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# Artificial insemination in South American camelids and wild equids

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## Abstract

An overview of the present status of the use of artificial insemination (AI) in South American camelids and wild equids is offered. Technical aspects of semen collection, dilution and cryopreservation have limited the development and use of AI in camelid and equid species. To-date, efficiency is low but progress has been made and viable offspring have been produced through the use of AI in domestic South American camelids using both fresh and frozen semen. The origin, composition, and function of the viscous component of camelid seminal plasma remain a mystery and an obvious area for future research. A better understanding of the normal constituents of seminal plasma will enable the rational design of semen extenders suitable for camelids. Post-thaw sperm viability is very low, and studies are needed to address questions of optimal freezing and thawing procedures as well as the insemination dose. The basis for differences in reported pregnancy rates with sexed and frozen semen in domestic equids, and the ultimate success of AI in wild equids will require continued research into the “stallion effect”, extenders and cryoprotectants, optimal volume and number of spermatozoa, temperatures during handling, processing and transport, and insemination techniques. In both camelids and equids, research on domestic species under controlled conditions provides an excellent opportunity to develop effective semen handling techniques for application in wild and endangered species of the respective families.

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*Keywords:* artificial insemination; semen; camelids; llamas; alpacas; equids; horse; donkey

## 1. Introduction

Artificial insemination (AI), particularly with frozen semen, has been by far the most effective reproductive technology for selective genetic improvement in farm animal production. The power of this technique for influencing the genetic make-up of a given population is

exemplified in Holstein cattle, where average milk production has nearly doubled in the span of only 40 y. The observation that AI has brought about a measurable narrowing of genetic diversity within the Holstein breed (i.e., rapid selection for a specific production trait) provides support for the notion that if applied for that purpose, AI is equally as effective for preserving and expanding genetic diversity in threatened populations. The potential for successful application of AI in wild camelids and equids, in particular, is high, because the abundance of domesticated camelid and equid populations provide an excellent opportunity for research and

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Table 1  
Semen collection methods and characteristics (mean  $\pm$  S.E.M. and/or range) in South American camelids.

Species [Ref.]	Method of collection <sup>a</sup>	Volume-mL (range)	Sperm $\times 10^6$ per mL	%Motility (range)	%Normal
Alpaca [3]	Vaginal sac	1.9 (0.4–6.6)	33.0	Low	41
Alpaca [9]	Electro-ejac	(1.1–1.8)	0.001–2.55	Low	–
Alpaca [13]	AV-dummy	1.7 $\pm$ 0.2 (0.4–4.3)	–	50.0	–
Alpaca [14]	AV-dummy	(0.6–2.7)	0.09–0.2	–	–
Alpaca [15]	AV-dummy	1.9 $\pm$ 0.4 (0.8–3.1)	82.5–250	85 $\pm$ 5.2	75.9 $\pm$ 2.1
Alpaca [16]	AV-dummy	3.0 (1.0–12.5)	600	–	–
Alpaca [17]	AV-dummy	2.6 $\pm$ 1.8	0.06 $\pm$ 0.03	49.7 $\pm$ 22.6	–
Alpaca [18]	AV-dummy	–	–	68.2	–
Alpaca [19]	AV- female	1.8 $\pm$ 0.8 (0.6–3.8)	17.6 $\pm$ 26.1 (0.05–92.9)	–	51.0 $\pm$ 12.4
Alpaca [20]	AV-dummy	(0.6–8.2)	63–250	55–75	55–75
Llama [7]	AV- female	2.4 (0.2–6.5)	106.8 (15.0–640)	15–50	90.6
Llama [21]	AV-dummy	3.0 $\pm$ 1.9 (0.2–7.9)	1.0 $\pm$ 0.8 (0–3.4)	23.7 $\pm$ 20.0 (0–65)	39.7 $\pm$ 18.5 (0–79.1)
Llama [22]	AV- female	3.5 $\pm$ 2.6	85 $\pm$ 89	25–33	32.5 $\pm$ 22.3
Llama [8]	AV-female	2.1 $\pm$ 1.4	80 $\pm$ 28	57.6 $\pm$ 22.3	–
Llama [11]	Electro-ejac	1.3–4.3	0.0–123.3	0.0–55.5	41.5–81.3
Vicuna [12]	Electro-ejac	1.0–2.5	0.01–0.14	10	66.0–75.0

