Faecal Concentrations of Progesterone and 17β-Oestradiol of Female Malayan Tapir from Different Faecal Hormone Extraction Methods

(Kepekatan Najis yang Mengandungi Progesteron dan 17β-Oestradiol Tapir Betina Malaya daripada Kaedah Pengekstrakan Hormon Najis yang Berbeza)

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ABSTRACT

Non-invasive hormone monitoring of endangered wildlife provides an essential tool to optimize breeding strategies, mainly in captive management. However, the protocol of reproductive hormone monitoring on pregnancy status using non-invasive faecal samples in Malayan tapir is still inconclusive. Therefore, we compared the metabolites reproductive hormones; 17β-oestradiol and progesterone in the faecal samples extracted using methods of Schwarzenberger et al. (1996) (Methods A and B), Brown et al. (2001) (Method C) and Shutt et al. (2012) (Method D) from pregnant and non-pregnant captive Malayan Tapir. Faecal samples from four female of Malayan Tapir (*Tapirus indicus*) were collected for five months at Sungai Dusun Wildlife Reserve (n = 2), Zoo Taiping (n = 1) and Zoo Negara (n = 1), Malaysia. Analysis by liquid chromatography-mass spectrometry (LCMS) confirmed that progesterone was detected in all fecal sample extracted by extraction Method A, while 17β-oestradiol was undetectable in all methods. The measurement of reproductive hormones for pregnancy status via Enzyme-Linked Immunosorbent Assay (ELISA) analysis showed no significant difference ($p > 0.05$) for progesterone between the fecal extraction methods. Yet the data obtained were not able to validate the pregnancy status, due to similarity in concentration for both pregnant and non-pregnant tapirs. While for 17β-oestradiol, significant results were observed in all extraction methods and in pregnancy status ($p < 0.05$). Method C was found to be the most reliable extraction method ($p < 0.05$) to indicate pregnancy status. From the results, it showed that solvent used, boiling process and multiphase extraction plays important role in fecal extraction process in Malayan Tapir.

Keywords: Malayan tapir; non-invasive fecal; oestradiol; pregnancy; progesterone

ABSTRAK

Pemantauan hormon tak invasif terhadap hidupan liar yang terancam penting untuk mengoptimumkan strategi pembiakan terutamanya dalam pengurusan haiwan tawanan. Walau bagaimanapun, protokol pemantauan hormon pembiakan terhadap status pembiakan dengan menggunakan tak invasif daripada sampel najis tapir Malaya masih tidak muktamad. Oleh itu, dalam penyelidikan ini kami mengkaji perbezaan pengukuran hormon pembiakan metabolit; 17β-oestradiol dan progesteron dalam kaedah pengekstrakan najis Schwarzenberger et al. (1996) (Kaedah A dan

Kaedah B), Brown et al. (2001) (Kaedah C) dan Shutt et al. (2012) (Kaedah D). Sampel najis daripada empat tapir betina Malaya (*Tapirus indicus*) dikumpul selama lima bulan di Hutan Simpan Sungai Dusun (n = 2), Zoo Taiping (n = 1) dan Zoo Negara (n = 1), Malaysia. Analisis dengan spektrometri kromatografi gas cecair (LCMS) mengesahkan bahawa semua sampel najis menunjukkan progesteron hanya daripada Kaedah Pengekstrakan A, sementara 17β-oestradiol tidak dapat dikesan daripada mana-mana Kaedah Pengekstrakan najis. Pengukuran hormon pembiakan untuk status kehamilan melalui analisis ujian imunosorben berkaitan enzim (ELISA) tidak menunjukkan sebarang perbezaan yang signifikan bagi progesteron antara Kaedah Pengekstrakan tinja. Namun data yang ditunjukkan dalam kajian ini tidak dapat mengesahkan status kehamilan kerana nilai progesteron untuk hamil dan tidak hamil adalah hampir sama. Sementara untuk 17β-oestradiol, ia menunjukkan hasil yang signifikan dalam semua kaedah pengekstrakan dan status kebuntingan (p < 0.05) dengan kepekatan tertinggi 17β-oestradiol dengan menggunakan Kaedah C, ini menunjukkan bahawa pelarut memainkan peranan penting dalam proses pengekstrakan najis dan nampaknya kaedah ini boleh digunakan untuk mendiagnos kehamilan tapir Malaya (p < 0.05). Kami mencadangkan untuk progesteron, pengesahan immunoasei diperlukan mungkin disebabkan oleh merendahkan kepekatan progesteron. Keputusan kajian menunjukkan jenis pelarut yang digunakan, proses pendidihan dan pengekstrakan multifasa memainkan peranan penting dalam proses pengekstrakan najis tapir Malaya.

