

Faecal steroid metabolites assay as a non-invasive monitoring of reproductive status in animals

Ashok Kumar, S Mehrotra, S S Dangi, G Singh, Subhash chand, Lakhanpal Singh, A S Mahla, Sachin Kumar, K Nehra

Division of Animal Reproduction

Indian Veterinary Research Institute, Izatnagar-243 122, Dist. Bareilly, UP, India

Corresponding author: Ashok kumar, email: drashokkumar39@gmail.com

Received: 08-05-2012, Accepted: 24-05-2012, Published online: 30-11-2012

How to cite this article: Kumar A, Mehrotra S, Dangi SS, Singh G, Chand S, Singh L, Mahla AS, Kumar S and Nehra K (2013) Faecal steroid metabolites assay as a non-invasive monitoring of reproductive status in animals, *Vet World* 6(1):59-63. doi: 10.5455/vetworld.2013.59-63

Abstract

Measurement of faecal steroids as a non-invasive technique is widely used to monitor reproductive hormones in captive and free-ranging wild animals. This method offers a great advantage over invasive techniques like blood collection and deserves to be used in domestic animals. Repeated blood sampling is stressful, having animal welfare issues, difficult to obtain in field condition. In the faeces of cow, estradiol 17-β predominated, whereas in mares and sows, estradiol 17-β and estrone are main estrogens. Steroids are metabolized in liver, excreted in faeces and extraction is done by using a variety of methods such as ethanol, methanol etc. Faecal estrogen and progesterone metabolites evaluations are well established approaches for monitoring of reproductive functions in a variety of mammalian species. So in future this method may be the most suitable for monitoring of reproductive status in farm animals particularly under field conditions.

Key words: Faecal steroids, non-invasive technique, estrogen, progesterone metabolites, reproductive status

Introduction

Determination of reproductive status is one of the most important factors for effective management in modern dairy industry. Captive management of any species benefits from understanding its basic reproductive biology, including endocrinology [1]. The non-invasive monitoring of hormones permit frequent sample collection and the alternatives are urine, milk and faeces. The estimation of urinary steroids has been used extensively to monitor the physiological status of animals [2,3]. The urine collection from farm animal species requires animal handling and fixing of catheters and milk can only be obtained from lactating animals, limits their uses for investigation particularly in heifers and dry-off animals. The faecal steroid metabolites opened up new windows for non-invasive monitoring of reproductive status. Therefore, faecal steroids are the most practicable choice for this purpose [4]. Studies of faecal steroid hormones were first conducted mainly on their metabolism and excretion [5], further studies done regarding the puberty, estrous cycle, pregnancy, abortion, reproductive behaviour, seasonality and the monitoring of treatment therapies in zoo and domestic animals [6].

Invasive Vs Non invasive methods

Invasive and non invasive monitoring of ovarian activity and pregnancy diagnosis have been done in domestic animals by determination of reproductive steroids in plasma/serum [7] and milk [8], urine [2], saliva [9] and faeces [10], respectively.

Excretion of faecal steroids

In general faecal estrogens are measured using specific estrogen antibodies or antibodies against total

unconjugated estrogens. Most species voided faeces containing a higher percentage of free than conjugated steroids. Estrogens are end products of steroid metabolism and therefore the compounds in plasma and faeces are similar [11]. Progesterone is mainly metabolized by liver prior to its faecal excretion in ruminants [6]. About 99% of excreted progesterone is unconjugated compounds [12] and the principal faecal progesterone metabolites are 5α and 5β pregnane series in cows. The steroid hormone metabolites quantified in faeces, widely used in studies of wild animals, as a non invasive, non stressor, economical and animal saving technique which allows longitudinal studies by permitting frequent sampling of the same individual [13]. Such characteristics justify the use of this technique with laboratory and domestic animals. The route of excretion varies considerably among the species as well as between steroids within the same species. For example, in sheep 77% of progesterone metabolites excreted via faeces [12] in contrast 97% in domestic cat [13]. Several studies comparing plasma and faecal steroids value during the estrous cycle indicated a delay of faecal steroids excretion compared to that in plasma having a lag time of about 12-24 hour in ruminants and about 24 to over 48 hour in hind gut fermenters like horse, pig, rhino, elephant, primates [14]. It is 12 hour between milk progesterone and its faecal metabolites in cows. The delay time between the circulation of steroids in plasma and their appearance in urine samples rather short less than 5 hr, but faecal steroid metabolites have an appreciable lag time which approximately correlates with lag time necessary for the intestinal passage of bile to the rectum [12]. The lag time is affected by digestibility of the forage, which influences the rate of passage of digesta [15]. Rabiee *et*

