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COMPARISON OF FECAL HORMONE EXTRACTION METHODS AND ANALYSIS ON REPRODUCTIVE HORMONES IN FEMALE MALAYAN TAPIRS

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COMPARISON OF FECAL HORMONE EXTRACTION METHODS AND ANALYSIS ON REPRODUCTIVE HORMONES IN FEMALE MALAYAN TAPIRS

By

MUHAMMAD NAJIB BIN RUSLAN

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COMPARISON OF FECAL HORMONE EXTRACTION METHODS AND ANALYSIS ON REPRODUCTIVE HORMONES IN FEMALE MALAYAN TAPIR

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Non-invasive hormone monitoring provides an important tool to optimize breeding strategies of wildlife species including tapir in captive managements. Previous studies stated that tapir sp. would have been excreted low amount of steroids (progesterone and 17β-oestradiol) in their feces. In addition, there is still no standardized fecal extraction protocol for Malayan Tapir. Therefore, it is important to detect that low amount of hormones (progesterone and 17β-oestradiol) through several fecal extraction protocols in Schwarzenberger et al. (1996a) (Method A and B), Brown et al. (2001) (Method C), and Shutt et al. (2012) (Method D). The fecal extraction method provides differences in the steps of the method and solvents that used to extract the steroid hormones. Fecal samples were collected from four female Malayan Tapir (N=4) at Sungai Dusun Wildlife Reserve (n=2), Zoo Taiping (n=1) and Zoo Negara (n=1), Malaysia. Results from Liquid Chromatography Mass Spectrometry (LCMS) confirmed the presence of progesterone but not for 17β-oestradiol in fecal samples. Meanwhile, results from Enzyme Linked Immunosorbent Assay (ELISA) showed that there was not a significant difference between all methods in progesterone and its relation to reproductive status ($p>0.05$). In contrary, there was significant difference between methods in 17β-oestradiol and its relation to reproductive status ($p<0.05$). These results suggest that, validating the progesterone immunoassay is needed as it might underestimate the actual progesterone concentration. Besides, all extraction protocols for 17β-oestradiol resulted in different concentration range between non-pregnant and pregnant animals. Nevertheless, fecal 17β-oestradiol analysis could be suitable for determining pregnancy status in female Malayan Tapirs.

Keywords: Malayan Tapir, fecal, progesterone, 17β-oestradiol, extraction.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERBEZAAN ANTARA PROTOKOL HORMON EKSTRAKS DAN ANALISIS TERHADAP HORMON PEMBIAKAN BETINA TAPIR MALAYA MELALUI SAMPEL NAJIS

Oleh

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Pemantauan hormon tidak invasif menyediakan satu alat yang penting untuk strategi pembiakan spesies hidupan liar dan pengurusan haiwan kurungan. Kajian sebelum ini menyatakan tapir berkemungkinan mengeluarkan sedikit steroid (progesteron dan 17β-oestradiol) dalam najis. Oleh itu, tujuan untuk kajian terkini adalah untuk mengesan dan mengukur analisis hormon pembiakan dengan membandingkan kaedah pengestrakan najis untuk kedua-dua hormon pembiakan (progesteron dan 17β-oestradiol) untuk empat Malayan Tapir (N=4). Sampel najis telah diambil dari Rizab Hidupan Liar Sungai Dusun (n=2), Zoo Taiping (n=1) dan Zoo Negara (n=1), Malaysia. Sampel kemudiannya diekstrak menggunakan empat protokol pengekstrakan najis yang berbeza oleh Schwarzenberger et al. (1996a) (Kaedah A and B), Brown et al. (2001) (Kaedah C), and Shutt et al. (2012) (Kaedah D). Setiap kaedah menyediakan perbezaan dalam langkah kaedah dan pelarut yang digunakan untuk mengeluarkan hormon steroid. Hasil daripada Kromatografi Cecair Spektrometri Jisim (LCMS) telah mengesahkan kehadiran progesteron tapi tidak terhadap 17β-oestradiol dalam sampel najis. Sementara itu, keputusan daripada Asai Imunoterap terangkini Enzim (ELISA) menunjukkan bahawa tidak ada perbezaan yang signifikan di antara semua kaedah progesteron dan kaitannya dengan status pembiakan ($p > 0.05$). Namun, terdapat perbezaan yang signifikan antara kaedah dalam 17β-oestradiol dan kaitannya dengan status pembiakan ($p < 0.05$). Keputusan ini menunjukkan bahawa, mengesahkan imunoterap progesteron masih diperlukan kerana ia mungkin memandang rendah kepekatan progesteron sebenar. Di samping, semua protokol perahan 17β-oestradiol menyebabkan julat kepekatan yang berbeza antara haiwan yang tidak bunting dan bunting. Walau bagaimanapun, analisis 17β-oestradiol najis mungkin sesuai untuk menentukan status kehamilan dalam betina tapir Malaya.

