



Concentration Of Cortisol Metabolites In Captive Sumatran Elephants At Elephant Conservation Facilities In Aceh

Rosa Rika Wahyuni¹, Sri Wahyuni², Al Azhar², Gholib²

¹*Program Studi Magister Kesehatan Masyarakat Veteriner, Universitas Syiah Kuala*

²*Dosen Fakultas Kedokteran Hewan Universitas Syiah Kuala, Banda Aceh*

Email for correspondence: sha_12024@yahoo.co.id

Abstract

This study was conducted to validate the DRG Cortisol ELISA EIA-1887 Germany kit for measure the concentration of stress hormone metabolites (cortisol) from the feces and its correlation to the stressor factor in captive elephants in PKG and CRU of Aceh. These factors are location, diet and presence of livestock. There is no special treatment, observation based on the activity, behavior or natural condition of the animals. The sampling technique was non invasive, fresh dung samples of each (± 20 gram) were collected from 25 elephants in CRU and PKG. Feces taken in the morning (before the animals are bathed) along with the observation of animal behavior. All samples were collected and stored at -20°C until the analysis process. The validation test are analytic (parallelism) and biological validation test. The analytic test result (parallelism), showed that the sample curve was not parallel to the standard curve, but crossed the standard curve. While the results of biological validation test, DRG Cortisol ELISA EIA-1887 Germany kit can measure the concentration of cortisol hormone feces of Sumatran elephant and able to describe the difference of cortisol concentration relation to physiological events (stress vs non-stress). The mean values of cortisol metabolite concentrations from PKG Saree (Komplek PKG and Hutan Seunapet), Sampoiniet CRU, Cot Girek, Das Peusangan, Meulaboh and Aceh Timur were (577 ng/g and 400 ng/g), 435ng /g, 419ng /g, 517ng / g, 401ng/g and 425ng /g. The measurement results correlate with the physiological conditions and observed factors.

Keywords: Validation, ELISA, Stress, Cortisol

Background

The Sumatran Elephant (*Elephas maximus sumatranus*) is one of Asia's endemic elephant subspecies on the island of Sumatra (Sukumar 1994), endangered (IUCN 2013). The life of the largest land mammal is protected by the state as stated in Law No. 5 of 1990 on Conservation of Biological Natural Resources and its Ecosystem. In an ex-situ elephant conservation effort, captive Sumatran elephants are maintained in Elephant Conservation Institutions (PKG), one of which is the Elephant Conservation Center (PKG) Aceh. The existence of PKG Aceh is determined based on Minister of Forestry Decree Number: 173/Kpts-II/1991 as conservation institutions where elephant conservation or preservation is outside their habitat to become extinct (including CRU). The management of this conservation institution should be implemented based on

ethical principles and animal welfare (PP Menhut, 2012).

Animal welfare can be achieved if the animal does not experience stress. Stress is a physiological, biochemical, and animal behavior response to a variety of physical, chemical, and biological factors that can lead to increased glucocorticoids, especially cortisol and corticosterone in blood and feces (Hoferdan East, 1999). In elephants, glucocorticoid metabolites are also excreted in the feces. Increased concentration of glucocorticoids (cortisol) in the feces is an indication of stress on these animals. Measurement of glucocorticoid hormone levels is used as a parameter of the animal response to stress (Wasser et al., 2000; Möstl and Palme 2002; Millspaugh and Washburn 2004; Palme et al., 2005; Sheriff et al., 2011).

Analysis of the concentration of glucocorticoid metabolites in feces can be

done by non-invasive methods aimed at measuring adrenocortical activity and studies of mammalian animal welfare (Keay et al., 2006), primates (Anestis, 2010) and domesticated animals (Hodges et al., 2010). The advantage of noninvasive methods is to allow long-term studies of animals to be conducted without direct contact so that they may cause bias of the research data (Brown et al., 1994; Schwarzenberger et al., 1996; Hamasaki et al., 2001; Möstl et al. , 2005).