al. [16] studied progesterone clearance with different diets and reported that changes in feed have less effect on faecal progesterone. Faecal progesterone metabolites after parturition did not decrease to basal value until 3 to 4 days [17] indicating the lag time. The difference in excretion time of steroid between the estrous cycle and postpartum is probably caused by the very high concentration present during pregnancy and by the enterohepatic circulation which retards the excretion [18]. Palme [19] addressed the main topic of concern in faecal steroid hormone metabolites such as sample collection and storage, time delay, extraction procedures, assay selection and validation as well as the confounding factors.

Extraction of faecal steroids

Extraction of the faecal steroids was done by Mostl *et al.* [11] and Hirata and Mori [20] in cattle by using diethyl ether while phosphate buffer solution was used by Hulten *et al.* [21] in gilts. Methanol was used in buffaloes [22], in cows [10,23] and ethanol by Capezzuto *et al.* [24] in goats. Recently extraction of fecal samples with petroleum ether has represented a valid alternative to other more time-consuming methods of determining faecal progesterone concentrations in the peripubertal female bottlenose dolphin (*Tursiops truncatus*) by Biancani *et al.* [25].

Faecal estrogen

Estradiol-17 is the primary and biologically active estrogen secreted both from theca and granulosa cells of growing ovarian follicles. Their effects on follicular growth are primarily mediated through estrogen receptor and expression of their receptor has been detected in granulosa cells of bovine antral follicles at various stages of its development [26]. Faecal estrogen determination was done in pregnant women [27], farm animals [11], bottlenose dolphin (*Tursiops truncatus*) [25], pichis *Zaedyus pichiy* (*Xenarthra: Dasypodidae*) [28], Gilbert's potoroo (*Potorous gilbertii*) [29], plains zebra mares [30], pygmy rabbits (*Brachylagus idahoensis*) [31], Snow Leopards (*Panthera uncia*) [32], Veiled Chameleons (*Chamaeleo calyptrotus*) [33], chinchilla [34]. Faecal estrogen concentration was at peak on day 4 post estrus and the lowest during 2nd week of estrous cycle in buffalo [35] indicating a lag time of days 4 between serum and faecal concentrations, the same observation was reported by Kumar [36]. Although some studies reported the determination of the preovulatory estrogen peak in mares, but it was unsuccessful in cows which could be due to low plasma concentrations. It is a reliable indicator of ovulation in feline and canine species [37,38]. Faecal steroids has been used to monitor estrous cycle of giant panda (*Ailuropoda melanoleuca*) [39] and to evaluate ovarian function in Javan Gibbons (*Hylobates moloch*) [40] and Black-and-Gold Howlers (*Alouatta caraya*) [41]. Serum estradiol measures are poor indicators of follicular activity in elephants, whereas urinary estrogen analyses

are capable of characterizing the two follicular waves that occur during the estrous cycle [42] and also possible that measurements of other steroid metabolites in serum, urine or faeces might reveal differences that were not evident by serum testosterone measures alone [43].

Estrone sulphate is the major estrogen produced by conceptus (feto-placental unit) and can be measured in maternal plasma, milk, faeces etc. in domesticated hoof stock, red buffalo, yak, Grevy's Zebra, Nubian ibex, musk ox, caribou, gorilla, orangutan, Mhor gazelle, Przewalski's mare and Malaysian tapir, yellow baboon, sable antelope, bison, cynomolgus monkey and wild felids [6,32,44,45] and could be a reliable indicator of fetal viability as well [11]. Pregnancy can be confirmed by both faecal and plasma estrogen after day 120 in cows and mares [46], after the 12th week in cows [47] and 14th weeks onwards in buffalo [36]. In sow, there is an estrogen peak in the blood between day 23-30 of gestation [48], which can be detected in faeces [49] as a method of pregnancy diagnosis. The applicability of non-invasive steroid hormones techniques for monitoring of reproductive status in wild boar with positive correlation to serum values, enabling a more informed and correct management of the species [50].

