertility in camelids that need to be addressed in the future.

Reproduction physiology in camelids

The reproductive efficiency in Old world camels is considered to be low, because of a late onset of puberty, long gestation period, low overall calving rates and high early embryonic mortality. Female camels usually reach puberty between 2-4 years of age, but usually are not

bred until they reach full maturity between 4-5 years; this results in a first calf at the age of 5 years or later (Al-Eknah 2000; Skidmore 2011). The majority of female alpacas become sexually active between 12-14 months, while males reach full maturity with around 5 years. Although they start showing sexual interest in females at the age of 1 year, they are incapable of mating due to the fact that the penis is adherent to the prepuce until puberty is reached (Brown, 1994). However, it is common practice to use males for mating from 3 years onwards. The onset of puberty in all camelids is also affected by environmental conditions and by the nutrition status, which apparently requires about 60%-70% of the adult body weight to be attained to come into puberty (Al-Eknah 2000; Smith et al.1994).

Camelids display unique features in their reproduction system, which makes the application of advanced breeding techniques routinely used in other livestock difficult or impossible. Contrary to other livestock species, camelids are induced ovulators, which usually only ovulate in response to mating; otherwise follicles start regressing after an initial phase of growth and maturity (Skidmore et al. 1996). Ovulation can be induced by treatment with gonadotrophic hormones, as manual stimulation of the cervix does not result in the release of LH (luteinizing hormone) from the pituitary gland to cause ovulation. Intramuscular injection of seminal plasma will induce ovulation in Bactrian camels, alpacas and llamas, the latter also being reported to ovulate spontaneously at the height of breeding season in 5% of the females (Brown 2000). Outside of the breeding season the ovaries are inactive or show a limited number of small follicles with irregular follicular wave patterns (Skidmore et al. 1996).

While the testes of Camelini are descended in the scrotum at birth, in Lamini they descend usually by 6 months of age. Testes vary in length from 7-10 cm in dromedaries, 10-12 cm in Bactrian camels and 3-5 cm in llamas and alpacas; during the sexual active rutting season they usually swell and increase in size (Bravo et al. 2000).

Reproductive problems in camelids

Reproductive efficiency in camelids is generally considered to be low, with varying calving rates ranging between 40% in nomadic and 70% in more intensively managed herds. Uterine infections, ovulation failure and management errors have been suggested as part of the causes of infertility (rev. in Ali et al. 2010). Among the described reproductive disorders in female camelids are conditions (i) concerning the ovarial system: persistent corpus luteum, cysts, ovulation failure, ovarian inactivity, oophoritis, salpingitis, hydro-/pyosalpinx, tumors; (ii) affecting the uterus: endometritis and fibrosis, metritis, muco-/pyometra; (iii) early embryonic death and abortion; and (iv) prolonged gestation. For male camelids, described

disorders concern the testes, including rete testis cysts, testicular hypoplasia, degeneration, cryptorchidism, orchitis, persistent penile attachment and preputial adhesion, as well as poor semen motility or azoospermia (Ali et al. 2010, Köhler-Rollefson et al. 2001).

Multifactorial causes for reproductive disorders have been identified, including genetics, management (*e.g.*, oestrus detection, mating practice), nutrition, and disorders as described above. Thus, a holistic approach to reproductive problems is necessary.

Concerning a genetic basis of fertility traits, several methods to identify genetic variants underlying infertility have been applied in other livestock species, including quantitative trait loci (QTL) and candidate gene approaches, whole-genome association studies (GWAS), gene expression and epigenetic studies. A brief overview is given in the following paragraph.

Genetic background of fertility traits identified in other livestock

Fertility is a complex trait, and the overall reproductive efficiency of a herd depends on a multitude of factors including onset of oestrous, conception, maintenance of pregnancy and successful calving. Fertility traits are generally considered to be based on a large number of genes, each with small individual effects. Heritability estimates in cattle vary from low h2 ∼ 0.10 for pregnancy rate and ∼0.01 for nonreturn rate) to moderately high (h2 ∼ 0.40 or 0.60 for scrotal circumference) with different estimates obtained in different breeds and populations (rev. in Fortes et al. 2013 and Kahtkar et al. 2014). Likewise, different genomic regions are associated with reproductive traits in *Bos indicus* compared to *Bos taurus* cattle. QTL, GWAS and genomics (GS) selection studies detected candidate genes for female fertility mainly on bovine chromosomes 1, 5, 14 and 16, which are published in specific databases (*e.g.*, http://genomes.sapac.edu.au/bovineqtl). Strong signals and causative mutations for fertility have been identified throughout the genome (see Figure 1; Fortes et al. 2013), and using next generation sequencing a number of embryonic lethal mutations and haplotypes were found (Kahtkar et al. 2014). These markers can be used for screening cattle populations and enhance genomic selection and genomic-assisted breeding management.

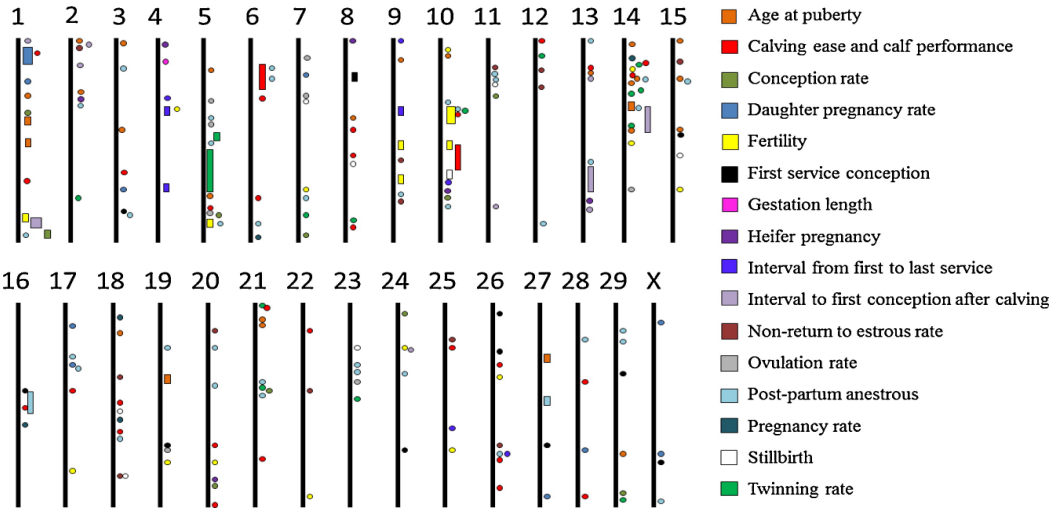

Figure 1: Schematic representation of quantitative trait loci (QTL) reported for female cattle fertility traits (Fig.1 re-print from Fortes et al. 2013; License agreement: 3873590298050).