The enzyme-linked immunosorbent assay method (ELISA) is commonly used for the measurement of hormone concentrations in various types of samples (analytes) (Kou et al. 2011; Gholib, 2011; Barja et al. 2012; Benhaïem et al., 2012; Rangel-Negrin et al 2014). The use of ELISA techniques in non-invasive research has the potential to help the wildlife conservation world by providing a better understanding of animal behavior and welfare (Graham et al.2002; Heistermann et al. 2004; Erika, 2015) in this case is the Sumatran elephant captive in Aceh. However, the application of this technique for the purpose of regular stress monitoring of captive Sumatran elephants in Aceh is constrained by the large analytical costs associated with the high cost of special analysis kits for animals.

Based on the phenomenon, it is necessary to examine the welfare status of captive elephant elephants in some conservation institutions such as PKG and CRU in Aceh by measuring cortisol levels and their correlation to a number of stressors in Aceh Elephant conservation sites such as location, feed and livestock.

Materials and Methods

The subjects were 25 captive elephant elephants in six conservation sites, namely PKG Saree (in the PKG complex and Tahuno Seunapet Forest), CRU Meulaboh, Sampoiniet CRU, Cot Girek CRU, East Aceh CRU, and Peusangan Bener Meriah CRU. From 20 gram in each elephant, fresh fecal samples were taken as much as the morning before the animals were bathed along with the observation of animal behavior. The metabolite of cortisol was extracted from feces using methanol

and determined its concentration by ELISA technique using commercial cortisol kit DRG Instruments GmbH Germany, catalog number EIA-1887. The ELISA kit used is a commercial ELISA kit DRG Cortisol ELISA EIA-1887 Germany, preceded by analytical (parallelism) and biological (Analogues et al., 2007; Rangel-Negrin et al., 2014) test. there are special treatments, observations based on the activity, behavior or natural condition of the animals The factors analyzed by correlation with stress hormone concentration are the location, feed and presence of livestock present in the captive elephant in PKG and CRU in Aceh.

Test Procedure

Sample Preparation.

Samples of frozen feces that have been removed from the freezer at thawing in the oven at 50°C for an hour then homogenized. The fecal sample is taken by the thumb and inserted into the labeled film tube. The sample is then dried in an oven with a temperature of 50 ° C until the sample is completely dry and then pulverized until it becomes powder and sieved.

Sample Extraction

The extraction of faecal samples was carried out in reference to Pettit et al. (2007), as follows. Fecal powder of 0.05-0.06 gram was put into 15ml tube, added 3ml methanol 80% and shaken (vortex) with multitube vortexer for 15 minutes. Next the sample was centrifuged at 3000 rpm for 10 min, 0.5 ml of supernatant was taken to microtube 1.5 ml, and dried in 37OC oven until the methanol solvent ran out (dry) and stored again in the freezer until hormonal analysis was performed.

Measurement of cortisol hormone metabolite with ELISA technique

a. Analytical Validation (Parallelism Test).

In this study, the Parallelism test was performed to represent the analytical validation test on the commercial catalyst Cortisol ELISA (EIA-1887 DRG Instruments GmbH, Germany). This test aims to determine the linearity of the sample curve and the standard curve used and to

determine the appropriate level of dilution. To obtain the appropriate level of dilution is to consider the position of the standard curve with the sample curve being tested. In this test the selected sample is G1 and G33 samples. Then do some comparison of dilution in stratified, that is 1: 2, 1: 4, 1: 8 and 1:16 using assay buffer. Furthermore, the similarity of slope test between standard curve and sample curve (Pettitt et al., 2007 and Zar, 1996).

b. Biological Validation

Biological validation is performed to determine whether the measured hormone matches the physiological picture of the individual or sample being tested. The sample used is a sample with ID G1-G8 from elephant individual named Paychit, Each sample is different the time of its taking and different conditions experienced by the animals. These different times and conditions will be a reference to the suitability of sample status to the high value of cortisol concentration obtained.

