Assisted reproductive technologies for endangered felids

Jason Herrick

Department of Reproductive Sciences, Omaha's Henry Doorly Zoo and Aquarium

Abstract

Assisted reproductive technologies (ART) could overcome many of the challenges associated with zoo-based conservation programs for endangered felids. Although felids are 1 of the most well-studied groups of zoo species, our understanding of feline biology and the protocols for ART in cats are still far behind what is available for rodents, livestock, and humans. The dangerous nature of these animals has shaped experimental designs to study nondomestic cats and determined which ART are the most relevant. Domestic cats have been produced by artificial insemination, in vitro fertilization, and embryo transfer, providing basic protocols that can be extrapolated to nondomestic cats. Variation in estrous cycle dynamics among species and variable rates of spontaneous ovulation between individuals necessitate the use of exogenous hormones for most ART procedures. For artificial insemination, pregnancies have been produced by vaginal, uterine, and oviductal inseminations. Oviductal inseminations may be the most broadly applicable based on the minimal number of sperm required, but these procedures are logistically challenging for large cats. In vitro fertilization is effective for producing viable, early-stage embryos, but culture conditions for blastocyst production remain suboptimal. In addition, performing long-term culture outside of the appropriate laboratory environment is problematic. Further optimization of oocyte and embryo vitrification would expand the applicability of these technologies. Other research priorities are the development of methods for real-time cycle monitoring and alternative gonadotropin products for stimulation of follicular development and ovulation. Finally, theriogenologists are encouraged to contact their local zoos if they are interested in providing their expertise to these efforts.

Keywords: Cat, artificial insemination, in vitro fertilization, embryo transfer

Introduction

The zoos in the US, accredited by the Association of Zoos and Aquariums (AZA), maintain 17 species of cats in managed breeding programs called Species Survival Plans (SSPs). The goal of these SSPs is to maintain genetically diverse populations (> 90% of the diversity present in the founder population), for 100 – 200 years, that could be used to supplement or reestablish wild populations that have significantly declined or gone extinct.[1] Given the small size of zoo populations that is dictated by the number of zoos exhibiting the species, reproduction is carefully managed to avoid inbreeding and maximize genetic diversity. In addition, periodic infusion of new genetics from wild individuals or unrelated captive animals from other regional populations (e.g. European zoos) is necessary. Unfortunately, animals recommended for breeding are not always located at the same zoo and, following transport, genetically matched individuals are not always behaviorally compatible. Similarly, the acquisition of new genetics is complicated by extensive regulations and permit restrictions even for international transport from 1 zoo to another. Assisted reproductive technologies (ART) could be used to overcome many of these challenges, but our understanding of basic reproductive biology in nondomestic species is severely lacking and access to the animals needed to conduct the necessary studies is limited.[2] The felids are 1 of the most well-studied groups of zoo species, but our understanding of feline biology and the protocols for ART in cats are still far behind what is available for rodents, livestock, and humans.

Many species of wild canids are housed in zoological institutions, with some species having endangered status. Captive breeding programs aim to maintain a healthy captive population and some work with wildlife agencies to support wild populations. Whereas some species reproduce well in captivity, others are less consistently successful. Some species have a predilection for pyometra and require methods to prevent, diagnose, and treat this potentially fatal condition. Breeding recommendations from species survival plan (SSP) committees sometimes involve older individuals that have not been reproductive in years or involve individuals that are not actively in a social pair bond. Techniques used in domestic dog theriogenology practice have provided a useful framework for addressing these challenges in captive wild canids.

As nondomestic felids are dangerous carnivores and routine handling for research is not possible due to both their size (Amur tiger, *Panthera tigris altaica*; 175 kg) and general temperament (black-footed cat, *Felis nigripes*, 3 kg), the domestic cat has proven to be an invaluable model for studies of basic feline biology.[3,4] As a result, most of what we know about reproduction in domestic cats, as well as many of the 'world's first' births from various ART in domestic cats, have come out of zoo-based

research programs interested in nondomestic cats.[5,6]