Faecal estrogen determination in the male can be applied to those species, which produce high amounts of estrogens in the testis, like stallion and boar. Faecal estrogen values of mature stallions can reach values compared to those of pregnant mares [46] and has been shown to be a reliable indicator of cryptorchidism in horses [51].

Faecal progesterone metabolites

Progesterone is principal steroid hormone secreted mainly by the corpus luteum (CL) that regulates estrous cycle and maintains pregnancy in all farm animals. Adrenal cortex and placenta also secrete some amount of progesterone during certain specific physiological stages like pregnancy. Faecal progesterone analysis has been successfully used for monitoring estrous cycle, pregnancy, abortion, puberty and seasonality in various animal species including primates [52], and wild animals mainly in caribous, rhinoceroses, felids, okapis, moose, minks, wolves, sable antelope etc. [6], brown brocket deer [53], Tsushima leopard cat [44] and recently in fishing cat (*Prionailurus viverrinus*) [54]. In African elephants, measures of progesterone and cortisol metabolites in faeces provide indices of reproductive function and physiological stress [55]. Monitoring of estrous cycle by faecal progesterone steroids in buffalo has been investigated by Arunji [35] who found pattern of faecal progesterone metabolites was virtually similar to that of plasma progesterone with delay time of days 2-4 and obtained faecal progesterone metabolites was basal on estrus and highest on day 8 of estrous cycle. Same correlation between serum and faecal progesterone was reported

by Kumar, [36] in buffalo. In buffalo, while studying the effect of Crestar™ on estrus synchronization, Hattab *et al.* [22] concluded that the progesterone concentration in blood clearly correlated with the concentration of metabolites in faeces, similar to finding of Kumar [36] and suggested that non-invasive method is a valuable tool for determining the luteal status.

Faecal steroid metabolites analysis has been successfully used for monitoring of reproductive status in mares [4], swine [21], cows [23], goats [24] and free ranging ungulates [56]. Rabiee *et al.* [57] studied plasma, milk and faecal progesterone concentration during estrous cycle of lactating dairy cows with different milk yields and concluded that milk production has no effect on faecal progesterone concentration. Isobe *et al.* [10] found that sensitivity and specificity of pregnancy diagnosis for beef and dairy heifers and beef cows was 71–79% at day 21–24 post-breeding by faecal progesterone metabolites which was comparable to the 72–77% in milk and serum progesterone profiles [7]. Kornmatitsuk *et al.* [23] measured faecal progesterone metabolites in dairy cows on day 19–22 post-breeding and found that it was 100% accurate in diagnosing non pregnant cows whereas accuracy of the pregnancy diagnosis was 67% and changes in faecal progesterone concentration were corresponding to serum values. Significance difference found between non-pregnant and pregnant buffalo on 3rd week was basis for as a tool for pregnancy diagnosis in buffalo by faecal progesterone assay [36] and Souza *et al.* [58] correlated faecal progesterone and estradiol metabolites with serum concentrations as well as physiological changes associated with pregnancy in *Cerdocyon thous*. Faecal progesterone metabolite analysis proved to be a reliable method for mapping oestrous cycle activity, but was not useful for the prediction of oestrus in captive southern hairy-nosed wombats (*Lasiorhinus latifrons*) [59]. Comparing vaginal cytology with faecal estrogen and progesterone metabolites, Frederick *et al.* [60] found that changes in genital appearance and behavior were consistent and complementary to hormonal data in sun bear (*Helarctos malayanus*). There was a significant positive correlation between plasma progesterone and dry faecal progesterone concentrations in goats [24], in buffalo [36] and in large hairy armadillo (*Chaetophractus villosus*) and crying armadillo (*Chaetophractus vellerosus*) [61]. The significant correlations between dry fecal and plasma progesterone concentrations validated this method for monitoring reproductive status in these species. Recently, Curry *et al.* [62] investigated the potential pregnancy biomarker proteins based on their increased abundance in the faeces of pregnant polar bears compared to pseudo-pregnant females (controls) promising a strong foundation for ensuing efforts to develop a non-invasive pregnancy assay for use in both captive and wild polar bears.