c. Measurement of cortisol hormone metabolites

The fecal extract was diluted with buffer assay with a ratio of 1:50. A total of 25 µl of standard solutions, controls, and samples were each inserted into a different microtiter plate (ELISA plate) and incubated for 5 min at room temperature. After that 200 µl enzyme conjugate was added into each well and reincubated for 60 min at room temperature. The ELISA plate was washed with 400 µl wash solution on each well three times, then dried by tapping the plate on an absorbant paper. The next stage added 200 µl substrate solution to each well and incubated for 15 minutes at room temperature. The enzymatic reaction was discontinued by adding 100 µl stop solution to each well plate. The abscission value of the solution is read immediately using an ELISA reader at a wavelength of 450 nm.

Results and Discussion

A. Analytical Validation (Parallelism Test)

Analytical validation is a validity test before performing hormonal analysis. The test aims to determine whether there is cross

reactivity between antibodies in the hormone and antigen (hormone) assays contained in the sample. It is important to know whether the tested sample contained substances that could disturb the binding process between the commercial cortisol ELISA kit antibody and the antigen from the sample to be measured (Gholib et al., 2014, 2016)

The ELISA cortisol kit (EIA-1887, DRG Instruments GmbH, Germany) is a kit designed to measure cortisol concentrations in serum or human plasma samples. There has been no report on the validity of the kit for measurement of cortisol metabolite concentrations contained in the captive elephant faeces. The selection of EIA-1887 cortisol kit in this study was based on various considerations, namely the price of the kit is cheaper than the price of other commercial cortisol kits such as EIA-K003-H5 cortisol kit, Arbor Assay production, Michigan, USA. The next consideration is a statement in the EIA-1887 kit manual that the EIA-1887 cortisol kit can also be used to measure cortisol concentrations in blood samples of experimental animals and primates. The good validity of the kit has been reported by Melinda (2016) for measurement of peanut cortisol concentrations of peanuts. Therefore, in this study, an analytical and biological validation test was conducted on EIA-1887 cortisol kit used to measure the concentration of cortisol hormone in captive Sumatran elephant in six conservation institutions (PKG and CRU) in Aceh.

Analytical validation test result that is parallelism test using two samples (1 and 2) shows the curve sample position and the parallel standard curve (Figure 3). According to Perez et al. (2004), the parallelism between the standard curve and the sample curve shows that there is no substance interfering with the antibody attachment process with the antigen in the assay system used. Thus, the position of the sample curve that is not parallel to the standard curve in this study indicates that in the sample of the elephant faeces tested (samples 1 and 2) there is a substance that interferes with the attachment of antibodies to the antigen-cortisol kit antigen EIA-1887.

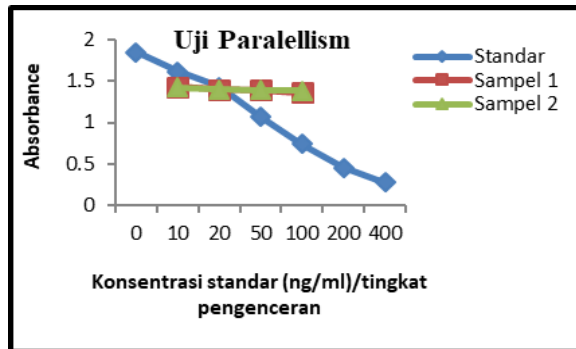


Figure 1. Parachelite cortisol test results of EIA-1887 DRG, Germany. The sample curve is not parallel to the standard solution curve.

The effect of the intruder component on the sample matrix can be minimized by diluting the appropriate sample (PB, 2007) or using a suitable solvent in the manufacture of the extract (AL, 2016). The best dilution rate and type of solvent for the purpose of measuring the concentration of Sumatran elephant cortisol present in the stool sample using the EIA-1887 cortisol kit should be further investigated.