Estrous cycle dynamics

Repeated collection of blood samples for hormone monitoring is not possible in nondomestic felids, so virtually all that is known about estrous cycle dynamics in nondomestic felids is based on longitudinal monitoring of hormone metabolites in fecal samples.[7-11] The opportunity to noninvasively collect daily samples during routine maintenance of the animal's holding and exhibit areas has provided information on the estrous cycles of > 12 species of cat and revealed a surprising degree of diversity. Perhaps most surprising was the realization that spontaneous ovulation occurs in at least 8 species of cats, including the domestic cat,[12] with the frequency varying between both species and individuals of the same species.[7-10,12] In addition, some species exhibit prolonged periods of ovarian inactivity that may be influenced by environmental factors.[13] The most extreme example of novel reproductive mechanisms within felids is the female lynx that appears to be seasonally monoestrous with the corpora lutea resulting from induced ovulation after breeding or spontaneous ovulation persisting and producing progesterone, well-beyond the length of pregnancy and continuing until the following breeding season.[14]

Ejaculate quality

Ejaculates from male cats are characterized by a high proportion (> 40%) of sperm with abnormal morphology, including several species in which the proportion of sperm with abnormal morphology, can exceed 60% (teratospermia).[3,15,16] High incidence of abnormal sperm morphology does not seem to affect fertility following natural breeding, at least in the cheetah.[17] In most cat species, males and females breed repeatedly during estrus perhaps making up for deficiencies in quality with quantity. For example, male lions have been reported to breed females > 40 times per day during estrus.[18] However, teratospermia likely has a more pronounced effect on ART, especially when cryopreserved sperm are used, since teratospermia has been correlated with functional differences in capacitation, zona pellucida penetration, tolerance of thermal and osmotic shock, and cellular metabolism.[19,20] Although a low incidence of sperm with normal morphology is prevalent in many felid species, this condition can be further exacerbated by both genetic and environmental factors. Ejaculate traits in Florida panthers[21] and lions from genetically-isolated populations[22] suggest that reduced genetic heterozygosity is associated with an increased incidence of abnormal sperm forms. Similarly, a recent study of snow leopards indicated that ejaculate traits were correlated with the dietary content of several nutrients.[23]

Exogenous hormones

Given the variability of the feline estrous cycle, ARTs require exogenous hormones to stimulate follicular development and ovulation at specific times. Follicular development is most often stimulated with equine chorionic gonadotropin (eCG, pregnant mare's serum gonadotropin) because this hormone has an extended half-life and is effective following a single dose, compared to follicle stimulating hormone (FSH) preparations that often require multiple, sometimes daily, injections.[24-26] Effective doses of eCG have been identified for most cat species and are not related to body size.[24] Following eCG treatment, ovulation can be induced with either human chorionic gonadotropin (hCG) or porcine luteinizing hormone (pLH), but fertility is greatly improved when pLH is used.[27] Recent studies used oral progestins (altrenogest, Regumate®, Merck Animal Health, Madison, NJ) to down-regulate endogenous ovarian activity prior to gonadotropin treatments. This treatment facilitates planning and allows eCG treatment in the absence of large follicles or functional corpora lutea.[28] The combined protocol of altrenogest, eCG, and pLH has resulted in excellent pregnancy rates in domestic cats following AI, as well as pregnancies in several nondomestic cat species.[25] However, the dose and duration of altrenogest, and the period between its cessation and the injection of eCG, may need to be optimized for each species.[29]

Artificial insemination

For AI in cats, the 2 big questions have been when and where. In eCG-hCG treated domestic cats, that were inseminated (laparoscopic uterine insemination) prior to ovulation, 14% became pregnant, and of those inseminated following ovulation, 50% became pregnant.[30] The conclusion was that periovulatory anesthesia may interfere with ovulation and/or sperm transport. This led further studies to focus almost exclusively on identifying the relative time of ovulation in each species, so that AI could be performed postovulation.[24] However, pregnancy rates in domestic cats induced to ovulate with hCG in a natural cycle were higher when AI was done preovulation.[31] Lions given a gonadotropin releasing hormone (GnRH) analogue (burserelin-acetate, Receptal®, Intervet, South Africa) to stimulate ovulation during natural estrus had excellent pregnancy rates (~ 33%) with preovulatory inseminations.[32]

Pregnancies have been produced in domestic cats following inseminations in which sperm were deposited in the vagina, uterus, and oviduct.[24,25,31,33] Most of the 'world's first' pregnancies in nondomestic cats were accomplished with laparoscopic uterine inseminations, but that was due to the almost exclusive use of that technique at the time.[24] Recently, oviductal inseminations were more widely used that resulted in higher success rates than previous uterine AI studies, due in part to the use of the improved eCG-pLH protocol.[25,34] Although direct comparisons of various insemination sites were not conducted in nondomestic cats, domestic cat studies suggest that acceptable (> 50%) pregnancy rates can be achieved with any of the possible sites of sperm deposition.