<sup>a</sup> AV-dummy = artificial vagina within a dummy (phantom) mount; AV-female = artificial vagina and a live receptive female mount.

development. The objective herein is to provide an overview of the current status and potential utility of artificial insemination in South American camelids and wild equids.

## 2. South American camelids

The llama (*Lama glama*) and alpaca (*Vicugna pacos*) are domesticated members of the four species of New World camelids. The other two are wild species, the guanaco (*Lama guanicoe*) and vicuna (*Vicugna vicugna*), and along with their domestic cousins, inhabit the Andean region of South America from Ecuador to southern Chile. Related Old World camelids include the dromedary (one-humped) and bactrian (two-humped) camels, that inhabit primarily the African and Asian continents, respectively. The International Union for Conservation and Natural Resources (IUCN) lists the vicuna and guanaco as threatened to some degree, and the wild subspecies of Bactrian camel as critically endangered. There appears to be more similarities than differences in the reproductive physiology of Old and New World camelids; however, the following discussion is confined to the latter.

### 2.1. Methods of semen collection

Historically, the inability to consistently collect semen has been one of the most serious impediments to the application of AI in camelids. The challenge stems from the recumbent mating posture in camelids, the duration of copulation (10–60 min in llamas and alpacas), intrauterine deposition of semen, and the viscous nature of the

ejaculate [1,2]. Attempts at semen collection in camelids have included the use of condoms or intravaginal sacs, vaginal sponges, electro-ejaculation, post-coital vaginal aspiration, and fistulation of the penile urethra Table 1. The use of intravaginal sacs [3] was cumbersome, hindered intromission, and was associated with vaginal injury. Similarly, recovery of semen samples from sterile sponges inserted into the cranial vagina before copulation was inefficient and of low quality, and samples were contaminated with blood and epithelial cells [4]. The impact and consequences of the surgical procedure of urethral fistulation [5] have not been evaluated.

The use of an artificial vagina (AV), similar to that used in sheep, was first reported in 1981 [6]. The AV included a coil to simulate the cervix and was mounted inside a dummy that the males were trained to mount. However, the lack of adequate heating equipment required repeated refilling of the hot water chamber to maintain a stable AV temperature during prolonged copulation. The technique was improved with the use of an electric heating pad wrapped around the AV [7]. Collection of semen by an AV mounted inside a dummy is more reliable than other methods and may provide a more physiological sample of semen [6]. An alternative to a dummy mount is the use of a receptive female; the penis of the male can be directed into an AV during the mount [7,8]. The disadvantage of semen collection with an AV is that the males must be trained to serve the AV; perhaps because of prolonged ejaculation, male llamas and alpacas do not serve an AV as readily as bulls and rams. However, consistent results can be obtained once the males become accustomed to the AV with a dummy or live mount.

Electro-ejaculation was attempted in alpacas more than four decades ago, using a custom-made electro-ejaculator and applying gradually increasing electrical stimulation (up to 40 V) in a periodic fashion (i.e., 4–6 s of stimulation, followed by 4–6 s of rest) [9,10]. From 1.1 to 1.8 mL of semen was collected, but sperm concentration (range: 1000–255,000 spermatozoa/mL) and quality were extremely variable, and contamination with urine was common. The use of electro-ejaculation in camelid species has since been nearly abandoned, due to inconsistent responses and the need for general anesthesia, but results of a recent study provide reason for reconsideration [11,12]. The distance between the prostate and the external anal sphincter was measured using transrectal ultrasonography in male llamas ( $n = 6$ ) and after general anesthesia, the electrodes of an electro-ejaculator probe designed for use in large dogs was placed directly over the prostate gland. Ejaculates were collected from all six males by electro-ejaculation, and samples were larger in volume with a greater percentage of motile sperm compared with semen collected from the same males by AV.