Recent gene expression profiling studies have shown possible genes and physiological processes underlying phenotypic fertility traits. Micorarray-based high-throughput technology has great potential in improving dairy cow fertility (rev. in Beerda et al. 2008).

Epigenomic studies associated with fertility processes in livestock are stills in early stages. A correlation between the genome-wide DNA methylation status and gene expression profiles in pathways linked to early pregnancy events, *e.g.*, failure of embryo implantation, was found. Hence, epigenetic markers could serve as potential biomarkers for predicting future fertility performance of an individual and enhance herd management (Khatkar et al. 2014).

Conclusions

There is an urgent need to investigate genetic factors underlying reproductive problems and fertility traits in camelids. With the development of the first draft genomes of New and Old World camelids (Burger & Palmieri 2014, Fitak et al. 2016, Wu et al. 2014), there are now initial tools available to launch more comprehensive studies. The precise recording of pathological phenotypes and the establishing of control groups of large sample size pose the next challenge to the joint community of camel breeders and scientists.

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Bridging the gap between the genotype and the phenotype: the role of *omics* technologies

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Introduction

The conceptual dichotomic scheme *genotype-phenotype* originated at least a hundred years ago and still plays a central role in modern biology. The terms *genotype* and *phenotype* were coined by the Danish scientist Wilhelm Johannsen (1857-1927), and both evolved in meaning, following subsequent scientific acquisitions. Today, we are deep into the *omic* revolution, and dramatic changes in the way biologist thinks are occurring. Development and diffusion of high-throughput technologies in several scientific disciplines has allowed large amount of data to be generated affordably. As a consequence, the reductionist (hypothesis-driven) approach to research is being progressively replaced by an 'holistic' (discovery-driven) vision of science. This evolution also interested the traditional *genotype-phenotype* conceptual frame, which is now entering in the *genome-phenome* phase. Currently, any morphological, developmental, biochemical or physiological property all the way down to the subcellular level (including epigenetic marks) is acknowledged as a phenotypic characteristic and belongs to the individual's *phenome*, just like the constitution of all of its genetic material belongs to its *genome* (Gjuvsland et al., 2013). By moving from a more traditional idea of phenotype ("easily distinguishable external characteristic"; in Lenartowicz, 1975) to what is referred to as *molecular phenotype* (Zhang et al., 2015), the *genome-phenome* dichotomy get attenuated. Molecular phenotyping allows pathway delineation in complex biological systems (Zhang et al., 2015), and a pathway-centric perspective is emerging as fundamental to the understanding of the mechanisms of complex traits, such as quantitative traits (Kim and Przytycka, 2012). Uncovering genotype-phenotype association is indeed only the first step in the understanding process, as the identified associations do not provide the explanation of the molecular mechanism behind the relationship, and have generally failed to explain more than a small proportion of the heritable variation, hence suggesting that the genotype–phenotype map is

more complex than expected. In what follows, a general, not exhaustive overview of the current *omic* tool box is provided.

Genomics

Though being the earliest domain going *omics*, genomics is a young discipline (term coined in 1986). Its development is tributary of (i) development of *in vitro* DNA amplification techniques during 80s and (ii) diffusion, in the 90s, of automated sequencing technologies. Firstly focused on gene mapping and *de novo* genome sequencing, it later assumed a more vast connotation, including structural genomics, analysis of polymorphisms (*variome*), evolutive and comparative genomics, functional genomics. Next Generation Sequencing (NGS) technologies, currently implemented in a plethora of different commercial platforms, all characterized by massive parallelization and decreased cost of analysis (Metzker, 2010; Glenn, 2011), represented a major breakthrough. Among the impact and consequences of NGS were the identification of millions of Single Nucleotide Polymorphisms (SNPs) and the development of SNP-chips for rapid and affordable genotyping of large amount of samples. Among the SNP-chip applications are: identification of Copy Number Variations (CNVs); study of evolutionary relationship among populations, genetic originality of populations and admixture/crossbreeding practices; detection of selection signatures; Genome-Wide Association Studies (GWAS); Quantitative Trait Loci (QTL) mapping; parentage testing; traceability/trackability of biological material to the corresponding individual and/or breed; genomic selection. By applying NGS to sample enrichment techniques, sequencing of whole exomes (*exomic*) is possible. By applying NGS to Random Amplified Digested (RAD) DNA, a reduced representation of the genome may be sequenced, making Genotyping By Sequencing (GBS) approaches affordable.

Transcriptomics

Literally, it refers to the systematic study of RNA molecules (mRNA, rRNA, tRNA, other non-coding RNAs) in given experimental conditions. NGS represented a true revolution in the field, marking the gradual switch from array-based transcription profiling techniques to RNAseq approaches, which allows the simultaneous accurate quantification of target RNAs, identification of start and stop transcription sites and exon/intron boundaries, mRNA splicing variant analysis (*spliceosomics*). Allele-specific expression analysis, with the aim of detecting genetic imprinting phenomena, is also possible via RNAseq technology. Unlike array-based technologies, RNAseq also allows *ex novo* transcript identification (including rare transcripts,

when adequate experimental sequence coverage is assured), and can be applied to species for which the genome sequence is not yet available or incomplete. A recent *omic* discipline, tightly linked to *transcriptomics*, is the RNA *degradomics*, whose goal is the systematic characterization of all possible mature RNA degradation products in a specific cell type or tissue, and the identification of the cleavage sites, via NGS techniques.

Epigenomics

It refers to the systematic study of all the epigenetic modifications able to alter gene expression levels without altering the genomic DNA sequence (thus contributing to phenotypic plasticity). DNA methylation, hystone modifications, and RNA interference phenomena are among the epigenetic mechanisms. Bisulfite-mediated conversion of unmethylated cytosine into uracile, combined to array-based systems or to NGS (bisulphite sequencing) are among the possible approaches of analysis. An alternative high-throughput approach could be the immunoprecipitation of methylated DNA (MeDIP), combined to array-based systems (MeDIP-chips) or NGS (MEDIP-seq). Chromatin immunoprecipitation (ChIP), combined to array-based systems (ChIP-on-chip) or NGS (ChIP-seq), also represents a possible approach (Mensaert et al., 2014). Genome-wide screening of RNA interference (RNAi) using synthetic RNAs may be adopted to systematically identify, through gene silencing, loci affecting specific phenotypes.