From a technical perspective, intravaginal AIs are the easiest to perform and, with appropriate training, could even be performed without sedation. In contrast, oviductal inseminations may be among the most challenging given the difficulty of catheterizing a ~ 1 mm opening via laparoscopy. Uterine inseminations, whether laparoscopic or transcervical also require extensive experience,

although that experience may be more widely available given the popularity of these techniques in small ruminants and domestic dogs. For the large cat species, there are logistical advantages to a transcervical procedure that could be performed in the female's holding area compared to a laparoscopic procedure that most often involves transport to the zoo hospital's surgical suite. In contrast, the need to transport females for laparoscopic procedures is a negligible factor for many of the small cats.

Ultimately, the most appropriate site of sperm deposition may be determined by the quantity and quality of sperm available. In domestic cats, intravaginal inseminations require $\sim 80 \times 10^6$ sperm compared to uterine inseminations in which 8×10^6 sperm are required and oviductal inseminations in which $2 - 4 \times 10^6$ sperm are effective.[25,31,35] Similar numbers of sperm appear to be effective for oviductal AIs in nondomestic cats.[36,37] In contrast, the limited data available on intravaginal or transcervical AIs in nondomestic cats suggest insemination doses require at least 100×10^6 sperm, with most pregnancies resulting from the use of $> 500 \times 10^6$ sperm.[32,38] For many of the cat species, especially the small cats, ejaculates rarely contain $> 100 \times 10^6$ sperm.[7,15,16] Snow leopards averaged 120×10^6 sperm per ejaculate that is sufficient for just a single transcervical AI compared to ~ 24 oviductal inseminations.[23] If cryopreserved sperm are used, the number of AIs per ejaculate would be even smaller. Additional studies are necessary to better define minimum sperm numbers needed for pregnancy that may be dependent on further refinements to synchronization protocols

In vitro fertilization and embryo transfer

With limited numbers of sperm available from a single ejaculate, IVF and embryo transfer (ET) could be a valuable ART for population management.[39] Offspring have been produced using these procedures in domestic cats, as well as several species of small cats, tigers, and cheetahs.[40-43] Exogenous hormone protocols stimulating follicular development can be easily adapted for oocyte collections and the laparoscopic follicular aspirations can be used to collect oocytes.[44,45] Sperm capacitation occurs readily in various culture media, fertilization rates are high, and resulting embryos will progress to the blastocyst stage.[41,46,47] Most pregnancies have resulted from the laparoscopic transfer of early cleavage stage embryos into the oviduct. Pregnancies from the transfer of later stage morulae or blastocysts into the uterus are far less common, which may be related to the reduced rate of cell division for in vitro cultured embryos compared to their in vivo counterparts.[41,47] Further optimization of culture conditions to support the development of viable blastocysts could facilitate transcervical embryo transfers.

In addition to the biological challenges, IVF and ET present a unique set of logistical challenges. First, few zoos in the US are equipped with suitable laboratories for IVF and embryo culture. In most cases, portable laboratory equipment was used to create 'mobile labs' that can be set-up in the corner of a zoo's veterinary hospital.[48] This approach has been successful, especially for short-term culture to cleavage stages,[42,48] but far from

an ideal environment for such sensitive procedures.[49] Second, most zoos only maintain a single female of each species. Two immobilizations of the same female for the oocyte collection and the subsequent embryo transfer may be overly stressful on the females (and staff) and the uterine environment of a gonadotropin-stimulated cycle is not always ideal for implantation.[50] Portable incubators may allow embryos to be transported short distances to other zoos for transfers of fresh embryos, but in most cases, IVF and ET will involve embryo cryopreservation. Recent advancements with oocyte and embryo vitrification in humans suggest it may be possible to vitrify oocytes immediately after collection at 1 zoo, warm those oocytes for IVF and embryo culture in a suitable laboratory, vitrify the resulting embryos, and warm just prior to transfer.[51] Domestic cat kittens were produced from vitrified oocytes and vitrified embryos, but further research is necessary to improve these procedures.[52,53.]