B. Biological Validation

Biological validation is a test performed to determine whether the measured hormone corresponds to the physiological picture of the individual or sample being tested (Heistermann, 2010). Biological validation of the EIA-1887 cortisol commercial kit was performed by extracting feces from a recently rescued elephant child from East Aceh and transferred to PKG Saree. The results of the examination showed that there was a difference in the concentration of cortisol metabolite on the elephant according to some measures given during translocation and treatment.

The highest cortisol concentrations were detected in the early adaptation period including post-treatment, which gradually decreased and stabilized when the elephant child was placed with the caregiver and the cortisol concentration began to rise again as the elephant child was separated from the caregiver (Figure 4). This condition indicates that the EIA-1887 cortisol kit used can measure various physiological changes

in animals as a result of the treatment of translocation, care, and placement that is a form of stressors that trigger stress. Changes in physiologic conditions associated with stress are indicated by increased concentrations of cortisol secreted in the feces. The presence of fluctuations in cortisol concentration closely matches the physiological conditions of the animal (stress and non-stress) due to the elephant's response to various events or actions given. This is in line with the statement of Heistermann (2010), that a biologically validated hormone analysis kit can detect hormone concentrations in accordance with the physiological conditions of the individual being tested. Therefore, based on the results of the biological validation test, the EIA-1887 cortisol kit has good biological validity and can be used to determine stress levels in captive Sumatran elephants.

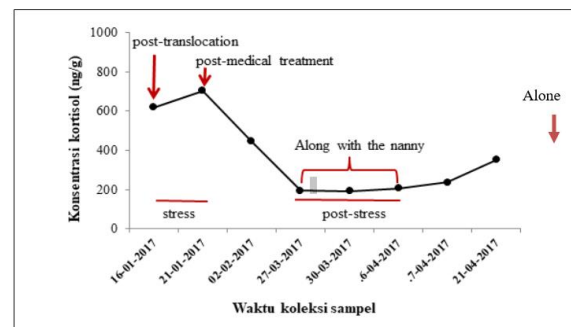


Figure 2. Graph of Biological Validity Test Result Result of biological validity test of EIA-1887 cortisol kit. Concentration of captive sumatran elephant cortisol on different physiological conditions, namely stress (post-displacement and treatment), post-stress (with parenting), and early stress (separation from nanny).

C. Measurement The concentration of stress hormone (cortisol) on Sumatran elephant using commercial kit Cortisol ELISA EIA-1887 Germany

The results of the measurement of cortisol hormone concentrations using the ELISA cortisol kit (DRG Instruments GmbH Germany, EIA-1887 catalog number) in the captive gajahsumatera in conservation institutions in Aceh varied according to individual elephants and the location of conservation agencies, with mean SDs of

462, 100,87 ng $\pm$ 59 The highest concentration of cortisol was found in elephant group kept in PKG Complex, that is 576,72 $\pm$ 29,37 ng while the lowest concentrations were found in elephants kept in the Seunapet Forest, 400.38 $\pm$ 28.86 ng (Figure 2). However, there was no statistically significant difference ($P > 0.05$). In addition, no statistically significant differences were found between the capillary cortisol concentrations of male and female sumatin, 475.1 $\pm$ 92.7 vs 444.7 $\pm$ 113.2 ng ($P > 0.05$).

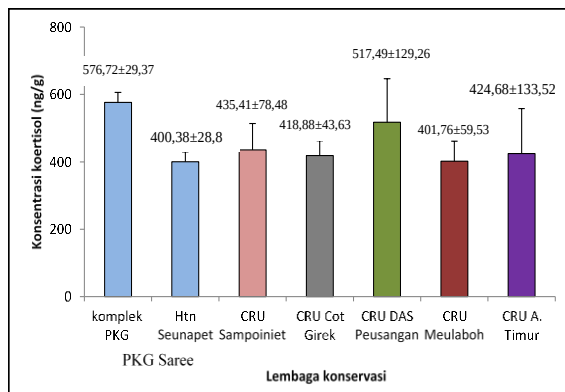


Figure 3. Concentration of captive Sumatran elephant cortisol in conservation institutions in Aceh. The highest concentrations were found in elephant group in PKG Complex and lowest in Seunapet Forest, but statistically no different ($P > 0.05$).