Proteomics and Metabolomics

Both disciplines address the systematic study of a more complex and dynamic array of molecules (the entire set of proteins and endogenous metabolites in a given cell type or organism) compared to genomics. 2D electrophoresis coupled to mass spectrometry has long been the main proteomic approach. Array-based system have been developed, which allow to screen for different interaction types (*interactome*) such as protein-DNA, protein-RNA, protein-protein, protein-lipid, protein-glycans, thus significantly empowering investigation ability in signaling pathways, drug-discovery, host/pathogen interactions, identification of prognostic and diagnostic biomarkers. Several mass spectrometry approaches have been developed, differing for the combination with distinct separation techniques, ionization methods, type of analyzer, refragmentation. These techniques are highly accurate, and permit the amino-acid sequence of a protein to be determined. Nuclear Magnetic Resonance (NMR) is largely adopted in proteomic and metabolomic studies. Main advantages are cheapness, easiness in sample preparation, rapidity and reproducibility of analysis, non destructiveness of

the sample, absolute and relative quantification of a large repertoire of metabolites (including unknown ones), also in complex mixtures (Simmler et al. 2014). As such, it is an ideal approach for metabolic profiling, although being less sensible than MS.

Conclusions and Perspectives

Infant "single-cell" *omics* technologies are emerging as most promising. High-throughput *phenomics* necessitates further technical development. Harmonization and standardization in high-throughput data production and analysis, as well as improvement of databases interactive access and data integration are needed, to follow the transition toward a "high-dimensional biology" and "system breeding". Collaboration among different groups of stakeholders will definitely be the key to success.

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First insight on the genetic structure of C*amelus dromedarius* populations through genome-wide SNP markers

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Introduction

Despite the worldwide relevance of the dromedary as a livestock species, no genome-wide screening tool (SNP chip) is today available, and no population genomics study has so far been published for *Camelus dromedarius*. In order to cope with this lack, we adopted a genome-wide Restriction site Associated DNA (RAD) sequencing approach, a method that combines traditional DNA shearing via endonucleases with the Illumina Next Generation Sequencing technology to simultaneously genotype tens to hundreds of thousands of single nucleotide polymorphism (SNP) markers in hundreds of individuals. The technology has the major advantages of not requiring substantial prior knowledge of both genome sequence and variability, while achieving per-site and per-individual costs below that of current SNP chip technologies.

Materials and Methods

A total of 120 animals from a wide geographic sampling area have been considered for this project. Here we will focus on 72 samples for which genotype data have been already produced, the remaining samples being currently under analysis. Seventy-one samples belonged to *C. dromedarius*, and were representative of thirteen different countries (Austria, 1; Iran, 9; Jordan, 10; Kazakhstan, 6; Kenya, 2; Libya, 1; Nigeria, 7; Pakistan, 5; Qatar, 7; Saudi Arabia, 9; Sudan, 3; Syria, 7; United Arab Emirates, 4). One *C. bactrianus* sample was included as outgroup. We adopted a double-digest approach (ddRADseq) to optimize the number of loci to be sequenced and maximize the number of sequence reads incorporated in

the analysis. The *in silico* analysis of the *Camelus dromedarius* (GCF_000767585) V1 whole-genome reference sequence highlighted the pair SphI-BstYI as the best combination of restriction enzymes able to produce fragments of 400-530 bp in size.

Gene diversity (H_E), inbreeding coefficient (F) and multidimensional scaling (MDS) analysis were performed using PLINK. Unsupervised Bayesian clustering was performed using STRUCTURE v. 2.3.4. (1-IBS) neighbor network was constructed using the Neighbor-Net algorithm implemented in the SplitsTree4 package v. 4.13.1.

Results

After applying filtering parameters (locus missingness ≤ 0.75, minimum coverage of 3), 38,886 SNPs were left. Average H_E on the whole dataset was 0.07 (ranging 0.01 to 0.5). As eighteen samples (25%) displayed genotype missingness ≥ 20%, inbreeding coefficient was only considered for the remaining fifty-four samples. The highest F value was observed for the two samples from Kenya (0.66 and 0.55, respectively). Forty-five samples (83%) displayed F values higher than 0.35. Negative or close to zero values were observed in the samples from Kazakhstan and in *C. bactrianus*. MDS analysis (Figure 1) clearly highlighted the outlier position of *C. bactrianus*, and interestingly detected an intermediate position of *C. dromedarius* samples from Kazakhstan. When the above samples were removed from the analysis, an eccentric position was observed for three *C. dromedarius* samples from Iran (Figure 2). Unsupervised Bayesian clustering (K = 2) consistently highlighted a clear differentiation of *C. bactrianus* from *C. dromedarius* and the presence of a *C. bactrianus* influence in Kazakh dromedaries (Figure 3, A). The STRUCTURE plot did not change after removal of the *C. bactrianus* sample and of the Kazakh dromedaries (Figure 3, B).

Discussion

In this study, a strong evidence for introgression of *C. bactrianus* into *C. dromedarius* animals from Kazakhstan is provided by analysis of the inbreeding coefficients, MDS analysis, and STRUCTURE. Hybridization between the two species has been practiced since the 1st millennium BC (rev. in Galik et al. 2015), as F_1-hybrids are recognized to display remarkable stamina and strength, perfectly suited for the transportation of goods along the multiple routes of the Silk Road, from China to the western fringes of Asia (Bernstein, 2009). Today, hybrid *Tulu* or *Nar* camels and their F_1-backcrosses are documented in Kazakhstan and Iran for improved milk and wool production (Faye & Konuspayeva, 2012). In our study, signals of *C. bactrianus* introgression were highly variable in extent, with low levels being detected in some

samples from Iran, Syria, Jordan, Saudi Arabia, United Arab Emirates and Pakistan, an higher proportions in four out of six Kazakh individuals (Figure 1).