## 2.2. Seminal plasma and semen handling

Effective use of AI requires the dilution and storage of semen, but difficulties in semen collection and handling in llamas and alpacas have been an impediment. The semen of llamas and alpacas is highly viscous [15,21] and as a result, assessment of sperm concentration, morphology, and motility, are difficult. Ejaculates vary in color from nearly clear to milky white, depending on sperm concentration [15,21,23], and a comparatively high proportion of morphologic abnormalities (e.g., 40%) is common [24]. Unlike the progressive motility of sperm seen in other domestic ruminants, only oscillatory movement is seen in the ejaculate of llamas and alpacas [13,16].

The role of high viscosity of camelid semen is not known, but it may act as a type of sperm reservoir or may be important for maintaining sperm viability within the uterus [25]. To facilitate handling and processing of semen, attempts have been made to

liquefy the ejaculate. In a study designed to test the effectiveness of enzymes for liquefying semen (i.e., collagenase, fibrinolysin, hyaluronidase, or trypsin), collagenase was effective in eliminating semen viscosity within 5 min, with little or no influence on sperm characteristics [18]. In a recent study, however, all enzymes effectively reduced viscosity, but satisfactory motility was maintained only with trypsin or papain; i.e., collagenase was toxic at all concentrations [26]. A mechanical technique of liquefying the ejaculate involved alternately aspirating and expelling the ejaculate through a needle; this effectively liquefied the ejaculate and had little influence on other characteristics of semen [27,28].

Only two reports were found regarding the chemical constituents of the seminal plasma of South American camelids—both on alpacas [13,14]. The concentration of components such as chloride, calcium, total proteins, inorganic phosphate, and glucose, were similar to that in other ruminants, but citric acid and fructose concentrations were much lower than that in bulls, horses and pigs—a feature thought to be consistent with the lack of vesicular glands in camelids. Although it is not yet clear whether the sugars act as an energy source for sperm metabolism or as signaling molecules to modulate sperm function (e.g., capacitation), fructose and glucose are routinely incorporated into the majority of extenders for the semen of bulls and rams.

## 2.3. AI technique, induction of ovulation and pregnancy rates

The first attempt at AI in llamas and alpacas [1] involved 42 female alpacas inseminated with fresh undiluted semen obtained from two vicunas and four paco-vicunas (cross between male alpaca and female vicuna). Semen was obtained by electro-ejaculation. Immediately before insemination, the females were mated with vasectomized males to induce ovulation. Semen was deposited in the area of the uterine bifurcation by means of a 35 cm-long plastic catheter, guided transcervically by rectal manipulation. Only one of 42 artificially inseminated females gave birth (Table 2).

Table 2  
Results of AI in alpacas and llamas using fresh, non-extended semen.

Species [Ref.]	Route of insemination	Diagnosed pregnancy rate (%)	Birth rate (%)
Alpaca [1]	Transcervical	–	1/42 (2%)
Alpaca [10]	Transcervical	27/58 (46.6%)	Not reported
Alpacas and Llamas [29]	Transcervical	37/94 (39%)	Not reported
Alpaca [15]	Transcervical and laparoscopy	27/40 (68%)	Not reported
Alpaca [28]	Transcervical	105/207 (51%)	Not reported