Conclusion

Determination of endocrine status by faecal

steroids is one of the important tools for efficient management, and efforts to use assisted reproductive technologies like artificial insemination, synchronization and embryo transfer; diagnosis of reproductive disorders depend on the knowledge of basic reproductive physiology of a particular species. Faecal steroid analysis needs to be explored as a non invasive tool to monitor reproductive status of the animals keeping in view of field application.

References

1. Wildt, D.E., Swanson, W.F., Brown, J., Sliwa, A., Vargas, A. (2010) Felids ex situ: managed programs, research and species recovery. In: Macdonald, D., Loveridge, A.J. (Eds.), *The Biology and Conservation of Wild Felids*. Oxford University Press, Oxford, pp. 217–236.
2. Lokutoff, N.M., Ott, J.E. and Lasley, B.L. (1983). Strategies for assessing ovarian function in exotic species. *J. Zoo. Anim. Med.*, 14: 3-12.
3. Steven, L.M., Caroline, M. and David, E.W. (1991). Urinary steroid metabolic profiles in female Pere David's deer (*Elophurus davidianus*). *J. Zoo. Wildl. Med.*, 22: 78-85.
4. Schwarzenberger, F., Mostl, E., Bamberg, E. and von Hegel, G. (1992). Monitoring of corpus luteum function by measuring progestagens in faeces of non-pregnant mares (*Equus caballus*) and Przewalski mares (*Equus przewalskii*). *Anim. Reprod. Sci.*, 29: 263-273.
5. Adlercreutz, H., Martin, F., Javenpan, P. and Fotsis, T. (1979). Steroid absorption and enterohepatic recycling. *Contraception*, 20: 201-223.
6. Schwarzenberger, F., Mostl, E., Palme, R. and Bamberg, E., (1996). Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. *Anim. Reprod. Sci.*, 42: 515-526.
7. Batra, S.K., Arora, R.C., Bachalus, N.K. and Pandey, R.S. (1979). Blood and milk progesterone in pregnant and non-pregnant buffalo. *J. Dairy Sci.*, 62: 1390-1393.
8. Gao, Y., Short, R.V. and Fletcher, T. P. (1988). Progesterone concentrations in plasma, saliva, and milk of cows in different reproductive states. *Br. Vet. J.*, 144: 262-268.
9. Kanchev, L.N., Marinova, C.H.P. and Stankov, B.M. (1988). Bovine salivary progesterone applications to the assesment of ovarian function and early pregnancy diagnosis. *Anim. Reprod. Sci.*, 17: 1-8.
10. Isobe, N., Akita, M., Nakao, T., Yamashiro, H. and Kubota, H. (2005). Pregnancy diagnosis based on the faecal progesterone concentration in beef and dairy heifers and beef cows. *Anim Reprod. Sci.*, 90: 211-218.
11. Mostl, E., Choi, H.S., Wurm, W., Ismail, M.N. and Bamberg, E. (1984). Pregnancy diagnosis in cows and heifers by determination of oestradiol-17 in faeces. *Br. Vet. J.*, 140: 287-291.
12. Palme, R., Fischer, P., Schildorfer, H. and Ismail, M.N. (1996). Excretion of infused "14 C-steroid hormones via faeces and urine in domestic livestock. *Anim. Reprod. Sci.*, 43: 43-63.
13. Brown, J.L., Wasser, S.K., Wildt, D.E. and Graham, L.H. (1994). Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured non invasively in faeces. *Biol. Reprod.*, 51: 776-786.
14. Wasser, S.K., Monfort, S.L., Souters, J. and Wildt, D.E. (1994). Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*) faeces. *J. Reprod. Fertil.*, 101: 213-220.
15. Adams, N.R., Abordi, L.A., Briegel, J.R. and Sanders, M.R. (1994). Effect of diet on the clearance of estradiol-17β in the ewe. *Biol. Reprod.*, 51: 668-674.