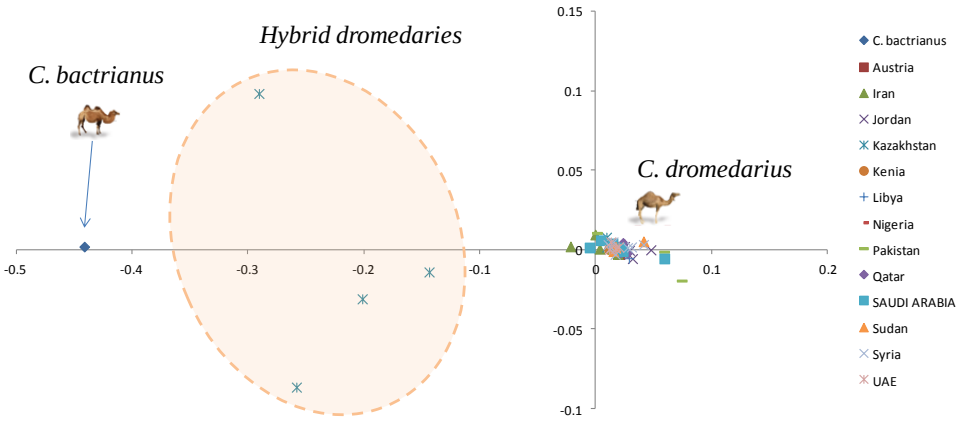

Figure 1: MDS plot of the pair-wise IBS distances among subjects from 13 different countries. A single *C. bactrianus* animal is also included in the analysis. First dimension on the x-axis; second dimension on the y-axis.

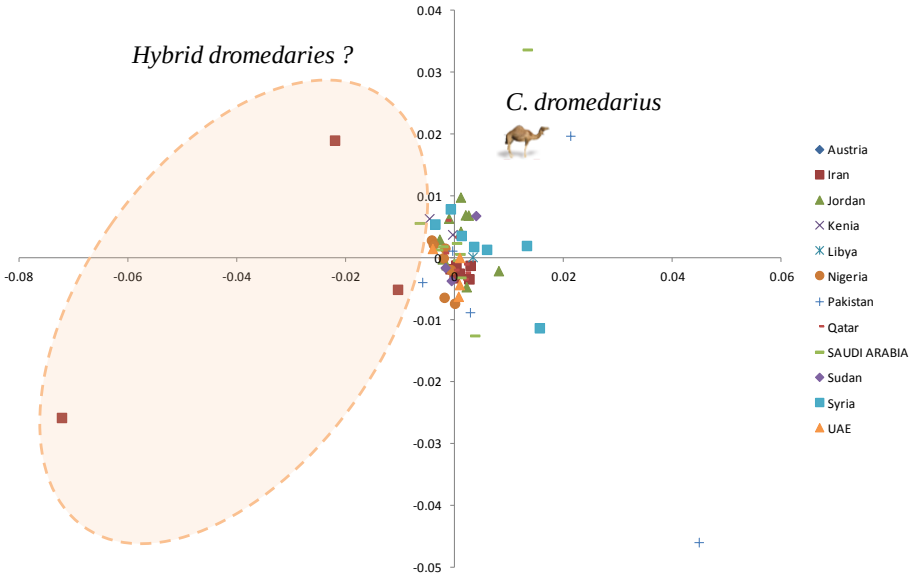

Figure 2: MDS plot of the pair-wise IBS distances among subjects from 12 different countries. First dimension on the x-axis; second dimension on the y-axis.

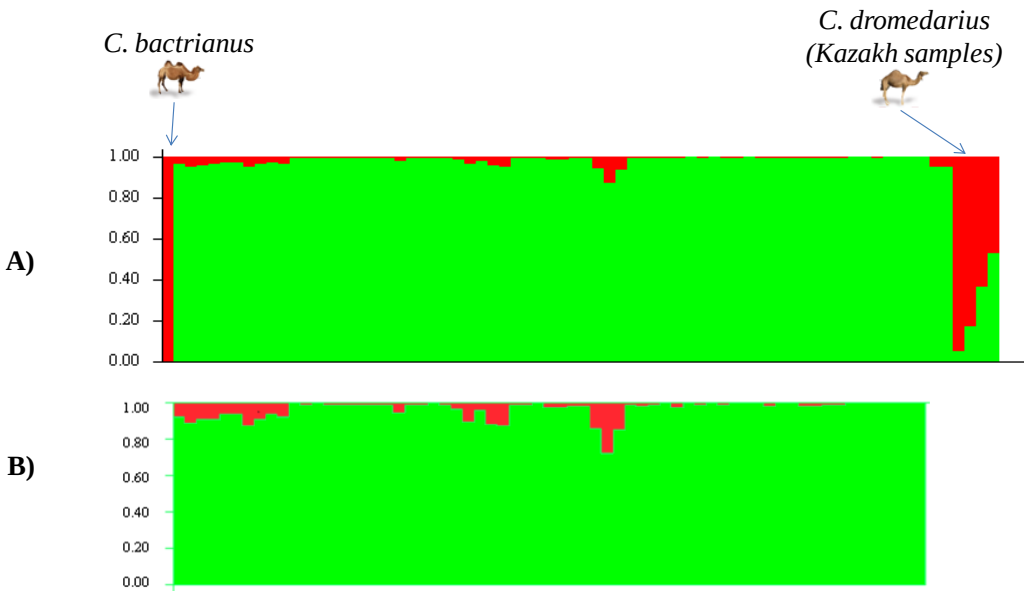

Figure 3: STRUCTURE plot at K = 2 for the whole dataset (A) and after removal of the *C. bactrianus* sample and the dromedaries from Kazakhstan (B).

No genetic sub-structuring was observed within the remaining dromedary samples, though they originated from twelve different countries (data not shown). These results are consistent with previous molecular evidences suggesting a peculiar genetic make-up of the *Camelus dromedarius* species (Almathen et al., 2016; Cherifi et al., submitted), shaped by centuries of use as pack animal along traditional caravan routes.

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Effect of management system on camel milk production in Western Sudan

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Abstract

Twenty four lactating she-Camels were selected, from the Sudanese Arabi type. The mentioned lactating she-camels were monitor and divided into two equal groups 12 she-camels in each. Group one (G1) was handled in a semi intensive system; supplementation consisted of concentrates and roughages and *Ad libitum* watering was practiced, the other group (G2) was hand out as a control, experimental animals managed traditionally within the same site of the experimental work. The collection of milk samples were started at 10 days postpartum and continued for 12 successive months during biweekly interval period. Hand milking was applied and Milking was practiced twice a day, approximately 12-hours interval; to prevent calf from suckling ***Sorar*** technique was used. Daily milk yield was estimated using different volume of graded cylinders. The data were subjected to statistical analysis programme using SIGMA-STAT. Soft ware computer Package described by Analysis of Variance (ANOVA). The results indicated that the averages daily milk yield for both farming system were 8.36 ± 1.64 lit/day and 3.24 ± 0.78 lit/day for semi-intensive and traditional system, respectively. The difference, both for daily and monthly milk yield, was highly significant ($P<0.001$).This difference was linked to the farming system adopted for camel husbandry, but also by the times of lactation from post-partum ($P<0.05$). The average daily milk yield obtained from the camels under semi-intensive system increased of 61% compared to camels managed under traditional system. The maximum average daily milk yield was attained in the (3^{rd}) third month post-partum in both systems, which were 11.3 ± 1.23 lit/day and 4.68 ± 0.76 lit/day in semi-intensive and traditional system, respectively. There was a prickly decreasing of daily yield in traditional system after reach the peak, in contrast to that in semi-intensive system which has a good persistency or stable for long time after reach the peak. The results indicated that the trend of daily milk yield

seemed to increase significantly (P<0.05) from the first day post-partum till reach the peak in third month and then declined gradually through the lactation period.