Kata kunci: Kehamilan; najis tak invasif; oestradiol; progesteron; tapir Malaya

Introduction

Rapid deforestation, habitat loss, and over-hunting have resulted in the population of Malayan tapir (*Tapirus indicus*) to decline rapidly and are consequently on the verge of extinction (Mace & Balmford 2000; Novarino 2005). Accordingly, the species has been listed as Endangered on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species (Traeholt et al. 2016). Given the current situation, it is therefore important to protect this species from completely vanishing in their ecosystem. Therefore, monitoring their breeding patterns is required for the survival of this species.

The assessment of reproductive performance in wildlife has been carried out through non-invasive faecal reproductive hormonal analysis through Immunological techniques such as radioimmunoassay (RIA) or enzyme immunoassay (Peter et al. 2018; Pukazenthi et al. 2013). This has also been reported in previous studies on oestradiol metabolism in other wildlife animals since more than 90% of this steroid metabolites were excreted in faeces (Hodges et al. 2010; Schwarzenberger 2007). The study of the faecal reproductive hormone metabolites such as progesterone and oestrogen are useful to monitor the reproductive physiology of wildlife such as in rhinoceros, felids, elephants, and primates (Conforti et al. 2017; Habumuremyi et al. 2014; Polegato et al. 2018; Thitaram et al. 2017). This approach could successfully improve the wildlife captive breeding program by build a control feeding and captive breeding managements. In addition, detailed information on reproductive behaviour (i.e. time of copulation, mating behaviour, signs of oestrous) and reproductive endocrinology (i.e. oestrous cycles and pregnancy) of wildlife could significantly contribute to reproductive success in captive settings and perhaps in the wild as well (Holt et al. 2014; Kumar & Umapathy 2019; Pukazenthi et al. 2013).

However, with respect to the reproductive physiology studies of Malayan Tapir, further information is also needed to build upon the existing understanding of the level of faecal progesterone and oestrogen concentrations related to reproduction phases. In the literature, the evidence of these two hormones and reproduction are frequently presented from invasive samples (i.e. plasma/serum); Malayan Tapir (Kusuda et al. 2007; Schaftenaar et al. 2006) and Bairdii tapir (Brown et al. 1994). However, non-invasive analysis is sparse and remains a significant concern among conservation biologists to ensure the long-term survival of this species both in the wild and captivity (Kumar & Umapathy 2019; Zemanova 2019). Even though non-invasive hormonal of oestrogens metabolites (i.e. oestrone sulphate, oestradiol) for Malayan tapir has been described through urine (Kasman et al. 1985) and faecal samples (Bamberg et al. 1991), limited studies have investigated and analyzed progesterone hormone due to the difficulty detecting its metabolite in faecal sample.

Apparently, selection of faecal extraction protocols is crucial owing to several metabolites that differ in polarities, thus requiring specific protocols and solvent to extract and determine the reproductive hormone metabolites (Murtagh et al. 2013; Palme et al. 2013). Different species execute their metabolism and excretion routes differently, even between the sexes of the same species (Palme et al. 2005; Touma et al. 2003). This indicates that an urgent need to develop hormonal

databases (i.e. progesterone and 17β-oestradiol) using faecal samples, where attention should be directed towards the methodological aspects of faecal extraction methods. Even though current extraction methods are available for other Perissodactyla species (Asa et al. 2001; Berkeley et al. 1997; Brown et al. 2001; Graham et al. 2001), limited data have been published with respect to Malayan tapir (Bamberg et al. 1991; Pukazenthi et al. 2013).