Future directions

Every aspect of ART in felids, even domestic cats, requires additional optimization. However, a few areas that would be particularly valuable. The first is improved methods for real-time monitoring of estrous cycles in females that do not exhibit overt behavioral changes. Fecal hormone monitoring is a great method for cycle characterization, but it only provides a retrospective indication of when a female was in estrus. Currently, vaginal cytology is the only method that can be used to determine cycle status in real-time, but it requires extensive training with the animal.[32,54] Behavioral training for voluntary blood collections (e.g. tails accessed under cage bars) would also allow the analysis of concentrations of hormones in serum that can be determined faster and with less 'noise' than a fecal hormone assay. Importantly, these techniques would be equally valuable to managers attempting to time animal introductions as it would be for researchers hoping to perform ART.

Although behavioral training and real-time cycle monitoring may allow for more ART procedures to be completed in the context of a natural cycle, there will still be a need for exogenous gonadotropins in some cases. Unfortunately, the 2 hormones that are the most widely used currently, eCG and pLH, are difficult to acquire. To the best of author's knowledge, eCG (PMSG) is only available from a single commercial source (ProSpec, East Brunswick, NJ) in the US and there are no longer any commercial sources for pLH, at least not in the quantities needed for in vivo use. In the very near future, new hormones have to be identified and tested for efficacy in felids. A variety of hormones are used within the human fertility industry, but many of these are designed for repeated, daily injections. Longer acting preparations would be ideal for cats. However, behavioral training for voluntary injections is becoming more common in zoos that could provide additional options that are already commercially available.

Finally, there is a need for more personnel to conduct ART procedures in cats. Currently there are only 3 research groups

in the US working on ART in nondomestic cats (Smithsonian Conservation Biology Institute, Cincinnati Zoo and Botanical Gardens' Center for Conservation and Research of Endangered Wildlife, and Omaha's Henry Doorly Zoo and Aquarium). Application of ART to cats housed in all of the zoos in the US requires extensive travel by these scientists, greatly increasing the costs of the research. However, it is possible that university- and/or private practice-based veterinarians and theriogenologists could be trained for these procedures and assist their local zoos. In addition, many of these theriogenologists already have extensive experience with techniques, such as vaginal cytology and endoscopic TCI, that are still new to many zoo-based researchers. Collaborations are common between zoo veterinarians and local specialists in veterinary dentistry, ophthalmology, orthopedics, etc. Expanding these collaborations between zoos and private and university-based theriogenologists would provide zoos, especially those without staff scientists, with valuable expertise to improve breeding efforts and advance the development of ART for felids and other endangered species.

Conflict of interest

There are no conflicts of interest to declare.

Acknowledgement

References

1. Soulé M, Gilpin M, Conway W, et al: The Millenium Ark: How long a voyage, how many staterooms, how many passengers? Zoo Biol 1986;5:101-113.

2. Herrick JR: Assisted reproductive technologies for endangered species conservation: developing sophisticated protocols with limited access to animals with unique reproductive mechanisms. Biol Reprod 2019;100:1158-1170.

3. Howard JG, Brown JL, Bush M, et al: Teratospermic and normospermic domestic cats: ejaculate traits, pituitary-gonadal hormones, and improvement of spermatozoal motility and morphology after swim-up processing. J Androl 1990;11:204-215.

4. Swanson WF, Roth TL, Wildt DE: In vivo embryogenesis, embryo migration, and embryonic mortality in the domestic cat. Biol Reprod 1994;51:452-464.

5. Goodrowe KL, Wall RJ, O'Brien SJ, et al: Developmental competence of domestic cat follicular oocytes after fertilization in vitro. Biol Reprod 1988;39:355-372.

6. Pope CE, McRae MA, Plair BL, et al: In vitro and in vivo development of embryos produced by in vitro maturation and in vitro fertilization of cat oocytes. J Reprod Fertil 1997;51(Suppl):69-82.

7. Herrick JR, Bond JB, Campbell M, et al: Fecal endocrine profiles and ejaculate traits in black-footed cats (Felis nigripes) and sand cats (Felis margarita). Gen Comp Endocrinol 2010;165:204-214.

8. Brown JL: Female reproductive cycles of wild female felids. Anim Reprod Sci 2011;124:155-162.

9. Putman SB, Brown JL, Franklin AD, et al: Characterization of ovarian steroid patterns in female African lions (Panthera leo), and the effects of contraception on reproductive function. PLoS One 2015;10:e0140373.