Keywords: Camel, Management, Milk Production, Daily Milk, Sudan.

Introduction

According to FAO statistics, camel population in Sudan ranks the second in the world after Somalia with 4.5 millions heads (Faye et al; 2011) North Kordofan state only has the highest camel population with more than one million heads (Faye, 2009). In some regions of Sudan camel milk is one of the main components of the diet (Bakheit, 2004). The dromedary produces more milk and for a longer period of time than any other species in the same environment (Farah and Younan, 2005). It is difficult to estimate the daily milk yield of camel under pastoralist conditions due to the inconsistency of milking frequency. Milk yield also varies with breed, stage of lactation, feeding status and management conditions (Farah and Fischer, 2004). Camels can produce milk under environmental and gives most milk near the beginning of lactation period (Bakheit *et al*, 2008). In many studies monitoring the camel milk yield estimated the daily yield at 21 litres in the 2nd week of lactation and 4.8 liters by the 16th week. The potential lactation yield of camels on irrigated pastures was calculated as 2847 Kg from results given by Knoess (1976). In Sudan Bakheit (1999) study the daily and total milk yield under traditional conditions and recorded that the daily milk varied between 1.39 – 3.30 liters and the total milk yield during lactation on 365 days varying between 507 – 1204 liters. The present study was initiated to estimate Sudanese camel milk production under improvement farming system and its influences by the supplemented feedstuff, watering regime, health care and internal and external parasites control.

Material and Methods

Study area

North Kordofan State lies between latitudes 11°:15/ and 16°:30/ N and longitudes 27° and 32° E at an altitude of 560 meters above sea level. Maximum temperatures range between 30 and 35°C, with peaks of above 40°C during the months of April, May and June, rainy season extends from July to October an August is greatest monthly rainfall. The study area can be categorized into two major soil groups, sandy and sandy loamy soils. The dominant trees species in the study area are composed of Acacia species, grasses and herbs are dominated as the under story vegetation in the study area (Technoserve, 1987)

Experimental animals identification

Twenty four lactating she-camels were selected, from the Sudanese Arabi Kabaishi breed (the animals pointed at late pregnancy randomly from nomadic herd). Each of the experimental selected animals was identified by plastic numerical tags which placed in ear of the animal. The mentioned lactated she-camel acquired and divided into tow equal groups 12 she-camel of each group one (G1) managed in a semi intensive system all animals were herded during night in closed pen and set free during the midday, supplementation consist of concentrates (2kg/day) and roughages (5kg/day) were used, *ad libtium* watering, health care and parasites control were practiced. Experimental animals in group two (G2) served as a control managed traditionally but within the site of the Experimental work, on traditional system the animals are brought to grazing areas where they selected the food by themselves from the available plants and allowing nothing as supplemented feeding, with the exception of offering salt as a brine or dry at wet season and watering regime every (6-7) days was applied.

Data collection

Milk samples collection were started in 10 days postpartum and continued for 12 successive months during biweekly interval period in all experimental animals in traditional and semi-intensive system. Hand milking was applied; the milker usually approaches she-camel from the left side, he stands on his right leg and balances the milking bowl on his left bent and uses all hands for milking. Milking was practical twice a day, approximately 12-hours interval; usually two teats were milked and leaving the remaining two teats for suckling by calf. ***Sorar*** technique, to prevent the calf from suckling was used for suckling control; two teats are tied up with a soft tape of cloth removed only at milking time. The estimates of milk yield were formulated from yield intervals for the whole year viz. 12 monthly yield were secured. Daily milk yield were estimated using different volume of graded cylinder involved 50 ml, 500 and 1000 ml. measuring cylinders. The milk yield was registered in the record of the milk production.

Statistical analysis

The data were subjected to statistical analysis programme using SIGMA- STAT. Soft ware computer Package described by Analysis of Variance (ANOVA) according to Snedecor and Cochran (1967). Duncan multiple range tests were used for means separations.

Results

The results indicated that the averages daily milk yield for both farming system were 8.36 ± 1.64 lit/day and 3.24 ± 0.78 lit/day for semi-intensive and traditional system, respectively. In both systems the daily and monthly milk yield, was highly significant difference ($P<0.001$). The total milk were 3009.6 litre and 1166.4 litre on semi-intensive and traditional system, respectively. the averages means from the analysis of variance for daily and monthly milk yield were highly significant ($P<0.001$) affected by the farming system which adopted for camel husbandry, but they affected significantly ($P<0.05$) by the times of lactation from post-partum. The maximum average daily milk yield was attained in the (3^{rd}) third month post-partum in both systems, or in 13^{th} week after calving, which were 8.7 ± 0.94 lit/day and 4.30 ± 0.59 lit/day in semi-intensive and traditional system, respectively. There was a sharp decrease of daily yield in traditional system after it reaches the peak, in contrast to that in semi-intensive system which has a good persistency or stable for long time after reach the peak, the trend of daily milk yield seed to increase significantly ($P<0.05$) from the first day post-partum till reach the peak in third month and then declined gradually through the lactation period, The maximum average daily milk yield was attained in the (3^{rd}) third month post-partum in both systems, which were 11.3 ± 1.23 lit/day and 4.68 ± 0.76 lit/day in semi-intensive and traditional system, respectively. It was evident that Farming system and management have significantly impact on the daily camel milk yield and milk yield which obtained from the camels under semi-intensive system was greater 53% than obtained from camels managed under traditional system. The study reflects clearly the significant contribution of improving systems on camel daily and total milk production. camels raised under semi-intensive system were able to produce more milk than the other reared under traditional system and these may be attributed to the forage availability and the supplementary diets, water availability and health care that were given to the camels in the semi intensive system, Further studies on camel milk production potential under different farming condition and controlled environmental factors to elucidate the potential of camel.