D. Correlation between welfare status and cortisol hormone metabolite levels in Sumatran captive elephant in Aceh.

Although there was no statistically significant difference ($P > 0.05$) regarding the concentration of captive Sumatran elephant cortisol in six conservation institutions (PKG and CRU), the concentrations of cortisol hormones in elephants at PKG Saree and Peusangan Peatland CRU showed a higher tendency compared with captive Sumatran elephants in other conservation areas. Concentration of cortisol on Sumatran captive elephant in PKG complex was highest, while the lowest cortisol concentration was found in captive elephant elephant in Seunapet Forest. This suggests that there is a difference in stress levels in Sumatran captive elephants among conservation agencies, indicating a different level of wellbeing, although there is no

statistically significant difference in hormone levels ($P > 0.05$).

Some of the factors that cause high levels of stress on elephants in PKG are the many interactions between elephants with humans and livestock, the limitations of natural feed and water sources. In addition, the location of PKG Saree is very close to the Banda Aceh-Medan highway and the large number of visitors and the presence of livestock (buffaloes, cattle, and goats) belonging to PKG officers or residents. The Sumatran captive elephant in the Saree PKG complex also received relatively monotonous foods such as coconut pelepah (83%) and natural grass (15%) and provided access to 2x1x1 cubic meter water reservoirs and limited water availability for drinking and bathing (Table 3, Figures 5 and 6). The presence of large numbers of livestock (buffaloes, goats, and cows) that surround PKG cause competition in getting source of feed. Free-released cattle can reach and feed on elephants, whereas elephants can not reach all places due to limited movement due to legs being tied up with limited length chains. Vegetation of trees for shade in PKG complex is very rare, so the animals are also often overheated in the hot day.

The above conditions are very different from the captive Sumatran PKG Saree elephant placed in the forest location of Seunapet or other conservation institution in Aceh. Although there are also buffaloes (buffaloes, cows or wild elephants) elephants at other conservation institutions in Aceh are fed with natural grass (80-100%) and abundant drinking water sources (rivers) and can be used to soak the animals (Table 3). Teduhan also still a lot while the competition to get the feed is not too often due to the availability of abundant feed.

Conclusion

The concentration of cortisol hormone metabolite as an indicator of stress on elephants maintained in six Aceh conservation agencies (PKG and CRU) can be measured by ELISA techniques using commercial cortisol kit EIA-1887 There is a difference in the level of welfare of Sumatral elephant captive in conservation

institutions in Aceh caused by various stressors at the site of maintenance.