10. Barnes SA, Andrew Teare J, Staaden S, et al: Characterization and manipulation of reproductive cycles in the jaguar (Panthera onca). Gen Comp Endocrinol 2016;225:95-103.

11. Reichert-Stewart JL, Santymire RM, Armstrong D, et al: Fecal endocrine monitoring of reproduction in female snow leopards (Uncia uncia). Theriogenology 2014;82:17-26.

12. Pelican KM, Brown JL, Wildt DE, et al: Short term suppression of follicular recruitment and spontaneous ovulation in the cat using levonorgestrel versus a GnRH antagonist. Gen Comp Endocrinol 2005;144:110-121.

13. Wielebnowski NC, Ziegler K, Wildt DE, et al: Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (Acinonyx jubatus). Animal Conservation 2002;5:291-301.

14. Jewgenow K, Painer J, Amelkina O, et al: Lynx reproduction--long-lasting life cycle of corpora lutea in a feline species. Reprod Biol 2014;14:83-88.

15. Pukazhenthi B, Laroe D, Crosier A, et al: Challenges in cryopreservation of clouded leopard (Neofelis nebulosa) spermatozoa. Theriogenology 2006;66:1790-1796.

16. Crosier AE, Marker L, Howard J, et al: Ejaculate traits in the Namibian cheetah (Acinonyx jubatus): influence of age, season and captivity. Reprod Fertil Dev 2007;19:370-382.

17. Lindburg DG, Durrant BS, Millard SE, et al: Fertility assessment of cheetah males with poor quality semen. Zoo Biol 1993;12:97-103.

18. Sunquist M, Sunquist F: Wild Cats of the World. Chicago, IL: The University of Chicago Press, 2002.

19. Pukazhenthi BS, Neubauer K, Jewgenow K, et al: The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology 2006;66:112-121.

20. Terrell KA, Wildt DE, Anthony NM, et al: Evidence for compromised metabolic function and limited glucose uptake in spermatozoa from the teratospermic domestic cat (Felis catus) and cheetah (Acinonyx jubatus). Biol Reprod 2010;83:833-841.

21. Roelke ME, Martenson JS, O'Brien SJ: The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Curr Biol 1993;3:340-350.

22. Brown JL, Bush M, Packer C, et al: Developmental changes in pituitary-gonadal function in free-ranging lions (Panthera leo leo) of the Serengeti Plains and Ngorongoro Crater. J Reprod Fertil 1991;91:29-40.

23. Herrick JR, Iske CJ, Santymire RM, et al: Factors affecting reproductive traits in male snow leopards (Uncia uncia). Reprod Fertil 2020;1:35-49.

24. Howard JG, Wildt DE: Approaches and efficacy of artificial insemination in felids and mustelids. Theriogenology 2009;71:130-148.

25. Swanson WF: Practical application of laparoscopic oviductal insemination for the propagation of domestic cats and wild felids. Reprod Fertil Dev 2019;31:27-39.

26. Thongphakdee A, Tipkantha W, Punkong C, et al: Monitoring and controlling ovarian activity in wild felids. Theriogenology 2018;109:14-21.

27. Conforti VA, Bateman HL, Schook MW, et al: Laparoscopic oviductal artificial insemination improves pregnancy success in exogenous gonadotropin-treated domestic cats as a model for endangered felids. Biol Reprod 2013;89:4.

28. Stewart RA, Pelican KM, Brown JL, et al: Oral progestin induces rapid, reversible suppression of ovarian activity in the cat. Gen Comp Endocrinol 2010;166:409-416.

29. Crosier AE, Comizzoli P, Koester DC, et al: Circumventing the natural, frequent oestrogen waves of the female cheetah (Acinonyx jubatus) using oral progestin (Altrenogest). Reprod Fertil Dev 2017;29:1486-1498.

30. Howard JG, Barone MA, Donoghue AM, et al: The effect of pre-ovulatory anaesthesia on ovulation in laparoscopically inseminated domestic cats. J Reprod Fertil 1992;96:175-186.

31. Tsutsui T, Tanaka A, Takagi Y, et al: Unilateral intrauterine horn insemination of fresh semen in cats. J Vet Med Sci 2000;62:1241-1245.

32. Callealta I, Ganswindt A, Malan M, et al: Non-surgical artificial insemination using a GnRH analogue for ovulation induction during natural oestrus in African lions (Panthera leo). Theriogenology 2019;139:28-35.