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Proteomic analysis of *Camelus* milks from Kazakhstan

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Introduction

Camel milk was reported to have medicinal and health-promoting benefits (Konuspayeva at al, 2007), such as anti-carcinogenic, anti-diabetic, anti-hypertensive, and has been recommended as an alternative for children and adults suffering from bovine milk allergy. Most of these properties which remain however insufficiently substantiated and unreliable, are undoubtedly dependent on milk composition which is still relatively unknown, particularly regarding the protein fraction. Variations observed in camel milk composition could be attributed to several factors such as geographical locations, seasonal variations, feeding conditions, genetics, in addition to other factors including stage of lactation, age and parity. Therefore, we combined different proteomic approaches to achieve a comprehensive description of the milk protein fraction from *Camelus bactrianus* and *Camelus dromedarius* and their hybrids, from different regions of Kazakhstan. Thus, coupling first SDS-PAGE and LC-MS/MS, and on the other hand LC-ESI-MS, has allowed identification, characterization and quantification of proteins in different milk samples (Saadaoui et al, 2014).

Materials and Methods

Milk samples

Raw camel milk was collected during morning milking from healthy two Kazakh dairy camel species *Camelus bactrianus* and *Camelus dromedaries*, and their hybrids at different lactation stages (30-90 days postpartum). In total 180 milk samples were obtained from local farms in Kazakhstan whose camels grazed on four various pastures depending on their geographical location: Almaty, Ontustik-Kazakhstan, Kyzylorda and Atyrau.

RP-HPLC/ESI-MS analysis

Separation of *Camelus* milk proteins and determination of their molecular masses were performed by interfacing an ESI-TOF mass spectrometer (micrOTOF ™ II Focus, Bruker Daltonics, Wissembourg, Germany) to a RP-HPLC a column. Twenty µL of skimmed milk samples first clarified by the addition of 230 µl of clarification solution: 0.1 M bis-Tris buffer pH 8.0, containing 8 M urea, 1.3% trisodium citrate, and 0.3% DTT, of which 15 µl were injected on a Discovery® BIOWide Pore (Supelco) C_5 (150 x 2.10 mm, 300A) column. The mobile phase of the column corresponded to a gradient mixture of Solvent A (H2O/TFA 100:0.25 v/v) and Solvent B (ACN/TFA 100:0.20 v/v). During the chromatography, the column is thermostatically controlled at 52ºC. The LC-MS system was controlled by the Hystar software version 2.3 (Bruker Daltonics). The charge number of the multi-charged ions, the deconvoluted mass spectra and the determination of the molecular masses were obtained from Data Analysis software (Bruker Daltonics).

SDS-PAGE and LC-MS/MS

Proteins of milk samples were pre-separated by subjecting camel milk to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 4.5% stacking and 12.5% resolving gels, running in 0.5 M Tris-HCl pH 6.8-SDS 0.4% and 1.5 M Tris-HCl pH 8.8-SDS 0.4% buffers, respectively. Samples were prepared with Laemmli Lysis-Buffer (Sigma-Aldrich). Finally, samples with more representative protein bands were cut for further analysis, using LC-MS/MS after tryptic digestion.

Gel samples were cut for 16 pieces ($1.5mm^3$). Namely, protein bands were excised and gel pieces placed into 96-well microtiter plates, equilibrated for 5 min with 100 µL of 50 mM ammonium bicarbonate (NH4HCO3) buffer, followed by the reduction (1h at 37°C, in 100 µL reduction buffer, containing 10 mM DTT) and alkylation. The electrophoretic bands were washed by buffer solution containing DTT/Iodoacetamide. Then gel samples were rehydrated and digested with 30 µL of loading buffer and 100 ng trypsine (sequencing grade Promega, Charbonnières, France).

The identification of the main milk proteins (Figure 1b) using mono dimensional electrophoresis (1D SDS-PAGE) was performed, followed by trypsin digestion. Then, tryptic peptides were analyzed by Liquid Chromatography (LC) coupled with tandem mass spectrometry (LTQ-Orbitrap, Thermofisher Scientific). The identification of proteins was carried out using X!Tandem pipeline search engine developed by PAPPSO platform (http://pappso.inra.fr/bioinfo/) with the UniprotKB ***Cetartiodactyla*** database.

Results and Discussion

LC-ESI-MS technique allows the detection of numerous genetic variants of milk proteins. Identified camel milk proteins were eluted in the following order shown on Figure 1a): κ-casein (peak I), whey acidic protein WAP (peak II), α_{s1}–casein (peak III), α-lactalbumin (peak IV), α_{s2}-casein (peaks V-VIII), peptidoglycan recognition protein PGRP (peak X), camel serum albumin CSA + lactoferrin (peak XI), and β-casein (peak XII). Moreover, using LC-ESI-MS provides a high sensitivity characterization of camel milk low-abundance proteins, post-translational and process-induced modifications, such as variations in the degree of phosphorylation and glycosylation (Kappeler, 1998). Degree of phosphorylation varied among the individual caseins. The measured molecular masses of αs1-, αs2- and β-casein were dependent of the number of phosphate group, which provides an increment accounting for 79.9 Da. For examples, we identified 5 different levels of phosphorylation for α_{S2}-casein (ranging between 7-11P), and the same splicing variants (short and long isoforms) for α_{S1}-casein in *Camelus bactrianus* as it was found in *Camelus dromedarius.* Besides, κ-casein is the only member of the casein family which is glycosylated.