References

- [AL] Ansh Labs. 2016. *Assay Validation for Animals*. <http://www.anshlabs.com-Assay-validation-for-animal>. 445 Medical Center Blvd, Webster, TX 77598.
- Anestis SF. (2010). Hormones and social behavior in primates. *Evolutionary Anthropology*. 2010;19: 66–78. doi: 10.1002/evan.20253. [Cross Ref]
- Barja, I., G. Escribano-Avila, C. Lara-Romero, E. Virgos, J. Benito. And E. Rafart. 2012. Non-invasive monitoring of adrenocortical activity in European badgers (*Meles meles*) and effects of sample collection and storage on faecal cortisol metabolite concentrations. *Animal Biology*. 62:419-432.
- Benhaiem, S., M. Dehnhard, R. Bonanni, H. Hofer, W. Goymann, K. Eulenberger, dan M.L. East. 2012. Validation of an Enzyme Immunoassay for the measurement of fecal glucocorticoid metabolites in spotted hyenas (*Crocuta crocuta*). *General and Comparative Endocrinology*. 178:265-271.
- Brown, J., 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 feces. *Biol. Reprod*. 51:776-86.
- Erika T. Stevenson. (2015). Validation of a Non invasive Technique for the Assessment of Physiological Stress in Coyotes (*Canis latrans*). Utah State University.
- Fulkerson WJ, Jamison P A 1982 Pattern of cortisol release in sheep following administration of synthetic ACTH or imposition of stressor agents. *Australian Journal of Biological Sciences* 35: 215–222.
- Gholib. 2011. Non-Invasive Hormone Monitoring: Faecal androgen and Glucocorticoid in Male Crested Macaques (*macaca Nigra*) in Relation to Seasonal and Social Factors. *Thesis*. Postgraduate School, IPB, Bogor
- Goymann W, Möstl E, Van't Hof T, East M, Hofer H 1999 Noninvasive fecal monitoring of glucocorticoids in spotted hyenas (*Crocuta crocuta*). *General and Comparative Endocrinology* 114: 340–348.
- Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. *Methods Ecol. Evol.* 3, 757–765.
- Graham, L. H., Webster, T., Richards, M., Reid, K., and Joseph, S. 2002. Ovarian function in the Nile hippopotamus and the effects of Depo-Provera (TM) administration. *Reproduction* 60:65-70
- Hamasaki, S., Yamauchi, K., Ohki, T., Murakami, M., Takahara, Y., Takeuchi, Y., & Mori, Y. (2001). Comparison of various reproductive status in sika deer (*cervus nippon*) using fecal steroid analysis. *The Journal of Veterinary Medical Science*, 63(2), 195-198
- Heistermann, M., Ademmer, C., and Kaumanns, W. 2004. Ovarian cycle and effect of social changes on adrenal and ovarian function in *Pygathrix nemaeus*. *Int. J. Primatol.* 25:689-708
- Heistermann, M., Palme, R., and Ganswindt, A. 2006. Comparison of different enzyme immunoassays for assessment of adrenocortical activity in primates based on fecal samples. *Am. J. Primatol.* 68:257-73
- Heistermann M. (2010). Non-invasive monitoring of endocrine status in laboratory primates: Methods, guidelines and applications. *Adv. Sci. Res.* 1: 1-9
- Hirschenhauser, K., Kotrschal, K., & Möstl, E. (2005). Synthesis of measuring steroid metabolites in goose feces. *Annals of the New York Academy of sciences*, 1046(1), 138-153.
- Hodges, K., Brown, J., Heistermann, M., 2010. Endocrine monitoring of reproduction and stress. In: Kleiman, D.G., Thompson, K.V., Baer, C.K. (Eds.), *Wild Mammals in Captivity: Principles and Techniques for Zoo Management*, second ed. University of Chicago Press, Chicago, IL, pp. 447–468
- Hofer H, East ML (1998) Biological conservation and stress. In Moeller A P, Millinsky M, Slater P L (eds) *Stress and*