16. Rabiee, A. R., Dalley, D., Borman J. M., Macmillan, K.L. and Schwarzenberger, F. (2001). Progesterone clearance rate in lactating dairy cows with two levels of dry matter and metabolizable energy intakes. *Anim. Reprod. Sci.*, 66: 35-46.
17. Schwarzenberger, F., Mostl, E., Bamberg, E., Pammer, L and Schmechlik, O. (1991). Concentrations of progestagens and oestrogens in the faeces of pregnant Lipizzan, Trotter and Thoroughbred mares. *J. Reprod. Fert., Suppl.*, 44: 489-499.
18. Schwarzenberger, F., Patzl, M., Francke, R., Ochs, A., Buitter, R., Schaftenaar, W. and DeMeurichy, W. (1993). Fecal progestagen evaluations to monitor the estrous cycle and pregnancy in the okapi (*Okapia johnstoni*). *Zoo Biol.*, 12: 549-559.
19. Palme, R. (2005). Measuring Faecal steroids: guidelines for practical application. *Ann. N.Y. Acad. Sci.*, 1046: 75-80.
20. Hirata, S. and Mori, Y. (1995). Monitoring reproductive status faecal progesterone analysis in ruminants. *J. Vet. Med. Sci.*, 57: 845-850.
21. Hulten, F., Zhang, B.R., Frosberg, M. and Dalin, A.M. (1995). Applying a progesterone assay to faecal samples collected from sows during the oestrous cycle. *Reprod. Dom. Anim.*, 30: 101-106.
22. Hattab, S.A., Kadom, A.K., Palme, R. and Bamberg, E. (2000). Effect of Crestar on estrus synchronization and the relationship between faecal and plasma concentrations of progesterone in buffalo cows. *Theriogenology.*, 54: 1007-1017.
23. Kornmatitsuk, B., Thitaram, C. and Kornmatitsuk, S. (2007) Measurement of faecal Progesterone Metabolites and its application for Early Screening of Open Cows Post-insemination. *Reprod. Dom. Anim.*, 42(3): 238-242.
24. Capezzuto, A., Chelini, M.O.M., Felipe, E.C.G. and Oliveira, C.A. (2008). Correlation between serum and fecal concentrations of reproductive steroids throughout gestation in goats. *Anim. Reprod. Sci.*, 103:78-86.
25. Biancani, B., Da Dalt, L., Lacave, G., Romagnoli, S. and Gabai, G. (2009). Measuring fecal progestogens as a tool to monitor reproductive activity in captive female bottlenose dolphins (*Tursiops truncatus*). *Theriogenology*, 72 :1282–1292.
26. Rowsenfield, C.N.S., Yuan, X., Manikkam, M., Calder, M.D., Gavernick, H.A. and Lubshes, D. B. (1999). Cloning, sequencing and localization of bovine estrogen receptor-beta within the ovarian follicle. *Biol. Reprod.*, 60: 691-697.
27. Adlerkreutz, H. and Martin, F. (1976). Oestrogen in human pregnancy faeces. *Acta Endocrinol.*, 83: 410-419.
28. Superina, M., Carreno, N. and Jahn, G.A. (2009). Characterization of seasonal reproduction patterns in female pichis *Zaedyus pichiy* (Xenarthra: Dasypodidae) estimated by fecal sex steroid metabolites and ovarian histology. *Animal Reproduction Science*, 116: 358–369.
29. Richardson, E.S., Bradshaw, D., Friend, T. and Fletcher, T. (2010). Monitoring reproduction in the critically endangered marsupial, Gilbert's potoroo (*Potorous gilbertii*): Preliminary analysis of faecal oestradiol-17b, cortisol and progestagens. *General and Comparative Endocrinology*, 165: 155–162.
30. Ncube, H., Duncan, P., Grange, S., Cameron, E.Z., Barnier, F. and Ganswindt, A. (2011). Pattern of faecal 20-oxopregnane and oestrogen concentrations during pregnancy in wild plains zebra mares. *General and Comparative Endocrinology*, 172: 358–362.
31. Shipley, L.A. and Brown, J.L. (2011). Characterizing gonadal and adrenal activity by fecal steroid analyses in pygmy rabbits (*Brachylagus idahoensis*). *General and Comparative Endocrinology*, 171: 373–380.
32. Kinoshita, K., Inada, S., Seki, K., Sasaki, A., Hama, N. et al. (2011). Long-Term Monitoring of Fecal Steroid Hormones in Female Snow Leopards (*Panthera uncia*) during Pregnancy or Pseudopregnancy. *PLoS ONE* 6(5): e19314. doi: 10.1371/journal.pone.0019314.
33. Kummrow, M.S., Gilman, C., Mackie, P., Smith, D.A. and Mastromonaco, G.F. (2011). Non invasive Analysis of Fecal Reproductive Hormone Metabolites in Female Veiled Chameleons (*Chamaeleo calyptratus*) by Enzyme Immunoassay. *Zoo Biology*, 30: 95–115.