In subsequent studies, pregnancy rates after AI with fresh raw or extended semen have varied from 39 to 68% (Table 2), but unfortunately birth rates have not been reported. In a study designed to determine the optimum time for AI after an ovulation-inducing stimulus (i.e., hormone treatment or copulation with a vasectomized male), 96 female alpacas were artificially inseminated 7–45 h post-stimulus. Fresh, undiluted semen collected by electro-ejaculation was infused transcervically by rectal manipulation or through a vaginal speculum [10] (Table 2). The alpacas were slaughtered 72 h after AI and the conception rate was 12, 53, 43, 75, and 58% at 7–18 h, 19–26 h, 27–34 h, 35–45 h, and 52 h after AI, respectively. No differences were observed between groups stimulated to ovulate by hormone treatment or through the use of a vasectomized male. In another study [29], 83 alpacas and 11 llamas were inseminated with fresh undiluted semen collected by electro-ejaculation from one vicuna and four paco-vicunas. Semen was deposited into the uterine horns, and ovulation was induced by administration of hCG or mating with a vasectomized male. The pregnancy rate was 48% in females induced to ovulate using hCG and 11% in females induced to ovulate using a vasectomized male. More recently [15], the pregnancy rate of female alpacas inseminated with fresh semen obtained by AV and deposited into the uterine horns transcervically or by laparoscopy (20 per group) was 73 and 67%, respectively. Similar results were reported using semen diluted with egg yolk (10%) and citrate (3%) by transcervical deposition and induction of ovulation with GnRH 24 h before AI [30]. In the most recent study [28], 51% of 207 alpacas were diagnosed pregnant after transcervical insemination using semen collected by AV and mixed 1:1 with BSA (30%) and glucose (60%); ovulation was induced with a GnRH analogue (Buserelin; Intervet) 24–26 h before AI.

The absence of data on birth rates in studies done to date is disturbing, and may reflect an underlying problem related to the method and timing of inducing ovulation. In a recent study in llamas examined

ultrasonographically every 4 h [31], the interval from ovulation-inducing stimulus (natural mating, LH, or GnRH treatment) to ovulation was  $29.4 \pm 0.6$  h and did not differ among groups. In previous studies, however, AI was done coincident with hCG treatment [15], or 24–26 h after GnRH treatment [28,30]. Systematic evaluation of the effects of semen extenders/handling and the timing of AI relative to ovulation are needed to determine the relative competence of the gametes to produce a normal conceptus and a live birth.

#### 2.4. Semen extender, cooling and freezing

Very little information is available on the use of semen extenders in camelids (Table 3). Most extenders, as well as refrigeration and freezing techniques, used on semen of alpacas and llamas over the last 20 y have been adopted from those developed for the bull and ram. Extenders used for llamas and alpacas have ranged from the simplest, such as sodium citrate in combination with egg-yolk, a sugar or skim milk, to a more complex media containing phosphate buffered saline, bovine serum albumin, and inorganic/organic buffers such as Tris and Hepes in combination with various sugars (lactose, fructose or glucose).

Motility of llama semen was conserved for 24 h post-collection when diluted with a solution of 30% BSA and 60% glucose and placed in a refrigerator [8]. Dilution with a Tris–glucose–egg yolk semen extender without elimination of viscosity resulted in poor motility (5%) after 3 h [17], but results with prior mechanical liquefaction and preservation at 5 °C improved motility. Others reported the use of semen diluted with 10% egg yolk and 3% citrate, and although no information was given about semen quality after dilution, a pregnancy rate of 60% after AI was reported [30]. In another study [32], semen collected by AV and diluted with Tris and EDTA extenders, with or without surfactant (Equex STM), was pre-warmed to 38–40 °C and maintained at that temperature until insemination; however, no pregnancies were reported.

Table 3  
Results of AI in alpacas and llamas using fresh-extended or frozen\* semen.

Species [Ref.]	Semen extender	Sperm concentration (10 <sup>6</sup> /mL)	Diagnosed pregnancy rate (%)	Birth rate (%)
Llama [42]	Tris–citric acid– fructose	28.9 ± 18.7	31.8	Not reported
Alpaca [43]	Egg–yolk–glucose citrate plus trypsin	–	64.3	Not reported
Alpaca [43]	Egg–yolk–glucose citrate plus 5 mg/mL collagenase	–	57.7	Not reported
Alpaca [43]	Egg–yolk–glucose citrate plus 5 mg/mL collagenase	8–12	61–67	Not reported
Alpaca and Llama* [40]	Egg–yolk– citrate–glycerol plus 1mg/mL collagenase	–	26 (5/19)	5
Llama* [41]	Egg–yolk–glucose plus DMSO	–	7.8 (3/38)	Not reported

Table 4

List of species and sub-species of threatened and extinct wild equids according to the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of 2007.