- behaviour: advances in the study of animal behaviour*. Academic Press, San Diego: 405–426
- IUCN, 2013. IUCN Red List of Threatened Species. 2013.2.
- Keay, J.M., J. Singh, M.C. Gaunt, and T. KAUR. 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature. Review. *Journal of Zoo and Wildlife Medicine* 37: 234–244.
- Kou, M.T., D. S. Jong, and W. S. Lai, 2011. A Biological validation procedur for the measurements of fecal outputs and fecal kortisol metabolites in male Syrian hamsters. *Chinese Journal of Physiology*. 54(5):347-355
- Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen. Comp. Endocrinol.* 138, 189–199.
- Möstl, E., and R. Palme. (2002). Hormones as indicators of stress. *Domestic Animal Endocrinology* 23:67–74.
- Möstl, E., E.S. Rettenbacher, and R. Palme. (2005). Measurement of corticosteron metabolit in birds' dropping: an analytical approach. *Ann. NY Acad. Sci.* 1046: 17-34
- Palme, R., S. Rettenbacher, C. Touma, S.M. EL-bahr, and E. Möstl. (2005). Stress hormones in mammals and birds comparative aspects regarding metabolism, excretion, and non-invasive measurement in fecal samples. *Annals the New York Academy of Sciences* 1040: 162–171.
- Palme, R., (2005). Measuring Fecal Steroids Guidelines for Practical Application. *Ann. N.Y. Acad. Sci.*, 1046: 75-80
- [PB] Pierce Biotechnology. 2007. *Spike-and-Recovery and Linearity of Dilution Assessment*. Thermo Fisher Scientific Inc. Rockford (USA). Tersedia pada: <http://www.piercenet.com/files/TR0058-spike-and-Recovery.pdf>
- Perez GC, Laita SGB, Illera del Portal JC, Leisa JP. 2004. Validation of an EIA technique for the determination of salivary cortisol in cattle. *J Agricultural Res.* 2 (1): 45-51
- Pettitt, B.A., C.J Wheaton, and J.M. Waterman (2007). Effect of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*). *General Comparative Endocrinol.* 150:1-11.
- P.G. Vimalraj and M.G. Jayathangaraj. (2012). Non-Invasive Monitoring Of Fecal Cortisol Metabolites Level In Free-Ranging Asiatic Elephants In Response To Stress Due To Environmental Factors. *Australian Journal of Basic and Applied Sciences*, 6(13): 154-158.
- PP Kemenhut RI. (2012). Peraturan Menteri Kehutanan Republik Indonesia. Nomor 31 Tahun 2012 Tentang Lembaga Konservasi
- Rangel-Negrin, A., E. Flores-Escobar, R. Chavira, D. Canales-Espinosa, and P.A.D. Dias (2014). Physiological and analytical validations of fecal steroid hormone measures in black howler monkeys. *Primates.* 5:213-220.
- Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? *Endocr Rev* 21:55–89
- Schwarzenberger, F., E., Mostl, R., Palme and E., Bamberg, 1996. Faecal steroid analysis for non-invasive monitor and of reproductive status in farm, wild and zoo animals. *Animal Reproduction Science*, 42: 5: 15-526.
- Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R., & Boonstra, R. (2011). Measuring stress in wildlife: Techniques for quantifying glucocorticoids. *Oecologia*, 166(4), 869-887.
- Shutt K, Setchell JM, Heistermann M (2012) Non-invasive monitoring of physiological stress in the Western lowland gorilla (*Gorilla gorilla gorilla*): validation of a fecal glucocorticoid assay and methods for practical application in the field. *Gen Comp Endocrinol* 179: 167–177.
- Sire J E, Blom A, Sjaatad O V, Ropstad E, Bergdahl Nilsen T A 1995 The effect of blood sampling on plasma cortisol in female reindeer (*Rangifer tarandus tarandus*). *Acta Veterinaria Scandinavia* 36: 583–587
- SK Menhut RI No: 173/Kpts-II/1991 tentang Penetapan lembaga konservasi

- SK Menhut RI No. 479/Kpts-II/1998 tentang tujuan utama lembaga konservasi *ex-situ*
- Sukumar, R. (1994), *Elephant Days and Nights: Ten Years with the Indian Elephant*, Oxford University Press, Delhi.
- Tizard. 1988. *Pengantar Imunologi Veteriner*. Airlangga University Press
- Touma, C., and Palme, R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. *Ann. N. Y. Acad. Sci.* 1046:54-74
- Velloso, A. L., Wasser, S. K., Monfort, S. L., & Dietz, J. M. (1998). Longitudinal fecal steroid excretion in maned wolves (*Canis chrysocyon brachyurus*). *General and Comparative Endocrinology*, 112(1), 96-107
- Wasser, S.K., K.E. Hunt, J.L. Brown, K. Cooper, C.M. Crockett, U. Bechert, J.J. Millspaugh, S. Larson and S.L. Monfort, (2000). A Generalized Fecal Glucocorticoid Assay for Use in a Diverse Array of Nondomestic Mammalian and Avian Species. *General and Comparative Endocrinology*, 120: 260-275.