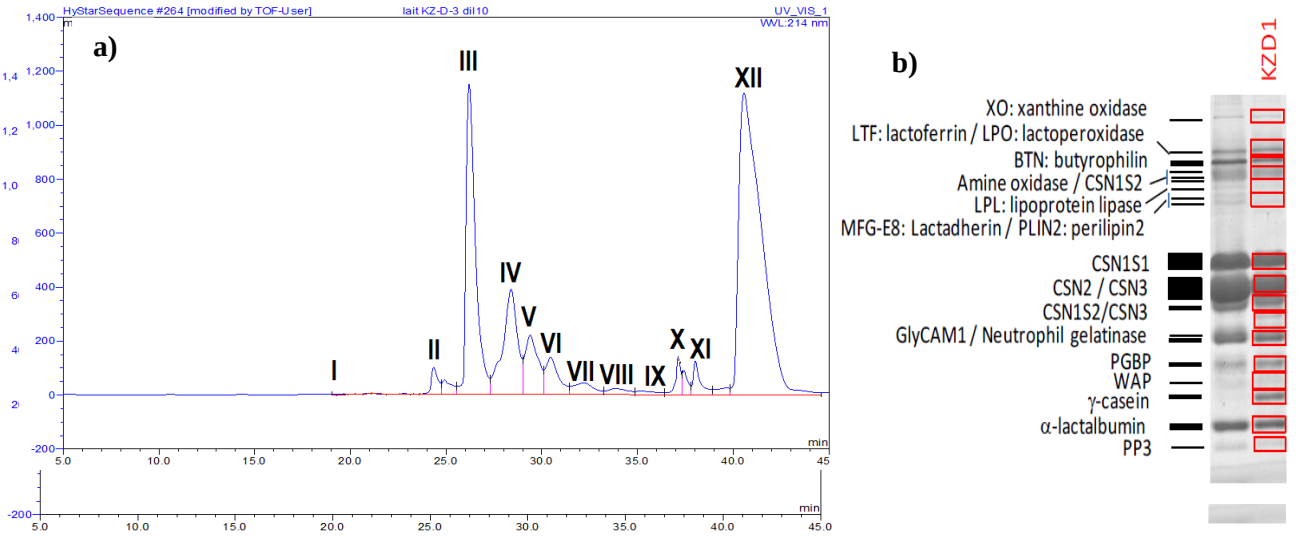

Figure 1: a) LC-ESI-MS and **b)** SDS-PAGE representative patterns of skimmed dromedary milk proteins fractions, sampled in Kyzilordinskaya region.

On first analysis by LC-MS/MS, it appears that on a single track of *C. dromedarius* milk sample, 209 proteins were identified, comparing with *ca.*200 proteins found in the same genus from Saudi Arabia and California (Alhaider et al, 2013): 14 matched with high certainty against entries in the *C. dromedarius* database (the same proteins as those found by Alhaider and co-workers, and the rest 195 to other mammalian databases. The number of total IDs arising from

all mammalian species fluctuated between 371 and 1005 in each milk sample. Analysis of these data is currently under processing.

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High polymorphic sites in 5´flanking region of beta-casein gene in Pakistani dromedary camel

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Introduction

Camel population is about 24 million and Pakistan ranked 8th among major camel raising countries in the world (FAO, 2013). Camel have unique physical characteristic of fatty deposition on its back known as "hump". Camel species can be categorized into Dromedary or Arabian with single hump native to dry deserts of West Asia and the other is Bactrian with two humps native to Gobi desert, central and East Asia. Camel milk is an important commodity and a major source of income for arid, semi-arid and desert areas. Camel milk is not appreciated properly as it values and its composition proved it as closer to human milk and digestible in individuals with lactose intolerance. Presence of nutritionally important constituents has drawn attention. It is rich source of vitamins, minerals, proteins and possesses antimicrobial as well as anti-tumor properties (Al-Ayadhi & Elamin, 2013). It contains 3% proteins and casein is the main constituent (Sawaya *et al.*, 1984). Distribution of milk proteins greatly differs between species and Casein protein contents are higher in camel milk and is considered as highly nutritive food compared to other dairy animals (Barłowska *et al.*, 2011). There are four main fractions of casein αs_1, αs_2, β and κ. These proteins have polymorphic sites in the related genome sequence in most of the animal species. Milk-specific genes express in the lactating mammary glands, and are regulated by hormonal and environmental stimuli which bind with the enhancer element located in the 5′ flanking region to the transcription site. Casein promoter have binding sites for signal transducers and transcription activators (Jeffrey *et al.*, 1999). Casein loci are indicated as candidate genes responsible for milk trait variations. We hypothesized that upstream region of beta-casein locus is not conserved in camels and to test this assumption we investigated the 5' flanking region of beta-casein gene in Pakistani dromedary Kachhi camel breed.

Materials and Methods

Sampling and genomic DNA extraction

Blood samples of Kachhi camel (n=21) were collected randomly from different areas of Pakistan in vacutainer. Genomic DNA was extracted from leukocytes (Sambrook & Russell, 2001). Concentrations were brought to the same level 30 ng/µL.

PCR amplification

Primers were designed with Primer3 software (Koressaar & Remm, 2007) using NCBI reference sequence with accession no. AJ409279.1. Two sets of overlapping primers (Table 1) were used to amplify the 5' flanking region (c.1-2147 to c.1-1073) of beta-casein gene. PCR reaction of 25 µL was incubated for initial denaturation at 95°C for 5 minutes, followed by 94°C for 30 seconds, annealing according to primers Tm (Table 1), then 72°C for 45 seconds, final extension at 72°C for 10 minutes. Amplicons were purified for sequencing through ABI 3100 Genetic Analyzer (Applied Biosystems, USA).

Table 1: Primers used for 5'flanking region of beta-casein gene.

Primer-ID	Sequence (5'->3')	Length	Tm	GC%	Product size
BCAS1-F	GGGGAAAGAGCTTTGACTA	19	54.05	47.37	594
BCAS1-R	CCCCAGTGTCGTAGGTATA	19	54.89	52.63	
BCAS2-F	CTTGGCAACCAAGAGCTA	18	54.48	50.00	550
BCAS1-R	CACAGGGAACCATATTCAGT	20	54.72	45.00	

Bioinformatics analysis

Sequence data was analyzed and edited with the help of CodonCode Aligner. Phylogenetic tree were constructed through MEGA 7 software package using the Neighbor Joining method with 1000bootstrap value (Kumar *et al.*, 2016).

Results and Discussion

Kachhi camel individuals (21) were sequenced for 5' flanking region of beta-casein gene. Sequenced data was aligned using NCBI blast tool and haplotypes were constructed. Aligned data showed two different haplotypes as VU-Kachhi_Cas_Haplotype-1 (n=16) and VU-Kachhi_Cas_Haplotype-2 (n=5). We observed ten polymorphic positions in both haplotypes and both were different for c.1-1999 and c.1-1156 in the upstream position of beta casein gene as given in the Table 2.

VU-Kachhi_Cas_Haplotype-1			VU-Kachhi_Cas_Haplotype-2		
Position	Sequence Change	Type of Change	Position	Sequence Change	Type of Change
c.1-1580	G>T	Transversion	c.1-1999	G>A	Transition
c.1-1450	A>G	Transition	c.1-1580	G>T	Transversion
c.1-1398	del. C	Deletion	c.1-1450	A>G	Transition
c.1-1391	del. C	Deletion	c.1-1398	del. C	Deletion
c.1-1371	del. C	Deletion	c.1-1391	del. C	Deletion
c.1-1330	del. C	Deletion	c.1-1371	del. C	Deletion
c.1-1200	del. C	Deletion	c.1-1330	del. C	Deletion
c.1-1160	C>G	Transversion	c.1-1200	del. C	Deletion
c.1-1157	C>T	Transversion	c.1-1160	C>G	Transversion
c.1-1156	ins. AT	Insertion	c.1-1157	C>T	Transversion

Table 2: Sequence variations in the 5' flanking region of beta casein gene in Kachhi camel breed.