34. Busso, J.M., Ponzio, M.F., Cuneo, F.D.M. and Ruiz, R.D. (2012). Reproduction in chinchilla (*Chinchilla lanigera*): current status of environmental control of gonadal activity and advances in reproductive techniques. *Theriogenology*, PubMed ID:22541170.
35. Arunji, J.T.K., (2008). Non-invasive monitoring of buffalo estrous cycle. M. V.Sc. Thesis, IVRI, Izatnagar.
36. Kumar, A., (2011). Faecal steroids as a tool of pregnancy diagnosis in buffalo. M. V.Sc. Thesis, IVRI, Izatnagar.
37. Gross, T.S. (1992). Development and use of faecal steroid analyses in several carnivore species. In: W. Schaftenaar, R.M. Buitter and S.J. Dielman (Editors), *The First International Symposium on Faecal Steroid Monitoring in Zoo Animals*, Rotterdam, pp. 55-61.
38. Herrick, J.R., Bond, J.B., Campbell, M., Levens, G., Moore, T., Benson, K., D'Agostino, J., West, G., Okeson, D.M., Coke, R., Portacio, S.C., Leiske, K., Kreider, C., Polumbo, P.J., Swanson, W.F., (2010). Fecal endocrine profiles and ejaculate traits in black-footed cats (*Felis nigripes*) and sand cats (*Felis margarita*). *Gen. Comp. Endocrinol.*, 165: 204–214.
39. Kersey, D.C., Wildt, D.E., Brown, J.L., Snyder, R.J., Huang, Y. and Monfort, S.L. (2010). Endocrine milieu of perioestrus in the giant panda (*Ailuropus melanoleuca*), as determined by non-invasive hormone measures. *Reproduction Fertility and Development*, 22: 901–912.
40. Maheshwari, H., Sjahfirdi, L., Astuti, P., Purwantara, B. et al. (2010). Fecal steroid profile of female Javan gibbons (*Hylobates moloch*) maintained in pairing-typed cage. *Hayati Journal of Biosciences*, 17: 43-49.
41. Kugelmeier, T., Rodrigo de l Rio doValle, Marcelo Alcindo de Barros Vaz Guim areas, et al. (2011). Tracking the Ovarian Cycle in Black-and-Gold Howlers (*Alouatta caraya*) by Measuring Fecal Steroids and Observing Vaginal Bleeding. *Int. J. Primatol*, 32:605–615.
42. Czekala, N.M., MacDonald, E.A., Steinman, K., Walker, S., Garrigues, N.M., Olson, D. and Brown, J.L. (2003). Estrogen and LH dynamics during the follicular phase of the estrous cycle in the Asian elephant. *Zoo Biol.*, 22:27–36.
43. Mouttham, L.L., Buhr, M., Freeman, E.W., Widowski, T.M., Graham, L.H. and Brown, J.L. (2011). Interrelationship of serum testosterone, dominance and ovarian cyclicity status in female African elephants. *Animal Reproduction Science*, 126:115–121.
44. Adachi, I., Kusuda, S., Nagao, E., Taira, Y., Asano, M., Tsubota, T. and Doi, O. (2010). Fecal steroid metabolites and reproductive monitoring in a female Tsushima leopard cat (*Prionailurus bengalensis euphilurus*). *Theriogenology*, 74: 1499–1503.
45. Brown, J.L. (2011). Female reproductive cycles of wild female felids. *Animal Reproduction Science*. 124: 155–162.
46. Bamberg, E., Choi, H.S., Möstl, E., Wurm, W., Lorin, D. and Arbeiter, K. (1984). Enzymatic determination of unconjugated oestrogens in faeces for pregnancy diagnosis in mares. *Equ. Vet. J.*, 16: 537-539.
47. Daniel, M., Desaulniers, A. K., Goff, K. J. Betteridge, J. E. and Rowell, P. F. F. (1989). Reproductive hormone concentrations in faeces during the oestrous cycle and pregnancy in cattle (*Bos taurus*) and muskoxen (*Ovibos moschatus*). *Canadian Journal of Zoology.*, 67:(5) 1148-1154.
48. Robertson, H.A. and King, G.J., (1974). Plasma concentration of progesterone, estrone, estradiol 17- and estrone sulfate in pig at implantation, during pregnancy and at parturition. *J. Reprod. Ferti.*, 40: 133-141.
49. Choi, H.S., Kiesenhofer, E., Gantner, H., Hois, J. and Bamberg, E. (1987). Pregnancy diagnosis in sows by estimation of oestrogens in blood, urine and faeces. *Anim. Reprod. Sci.*, 15: 209-216.
50. Macchi, E., Starvaggi Cucuzza, A., Badino, P., Odore, R., Re, F., Bevilacqua, L. and Malfatti, A. (2010). Seasonality of