Genus	Species	Common name	Sub-species	Red List category	Year assessed
<i>Equus</i>	<i>burchelli</i>	Plains zebra	<i>E. b. burchelli</i>	Extinct	1996
			<i>E. b. antiquorum</i>	Least Concern	2002
			<i>E. b. chapmani</i>	Data Deficient	1996
			<i>E. b. boehmi</i>	Least Concern	2002
			<i>E. b. zambeziensis</i>	Data Deficient	1996
			<i>E. b. crawshayi</i>	Data Deficient	1996
<i>Equus</i>	<i>grevyi</i>	Grevy's zebra	none identified	Endangered	1996
<i>Equus</i>	<i>zebra</i>	Mountain zebra	<i>E. z. zebra</i>	Endangered	1996
			<i>E. z. hartmannae</i>	Endangered	1996
<i>Equus</i>	<i>africanus</i>	African wild ass	<i>E. a. africanus</i>	Critical	1996
			<i>E. a. somaliensis</i>	Critical	1996
<i>Equus</i>	<i>hemionus</i>	Asiatic wild ass	<i>E. h. hemionus</i>	Vulnerable	2002
			<i>E. h. hemippus</i>	Extinct	2002
			<i>E. h. kulan</i>	Critical	2002
			<i>E. h. khur</i>	Endangered	2002
			<i>E. h. onager</i>	Critical	2002
			<i>E. h. luteus</i>	Vulnerable	2002
<i>Equus</i>	<i>ferus</i>	Przewalski's horse	<i>E. f. przewalskii</i>	Extinct in Wild	1996
<i>Equus</i>	<i>kiang</i>	Kiang	<i>E. k. kiang</i>	Data Deficient	1996
			<i>E. k. polyodon</i>	Data Deficient	1996
			<i>E. k. holderi</i>	Least Risk	1996

Sperm collected from the caudal epididymus has been used to avoid the complications of the viscous ejaculate, and for use in IVF. Spermatozoa from llama epididymides submitted to slow refrigeration to 5 °C after dilution with Kenney, Tris–yolk or Colorado semen extender, maintained a progressive motility of 50, 30 and 20%, respectively after 72 h [33]. In a recent alpaca study [34], epididymal alpaca spermatozoa diluted with a lactose-based extender and frozen in pellets had the highest percentage of motility and intact acrosome morphology.

Reports on the use of frozen semen are also limited (Table 3). Llama semen collected by electro-ejaculation and diluted with Tris–egg yolk– glycerol had only 10% motility after freezing and thawing [35]. Ejaculates collected from two llamas and three alpacas by AV were liquefied with collagenase (1 mg/mL) and diluted first with a mixture of sodium citrate (2.9%) and egg yolk (10%) and then with 7% glycerol added at 15 min intervals in three equal parts prior to freezing [36]. Sperm motility was estimated at 80% before freezing, 60% after final dilution and cooling (before freezing), and 30–40% after thawing. Most recently [37], ethylene glycol was used as a cryoprotectant for alpaca semen collected by AV, liquefied by mechanical action, and diluted with skim milk and fructose. Post-thaw motility

was 30% and highly correlated with estimates of live sperm and the acrosome reaction. Most studies have reported sperm motility ranging from 15 to 35% after thawing [32,38,39]. In a report of the first live births of South American camelids after AI with frozen semen [40], the semen of alpacas and llamas was treated with 1 mg/mL of collagenase, followed by a two-step dilution in sodium citrate–egg yolk extender, and frozen over liquid nitrogen vapor. After thawing, sperm motility ranged from 40 to 50%, and 5/19 inseminations resulted in live births (Table 3). The inclusion of DMSO as a cryoprotectant had a negative effect on sperm motility and viability of llama semen [41].