Kata kunci: Tapir Malaya, najis, progesteron, 17β-oestradiol, pengestrakan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

^{O}C	Degree celcius
µl	Microlitre
µm	Micrometre
ANOVA	Analysis of variance
B_O	Zero binding
cpm	Count per minutes
ELISA	Enzyme Linked Immunosorbent assay
g	Gram
g/mol	Gram per mol
HPLC	High Performance Liquid Chromatography
IACUC	Institutional Animal Care and Use Committee
IUCN	International Union for Conservation of Nature
kg	Kilogram
LCMS	Liquid Chromatography Mass Spectrometry
m/z	Mass per charge
min	minutes
mm	Millimetre
MS	Mass spectrometric
ng/g	Nanogram per gram
ng/ml	Nanogram per millilitre
nm	Nanometer
NSB	Non-specific binding
OD	Optical density
PERHILITAN	Jabatan Perlindungan Hidupan Liar dan Taman Negara
pg/ml	Picogram per millilitre
pNpp	p-nitrophenyl phosphate
RIA	Radioimmunoassay
rpm	Rotation per minute
RT	Retention time
SEM	Standard error of mean
SIM	Selected Ion Monitoring
S_{int}	Initial standard
sp.	Species
SPE	Solid Phase Extraction
TA	Total activity
UPM	Universiti Putra Malaysia
v/v	Volume per volume
WR	Wildlife Reserve

CHAPTER 1

INTRODUCTION

1.1 Background of study

Malayan Tapir (*Tapirus indicus*) belong to the Family Tapiridae. This species can be found in the tropical rainforest of Southeast Asia such as Peninsular Malaysia, Indonesia, Myanmar and also Thailand (Lekagul and McNeely, 1997). This species is listed as Endangered in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Traeholt et al., 2016). A rapid deforestation, habitat loss, and over-hunting make the population to decline and on the verge of extinction (Mace and Balmford, 2000; Novarino and Grant, 2005). Ecology of *Tapirus* species in the wild has been studied mainly on the size of home range (Foerster & Vaughan, 2002) diet (Tobler et al., 2006) and habitats (Salas, 1996; Tobler et al., 2006). As mentioned earlier, due to current status of tapirs in wild, it is also important to monitor their reproduction. However, there are still lack of information on their reproductive physiology. This remain a concern among conservation biologist to ensure the long-term survival of this species in wild and captivity (Pukazenthi et al., 2013).

The study on reproductive physiology of tapir species (i.e. oestrous cycle, pregnancy) is essential to improve their breeding management mainly in captivity (Pukazenthi and Wildt, 2003; Hodges et al., 2010; Pukazenthi et al., 2013). This is important as a long term approach to sustain the population size of this species both in wilds and captivity. Besides, breeding management through monitoring reproductive performances (i.e. pregnancy diagnosis) is vital for effective captive management, so that better approaches can be implemented to facilitate reproduction in tapir species (Pukazenthi et al., 2013; Holt et al., 2014). To have better understanding on reproductive performance of Malayan Tapir, there are invasive and non-invasive approaches. However, non-invasive approach is more useful to minimize the effects of animal handling and restraint, thus improve animal welfare. Non-invasive endocrinology monitoring has proven to be useful to study basic reproductive hormonal and this seen in species such as in rhinoceros and horses (Brown et al., 1994; Schwarzenberger et al., 1996a; Asa et al., 2001; Brown et al., 2001; Hodges et al., 2010). However, there is still inadequate of information on hormonal data of tapir species, particularly in Malayan Tapir (Kasman et al., 1985; Bamberg et al., 1991; Kusuda et al., 2007; Pukazenthi et al., 2013).

Hormonal data using plasma or serum samples have been done in almost tapir species (Pukazenthi et al., 2013). Although blood samples provide the most immediate information on reproductive hormones (i.e. progesterone and 17β-oestradiol), this approach is highly invasive towards animals and could affected the end results for hormone concentration (Welch, 1992). Meanwhile, fecal samples provide more accurate hormone measurement as it represents the pooled value of hormone metabolites that reflects specific time (Whitten et al., 1998; Mostl and Palme, 2002). However, limitation exist as the main source of steroid metabolism occur due to

presence of bacterial enzyme in fecal samples (Whitten et al., 1998; Touma and Palme, 2005). Therefore, methodological concerns such as samples storage, handling and extraction process are necessary to ensure hormone of interest are not lost during experiment (Palme et al., 2005; Palme et al., 2013). Besides, active compound of respective hormones such as progesterone and oestrogen are virtually absent in feces, with feces excreted only contain the fecal hormone metabolites or the end-product of the hormones itself (Palme et al., 2005; Hodges et al., 2010; Pukazenthi et al., 2013).