- reproduction in wild boar (*Sus scrofa*) assessed by fecal and plasmatic steroids. *Theriogenology*, 73:1230–1237.
51. Palme, R., Holzmann, A. and Mitterer, T. (1994). Measuring fecal estrogens for the diagnosis of cryptorchidism in horses. *Theriogenology*, 42: 1381 - 1387.
 52. Heistermann, M., Tari, S. and Hodges, J.K. (1993). Measurement of faecal steroids for monitoring ovarian function, in New World primates, Callitrichidae. *J. Reprod. Fertil.*, 99: 243-251.
 53. Pereira, R.J., Polegato, B.F., de Souza, S., Negro, J.A. and Duarte, J.M. (2006). Monitoring ovarian cycles and pregnancy in brown brocket deer (*Mazama gouazoubira*) by measurement of faecal progesterone metabolites. *Theriogenology*, 62: 387-399.
 54. Santymire, R.M., Brown, J.L., Stewart, R.A., Santymire, R.C., Wildt, D.E. and Howard, J. (2011). Reproductive gonadal steroidogenic activity in the fishing cat (*Prionailurus viverrinus*) assessed by fecal steroid analyses. *Animal Reproduction Science*, 128: 60–72.
 55. Foley, C.A. H., Papageorge, S. and Wasser, S. K. (2001). Non invasive stress and reproductive measures of social and ecological pressures in free-ranging African elephants. *Conservation Biology*, 15: 1134-1147.
 56. Borque, C., Sonia, S., Garnelo, P., Delclaux, M., Martínez, E. and Fuente, J.D.I. (2011) Fecal Steroid Evaluation to Monitor Reproductive Status in Wild Ungulate Females Using Enzyme Immunoassay Commercial Kits. *Journal of Zoo and Wildlife Medicine*, 42: 537-551.
 57. Rabiee, A. R., Macmillan, K.L. and Schwarzenberger, F. (2002). Plasma, milk and faecal progesterone concentrations during the estrous cycle of lactating dairy cows with different milk yields. *Anim. Reprod. Sci.*, 74: 121-31.
 58. Souza, N. P., P. Furtado, V. and Rodrigues da Paz, R.C. (2012). Non-invasive monitoring of the estrous cycle in captive crab-eating foxes (*Cerdocyonthus*). *Theriogenology*. 77 :233–239.
 59. Hogan, L.A., Phillips, C.J.C., Lisle, A., Keeley, T., Horsup, A.B., Janssen, T. and Johnston, S.D. (2010). Non-invasive methods of oestrus detection in captive southern hairy-nosed wombats (*Lasiorhinus latifrons*). *Animal Reproduction Science*, 119:293–304.
 60. Frederick, C., Kyes, R., Hunt, K., Collins, D., Durrant, B. and Wasser S.K. (2010). Methods of estrus detection and correlates of the reproductive cycle in the sun bear (*Helarctos malayanus*). *Theriogenology*, 74:1121–1135.
 61. Luaces, J.P., Ciuccio, M., Rossi, L.F., Faletti, A.G., Cetica, P.D., et al. (2011). Seasonal changes in ovarian steroid hormone concentrations in the large hairy armadillo (*Chaetophractus villosus*) and the crying armadillo (*Chaetophractus vellerosus*). *Theriogenology*, 75:796–802.
 62. Curry, E., Stoops, M.A. and Roth, T.L. (2012). Non-invasive detection of candidate pregnancy protein biomarkers in the feces of captive polar bears (*Ursus maritimus*). *Theriogenology*, PMID: 22538002.