### 3. Wild equids

The Equidae family includes the zebras, asses and horses. Although domestic and feral horses and donkeys, or burros, exist on all continents except Antarctica, wild equids are confined to the Old World (Asia and Africa). The IUCN lists seven wild species with several subspecies, the majority of which are threatened to some degree and even considered extinct in the wild (Table 4). In 2002, a Status Survey and Conservation Action Plan was published [44] that brought together the full range of what is known about

the conservation status and biology of each of the seven species of zebras, asses and horses, including what little is known of reproductive physiology in wild equids [45].

### 3.1. AI and related technologies in wild equids

Several recent reviews [46–48] have addressed the application of reproductive technologies for the conservation of threatened and endangered species, including the use of AI. Although AI and related technologies have been used in the domestic horse industry for decades [49], the technology has only recently gained momentum for use in wild equids, in part, because of the design and development of safe and effective practices of manual restraint for handling stallions and mares on a routine basis. In species such as the Przewalski's horse (*Equus ferus przewalskii*), AI technology is especially important, since the current population is based on 14 founder animals. With extinction in the wild (Table 4), genetic management is critical for overall conservation of the species *ex situ* and *in situ*. Hence, AI and related technologies will facilitate breeding with founder genetics without transportation of animals, since moving and maintaining animals can be life threatening, expensive, and logistically impractical, considering that many zoological institutions are not equipped to house and manage Przewalski's stallions and other wild equid males.

A major impediment in the development of AI and related technologies to produce pregnancies in wild equids, is the limited knowledge base of reproductive physiology in those species. Characteristics of the estrous cycle in Przewalski's horse [50] and Grevy's zebra (*Equus grevyi*) [45] have been determined by monitoring hormone metabolites in urine and feces. Reproductive characteristics in other wild equids, such as Plains zebras (*Equus burchelli*) [51,52], Persian onagers (*Equus hemionus onager*) [53], and kulan (*Equus hemionus kulan*) [54] have been determined by observing behavior. Although reproductive ultrasonography is used routinely in domestic equids, it has had limited use in wild equids. In an early study [55], transrectal ultrasonography was used to monitor follicular development in anesthetized Przewalski's mares a few times throughout the breeding season. More recently, scientists at the Smithsonian's *National Zoological Park* and the *Wilds* have developed a technique where Przewalski's and Persian onager females can be manually restrained without anesthesia and examined using transrectal ultrasonography several times per week during the breeding season (Collins, personal communication).

Although some wild equid males have been trained to an AV for semen collection [56,57], collection of semen has been done primarily with electro-ejaculation under anesthesia. In domestic stallions, electro-ejaculation apparently has not been successful [58] and has had limited success in the Przewalski's horse [56,59,60] and Persian Onager [61]. Since the latter studies involved a limited number of animals, knowledge of semen characteristics in wild equids is still rudimentary. Nonetheless, more recent progress with the use of electro-ejaculation on Przewalski's stallions has resulted in the successful collection of semen more than 85% of the time [62].

Although no pregnancies have been reported through the use of AI in wild equids, there is a renewed interest in conducting research in the Przewalski's horse at the Smithsonian's *National Zoological Park* and the *Wilds* using ultrasonography and reproductive hormones for synchronizing the estrous cycle and inducing ovulation for fixed-time AI with semen collected via electro-ejaculation. With these efforts, combined with knowledge of AI and related technologies gained from domestic equids, pregnancies in wild equids through the use of AI may soon be realized.