Phylogenetic tree of dromedary camel from Pakistan was constructed with the corresponding genetic sequences of other species available including Camelus dromedaries, Camelus bactrianus, Bos taurus, Bubalus bubalis, Sus scrofa and Capra hericus to see the biological positioning of Pakistani dromedary camel. Phylogenetic tree constructed through MEGA 7 software package using the Neighbor Joining method with 1000bootstrap value (Kumar et al., 2016) reconfirmed the classical biological classification of Pakistani camel breed in comparison to other mammals (Figure 1).

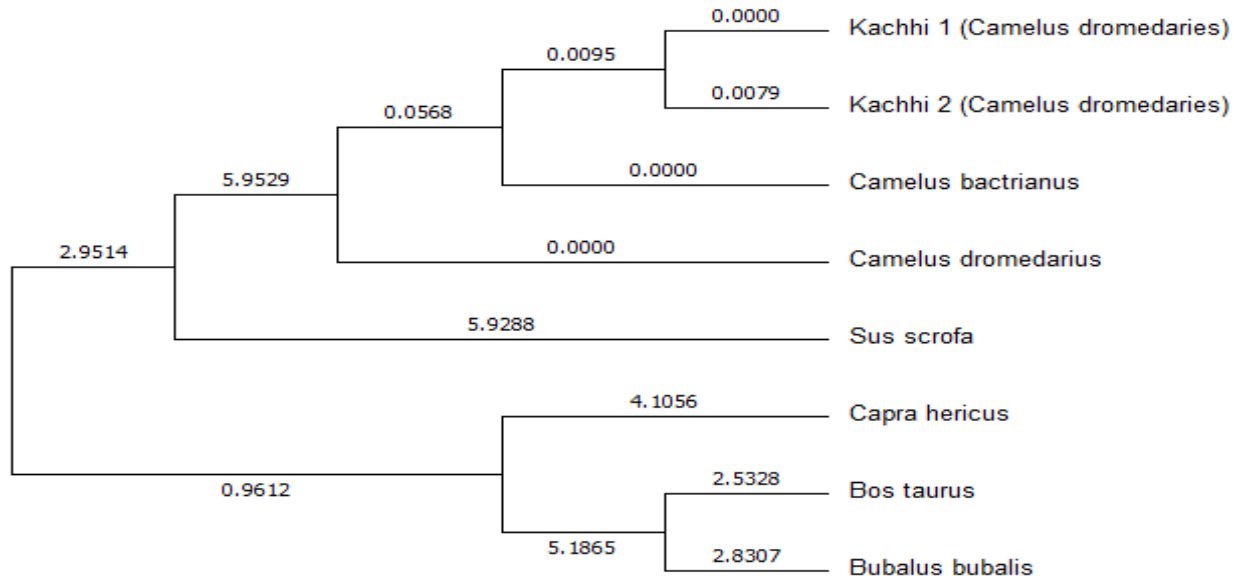

Figure 1: Phylogenetic tree of Kachhi haplotypes on the basis 5´flanking region of beta-casein gene.

Camel is animal of less watery environment and has adopted to face different immunological challenges. Beta-casein is an important milk protein expressing the lactating mammary tissues. We sequenced the 5′-upstream region of beta casein gene in Kacchi camel breed. This DNA segment is shown to be a regulatory sequence for tissue specific expression of corresponding gene in other mammals (Rijnkels *et al.*, 1998). Beta-casein (CSN2) is considered as a marker for milk yield in dairy animals. Upstream region of beta-casein gene in camel is found as polymorphic to the reference sequence of dromedary breed. Five single nucleotide substitutions and five deletion of cysteine nucleotide identified. Sixteen of the individuals showed insertion of two bases AT in c.1-1156 position. Animals bearing this insertion showed a significant different position in the clade. Phylogenetic tree analysis suggests this class a different group (Figure 1) from reference sequence of Camelus dromedaries and Camelus bactrianus. Insertion of two bases in (c.1-1156) very close to the regulatory sequence might be responsible in mRNA yield and milk trait consequently. However we could not determine the mRNA expression level in the mammary tissues. One substitution C>T in the -1578, located in the gene enhancer region has been documented for milk and milk protein yield in Holstein cattle. Effect of beta-casein haplotypes with more milk and protein, and significant reduced fat contents are also reported (Lipkin *et al.*, 2008). These findings indicate that beta-casein locus is highly polymorphic in Kachhi camel breed and can be considered useful for molecular marker assisted selection.

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Advanced Scientific Group

About Us:

The Advanced Scientific Group was established in 1990 under instructions and with support of his Highness / Sheikh Hamdan bin Zayed bin Sultan Al Nahyan, the representative of the ruler in the western region and his Highness/ Sheikh Hazza bin Zayed Al Nahyan, Vice President of the Abu Dhabi executive council, with the purpose of preserving and improving the Arabian camel breed through modern scientific technologies as applied in horses and cows. The Advanced Scientific Group has the most demand in the Arab Gulf Region with regard to embryo transfer in camels, as it produces about 1000 pregnancies a year.

Objectives

- To ensure achieving better level for breeds originated from purebred. The Advanced Scientific Group is the first center that received camel owners in the Gulf Region to make use of the expertise of the center.
- To increase opportunities for obtaining heritage materials and to encourage increasing participation in folklore festivals.
- To foster and support long-run initiatives with concerned establishments.
- To make best scientific researches that serve camels.
- To provide consultancy to camel' owners and present best methods for raising and treatment of camels.
- The production of Advanced Scientific Group is deemed the best -after 25 years of efforts- constituting 1% of the state production of camels and winning 30% of races (symbols- cups – cars).

Vision

- We focus on having confidence of the society and distinct efficiency in objectives achievement as well as being a reference for camel researches in both the Arab Gulf Region and the Arab World.
- The Advanced Scientific Group wrote a book on camels, which is deemed as a key reference in camel reproduction.

Identity

To provide scientific services with heritage trait, which services are formulated through experience and distinguished with honesty as well as being pulsed with interest. We assess our quality on the basis of the effective fulfillment of your needs.

Our Services

- Embryos transfer for Arabian originated camels to produce several camel calves a year instead of only one per two years.
- Infertility check up : we provide advice to owners of camels for treatment of camel infertility as well as receiving critical cases.
- DNA Analysis
- Disease Diagnosis & Blood analysis
- Offer Arabian originated breeds at annual auctions.
- Provide advice to owners concerning raising, feeding and treatment of their camels.
- Organizing personal production auctions for camel owners.

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