33. Chatdarong K, Axner E, Manee-In S, et al: Pregnancy in the domestic cat after vaginal or transcervical insemination with fresh and frozen semen. Theriogenology 2007;68:1326-1333.

34. Tipkantha W, Thuwanut P, Maikeaw U, et al: Successful laparoscopic oviductal artificial insemination in the clouded leopard (Neofelis nebulosa) in Thailand. J Zoo Wildl Med 2017;48:804-812.

35. Tanaka A, Takagi Y, Nakagawa K, et al: Artificial intravaginal insemination using fresh semen in cats. J Vet Med Sci 2000;62:1163-1167.

36. Lambo CA, Bateman HL, Swanson WF: Application of laparoscopic oviductal artificial insemination for conservation management of Brazilian ocelots and Amur tigers. Reprod Fertil Develop 2014;26:116.

37. Vansandt LM, Adania CH, Yanai PR, et al: Ovarian synchronization, ovulation induction, and successful artificial insemination in the jaguar (Panthera onca). 51st Annual Conference of the American Association of Zoo Veterinarians 2019.

38. Silva JNC, Leitão RM, Lapão NE, et al: Birth of Siberian tiger (Panthera tigris altaica) cubs after transvaginal artificial insemination. J Zoo Wildl Med 2000;31:566-569.

39. Swanson WF, Johnson WE, Cambre RC, et al: Reproductive status of endemic felid species in Latin American zoos and implications for ex situ conservation. Zoo Biol 2003;22:421-441.

40. Donoghue AM, Johnston LA, Seal US, et al: In vitro fertilization and embryo development in vitro and in vivo in the tiger (Panthera tigris). Biol Reprod 1990;43:733-744.

41. Pope CE, Gomez MC, Dresser BL: In vitro production and transfer of cat embryos in the 21st century. Theriogenology 2006;66:59-71.

42. Herrick JR, Mehrdadfar F, Campbell M, et al: Birth of sand cat (Felis margarita) kittens following in vitro fertilization and embryo transfer. Biol Reprod 2010:6-7.

43. Pope CE: Aspects of in vivo oocyte production, blastocyst development, and embryo transfer in the cat. Theriogenology 2014;81:126-137.

44. Herrick JR, Campbell M, Levens G, et al: In vitro fertilization and sperm cryopreservation in the black-footed cat (Felis nigripes) and sand cat (Felis margarita). Biol Reprod 2010;82:552-562.

45. Crosier AE, Lamy J, Bapodra P, et al: First birth of cheetah cubs from in vitro fertilization and embryo transfer. Animals (Basel) 2020;10.

46. Murakami M, Dong YJ, Suzuki T, et al: Development and subsequent cryotolerance of domestic cat embryos cultured in serum-free and serum-containing media. Cryobiology 2011;63:170-174.

47. Herrick JR, Lyons SM, Greene-Ermisch AF, et al: A carnivore embryo's perspective on essential amino acids and ammonium in culture medium: effects on the development of feline embryos. Biol Reprod 2018;99:1070-1081.

48. Swanson WF: Research in nondomestic species: experiences in reproductive physiology research for conservation of endangered felids. ILAR J 2003;44:307-316.

49. Wale PL, Gardner DK: The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. Hum Reprod Update 2016;22:2-22.

50. Shapiro BS, Daneshmand ST, Garner FC, et al: Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. Fertil Steril 2011;96:344-348.

51. Cobo A, Castello D, Vallejo B, et al: Outcome of cryotransfer of embryos developed from vitrified oocytes: double vitrification has no impact on delivery rates. Fertil Steril 2013;99:1623-1630.

52. Tharasanit T, Manee-In S, Buarpung S, et al: Successful pregnancy following transfer of feline embryos derived from vitrified immature cat oocytes using 'stepwise' cryoprotectant exposure technique. Theriogenology 2011;76:1442-1449.

53. Pope CE, Gomez MC, Galiguis J, et al: Applying embryo cryopreservation technologies to the production of domestic and black-footed cats. Reprod Domest Anim 2012;47(Suppl 6):125-129.

54. Asa CS, Junge RE, Bircher S, et al: Assessing reproductive cycles and pregnancy in cheetahs (Acinonyx jubatus) by vaginal cytology. Zoo Biol 1992;11:139-151.