## 4. Domestic equids

The application of AI and related technologies in domestic horses has been reviewed [63–65]. Historically, studies on the collection, processing and insemination with stallion and jack semen were initiated in Russia in the late 1800s, Japan in the early 1900s, and in the United States around 1940 [66]. Although the basic concepts of collecting semen in domestic horses for AI are essentially unchanged since the AV was introduced in the 1930s, more efficient AV design as well as alternative protocols for preparing and using extenders with fresh and fresh-cooled semen are in routine use throughout the equine industry today as reviewed [67]. The following is an overview of some current areas of research in equine andrology (i.e., frozen semen, sexed semen and low-dose insemination) that have the potential to be adapted for application in wild equids.

### 4.1. Frozen semen

The first foal born from AI with frozen/thawed spermatozoa (collected from the epididymus) was in 1957 [68]. Only now, more than 50 y later, is the use of frozen semen gaining widespread use within the equine industry, because of increasing acceptance by breed

registries and advantages over the use of fresh or fresh-cooled semen [63–65]. In general, pregnancy rates per cycle following AI with frozen/thawed semen are lower (range, 25–45%) than with fresh and fresh-cooled semen (range, 20–80%) [63]. Although there are many factors influencing pregnancy rates associated with fresh, fresh-cooled and frozen semen (e.g., semen processing and handling, reproductive health of mare, timing and site of intrauterine insemination, experience of the inseminator), the single most important factor with frozen semen appears to be the individual stallion. Spermatozoa from approximately 25% of stallions do not survive the freezing and thawing processes [69].

The nature of the “stallion effect” on freezing and thawing of spermatozoa is not well understood, but is an area of intense research. Some areas of investigation involve identifying aspects of spermatozoa and constituents of seminal plasma of different ejaculates within and among stallions during different times of the year that may be associated with cold shock, ice crystal formation, dehydration and swelling of spermatozoa during the freezing and thawing processes [49]. In addition, *in vitro* combined with *in vivo* experiments are being done to evaluate modifications to semen extenders and alternative cryoprotectants and time intervals associated with the freezing and thawing processes as well as the removal of the cryoprotectant prior to insemination. Although various methods for freezing and thawing equine spermatozoa have been described [63–65], there will likely be no standard protocols until the results of research provide a better understanding of the “stallion effect” on post-freezing survivability of equine spermatozoa.

Cryopreservation of germplasm of threatened or endangered species has been proposed as an approach to slow or halt the rate of species decline [46,48]. One mission under this approach is for organized repositories such as Genome or Genetic Resource Banks (GRB) to cryopreserve spermatozoa from every genetically valuable male, thus providing insurance against loss of a particular species. More immediately, AI with frozen semen would allow infusion of genetic material from free-ranging and captive animals across zoos and, perhaps, back into the wild to maintain diversity and vigor. Apparently, the only documented success of cryopreservation of spermatozoa in an endangered equid is for the “Poitou” donkey (*Equus africanus assinus*) [70]. This sole documentation, however, does not reflect the substantial amount of research that is ongoing with wild equids, especially the Przewalski’s horse (Collins, personal communication).

#### 4.2. Sex-sorted semen

The first foal born with sex-sorted semen was in 1998 [71]. Currently, the commercial application of flow cytometric sexing of equine spermatozoa is limited, primarily because of cost, time, and effort involved in the sorting and insemination processes [72]. In general, sex-sorting spermatozoa by flow-cytometry is based on the difference in DNA content between sperm carrying the X or Y chromosome. Differences in DNA content of X- versus Y-bearing sperm have been determined for at least 23 mammalian species, including the stallion in which an X–Y difference of 3.7% has been reported [72]. Although the accuracy of sorting X- and Y-bearing spermatozoa ranges from 85 to 95%, the efficiency of sorting is relatively low. Currently, only ~33% of the sperm passing through the system can be sorted to achieve a high degree of accuracy, and ~20% of the sex-sorted sperm are lost during the sperm concentration and packaging processes. Hence, approximately 13% of the sperm from an ejaculate are available as sorted X- and Y-bearing spermatozoa. Moreover, the current technology can only sort 1000–1500 spermatozoa/s. Hence, 4–5 d of continuous operation would be required to produce enough equine sex-sorted spermatozoa ( $500 \times 10^6$  progressively motile sperm) for one conventional insemination dose [63–65].