Technique such as Liquid Chromatography Mass Spectrometry (LCMS) have been used for hormone detection in biological samples (i.e. blood, urine and fecal extracts) of wildlife animals (Hauser et al., 2008; Kaklamanos et al., 2009; Hauser et al., 2011; Weltring et al., 2012). Liquid Chromatography Mass Spectrometry (LCMS) is a highly specific method that involve the separation of the compound using high performance liquid chromatography (HPLC) system with mass spectrometer (MS) equipment (Honour, 2006). In LCMS, separation of the compounds was made based on polarity during chromatography separation, which resulted in different retention times (Meyer, 2013; Murtagh et al., 2013). Selection of mobile phase with polar solvent is necessary, with methanol and acetonitrile are widely used for separation of steroids compound (Honour, 2006). This is important as steroid hormones (i.e. progesterone and oestradiol) are polar steroid, therefore separation of compound during LCMS can be achieved (Murtagh et al., 2013). Previous studies also stated that difficulty to detect hormone through fecal might be due to selection of extraction protocol (Murtagh et al., 2013). Palme et al. (2013) stated that the fecal extracts comprised of several metabolites that differ in polarities. Thus, extraction with right solvents and protocols are important.

Thus, a research study on extraction methods of fecal progesterone and 17β-oestradiol for Malayan Tapir is needed to improve methodological aspects of hormone monitoring. The fecal extraction methods are almost available for other animals, yet only few data have been published for Malayan Tapir as reported in Bamberg et al. (1991). Fecal extractions need to be well validated before longitudinal studies can be conducted. This is because different animals have different ways of hormone metabolism and excretion routes (Touma et al., 2003; Palme et al., 2005). As for previous studies, selection of extraction protocols was made based on hormone of interest, practicality and personal preferences (Graham, 2004; Hodges et al., 2010). In this study, previous extraction protocols used in felid species (Schwarzenberger et al., 1996; Methods A and B), rhinoceros (Brown et al., 2001; Method C) and primates (Shutt et al., 2012; Method D) were used to investigate the ability for this protocol to be implemented in Malayan Tapir species. In Methods A and B, selection of polar and non-polar solvents was chosen to investigate the influence of polarity to extract both progesterone and oestradiol. Most of the studies on mammalian species used 80% methanol as solvent as methanol is more polar than petroleum ether. Meanwhile, Method C was applied to extract hormones of interest by using multiple phase extraction protocol. According to Brown et al. (2001), the involvement of boiling and evaporation process in this protocol could enhance the extraction process. Extraction process in Method D provide the advantage compared to other methods as this protocol can be applied in field stations. Therefore, problems such as steroid metabolism can be reduced to minimal as extraction process takes place immediately at field.

This study hypothesized that both progesterone and 17β-oestradiol could be identified by using LCMS. In addition, the study also hypothesized that progesterone could be detected in LCMS but not quantifiable as reviewed by Pukazenthi et al. (2013), which stated that progesterone could be present in low amount in feces of Malayan Tapir. Meanwhile, 17β-oestradiol should be detected as animals including Malayan Tapir, excreted this hormone in fecal samples (Bamberg et al., 1991; Kuckelkom, 1994; Hodges et al., 2010). Since different extraction protocols could also influence the hormone to be extracted, this study hypothesized that the concentration between protocols to be significantly differ, due to relative ability of solvent and steps to extract progesterone and 17β-oestradiol. Besides, the study also hypothesized that concentration values for both hormones in all extraction protocols of pregnant individuals in different reproductive stages to be significantly higher than non-pregnant individuals.

Therefore, the study should be able to help in determining the reproductive hormone metabolites in fecal samples of Malayan Tapir. Besides, considerations regarding fecal extraction methods to be implemented in tapir species can be studied. The data obtained should also be able to determine reproductive status of Malayan Tapir by using fecal samples as medium to monitor their reproductive hormones.

1.2 Objectives

i. To determine the presence of compounds of progesterone and 17β-oestradiol hormones in fecal samples of female Malayan Tapir by using LCMS method

ii. To compare fecal extraction methods for progesterone and 17β-oestradiol hormone concentrations in pregnant and non-pregnant female Malayan Tapir

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LIST OF PUBLICATIONS

M. N. Ruslan., M. Shikh Maidin., and R. Salfarina. (2017). LCMS/MS detection of progestogens in female Malayan Tapir from different fecal extraction methods. *Fundamental Science Congress*. (Accepted).

M. N. Ruslan., M. Shikh Maidin., and I. N. Azhar. (2017). Concentrations of reproductive hormones in female Malayan Tapir within different fecal hormone extraction methods. *Malaysian Science and Technology Congress*. (Published).

M. N. Ruslan., and M. Shikh Maidin. (2018). Monitoring fecal progesterone metabolites of Tapir species. *Seminar Ekologi Malaysia*. (Accepted)

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