Pre-selection of the sex of the offspring through the use of sex-sorted spermatozoa has great potential as a tool for breeding management and conservation of endangered wildlife, especially since re-population may be accelerated by producing predominantly female offspring [46,48]. Although sex-sorted spermatozoa has yet to be applied to wild equids, sorting of X- and Y-bearing spermatozoa has been applied to other wild species (e.g., cetaceans, cervids) [46,47,72]. Although the technological process of sexing semen will continue to be optimized for domestic and non-domestic species [72], the limitation of sorting spermatozoa in high enough numbers in a reasonable amount of time has sparked renewed interest in developing low-dose insemination techniques.

#### 4.3. Low-dose insemination

Three methods of insemination using unconventionally low numbers of equine spermatozoa have been developed [71,73]: (1) surgical insemination into the oviduct; (2) hysteroscopic insemination at the uterotubal junction; and (3) rectally-guided deep-intrauterine insemination near the uterotubal junction. Although with the first method pregnancies have been achieved



with as few as 50,000–150,000 spermatozoa, it is invasive and considered impractical in most circumstances. The hysteroscopic technique is more practical, but requires specialized equipment and expertise, and at least two operators. The endoscope is guided visually without any physical manipulation of the uterus to the uterotubal junction where the spermatozoa are deposited on the oviductal papilla. The deep-intrauterine insemination technique is the most practical technique, and was initially developed for AI with sex-sorted semen. In general, the deep-intrauterine insemination procedure involves the use of a flexible pipette to negotiate the bifurcation between the left and right uterine horns. The pipette is guided by digital transrectal manipulation, and physically maneuvered to the cranial aspect of the uterine horn ipsilateral to the preovulatory follicle where the spermatozoa are deposited near the papilla.

Typically, AI with frozen equine semen using these techniques involves a volume of 0.5 mL with 50–100 × 10<sup>6</sup> spermatozoa. With the hysteroscopic technique, however, higher pregnancy rates were reported with 3 × 10<sup>6</sup> versus 14 × 10<sup>6</sup> frozen-thawed spermatozoa [74]. Although there is a need to directly compare the efficacy of the hysteroscopic and deep-intrauterine AI techniques using frozen semen, a relatively recent study has compared the two techniques with sexed equine semen [75]. Insemination with 20 × 10<sup>6</sup> pre-cooled, sex-sorted spermatozoa resulted in pregnancy rates from 55 to 72% using the hysteroscopic AI technique compared to a pregnancy rate of 38% using the deep-intrauterine AI technique. However, hysteroscopy is more complicated in wild equids, primarily because of time and effort associated with general anesthesia. The deep-intrauterine insemination technique is more readily applicable and has been used recently with Przewalski's mares (Collins, personal communication).

## 5. Conclusion

Difficulties with semen collection, dilution and cryopreservation have limited the development and use of AI in camelid species. However, progress has been made and viable offspring have been produced through the use of AI in domestic camelids, using both fresh and frozen semen. A better understanding of the normal constituents of seminal plasma will enable the rational design of semen extenders suitable for camelids. The origin, composition, and function of the viscous component of camelid seminal plasma remain a mystery and an obvious area for future research. To improve efficiency and maximize the use of valuable males, studies are also needed to address questions of optimal

freezing and thawing procedures and insemination dose. The basis for differences in reported pregnancy rates with sexed and frozen semen in domestic equids, and the ultimate success of AI in wild equids, will require continued research into the “stallion effect”, extenders and cryoprotectants, optimal volume and number of spermatozoa, temperatures during handling, processing and transport, and insemination techniques. In both camelids and equids, research on domestic species under controlled conditions provides an excellent opportunity to develop effective semen handling techniques for application in wild and endangered species of the respective families.

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