In examining these issues in more detail, we firstly detect the presence of progesterone and 17β-oestradiol in the faecal samples of Malayan tapir using LCMS, and secondly, assess the hormone concentration in different extraction protocols to extract progesterone and 17β-oestradiol in faecal samples of known pregnant and non-pregnant Malayan tapirs in captive from immunoassay. Different solvents (methanol, ethanol, and petroleum ether) and steps of extraction protocols from previous studies by Brown et al. (2001), Schwarzenberger et al. (1996), and Shutt et al. (2012) were adopted in this study as extraction parameters. It was anticipated that by comparing the faecal extraction methods for both hormones, the effect of extraction towards concentration values could be understood (Kuckelkom 1994). As such, consideration is made in choosing the optimum faecal extraction method to implement in the tapir species, particularly for Malayan tapir. The information and results obtained from this research can be applied to validate the relationship between circulated and excreted hormone profiles; furthermore, the reproductive biology of tapir species could be well understood (Berkeley et al. 1997; Pukazenthi et al. 2013).

MATERIALS AND METHODS

Faecal samples were collected from four female Malayan tapirs at Sungai Dusun Wildlife Reserve, Selangor (n = 2), Zoo Taiping, Perak (n = 1) and Zoo Negara, Kuala Lumpur (n = 1), Malaysia. The faeces for each animal were sampled three to four days' interval for five months between November 2016 and March 2017. Samples were collected between 0800 and 1000 before their daily feeding. The faecal samples were homogenised, and any undigested materials (i.e. plant parts, seeds and insects) were removed using forceps to reduce interferences during extraction process. The homogenised samples were placed into a labelled 50 mL falcon tube where all samples were transported in an icebox back to the laboratory, Universiti Putra Malaysia, Serdang, Malaysia for fecal extraction before keep in -20 °C for progesterone and 17β-oestradiol.

ANIMAL ETHICS AND APPROVAL

Approval for the study was granted by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM/IACUC/AUP-R033/2016), which also included a permit granted by the Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN) (NRE 600-2/2/21 JLD 4 (34), B-00441-6-16).

EXTRACTION OF FAECAL SAMPLES

A total of 48 fresh homogenised samples of pregnant (n = 2) and non-pregnant (n = 2) Malayan tapir faeces were collected from the sampling location as mentioned earlier. Each sample was divided into three replicates. All samples were used in extractions Methods (A, B, C and D). In this study, the selection of fecal extraction protocols and solvents were based on previous studies. The details of each step of faecal extraction are presented in the following sub-sections.

PROTOCOL OF METHODS A AND B

These two methods, (A and B), were adapted from Schwarzenberger et al. (1996). The difference between these methods is the solvent used. 80% of methanol and petroleum ether was used in Method A and Method B, respectively. One gram of each fresh faecal sample was placed in 10 mL of solvent (Method A, 80% methanol; Method B, petroleum ether) and vortexed for 1 min. The samples were then homogenised overnight at room temperature. Then, the samples were centrifuged at 1500 rpm for 15 min. The supernatants were recovered and stored at -20 °C for later analysis.

PROTOCOL OF METHOD C

This method was derived from the method employed in Brown et al. (2001). In Method C, 90% of ethanol was used followed by boiling and evaporation process. One gram of each fresh faecal sample was suspended in 5 mL of 90% ethanol and vortexed. The samples were then boiled in a water bath (90 °C) for 20 min and centrifuged at 1500 rpm for 20 min. The supernatant was poured into the new labelled tube, whereas 5 mL of 90% ethanol was added to the precipitate and centrifuged for 15 min. The extracts were pooled in the labelled tube and dried using a rotary evaporator. The dried extracts were reconstituted in 2 mL of methanol and briefly vortexed and then stored at -20 °C for later analysis.

PROTOCOL OF METHOD D

This method was adapted from Shutt et al. (2012) in which 90% of ethanol was used but with no involvement of equipment such as a centrifuge and vortex. This method is suitable for field extraction. Next, 1 g of each fresh faecal sample was placed in 10 mL of 90% ethanol and hand-shaken for 3 min without the involvement of a vortex or centrifuge machine. After 30 to 40 min, the supernatant was recovered and stored at -20 °C for later analysis.

HORMONES ANALYSIS

The detection of hormones; progesterone and 17β-oestradiol was carried out using Liquid Chromatography-Mass Spectrometry (LCMS). Whereas, the hormonal concentrations of progesterone and 17β-oestradiol were measured using Enzyme-Linked Immunosorbent Assays (ELISA).

CHEMICALS AND REAGENTS FOR LCMS

Progesterone (4-pregnene-3,20-dion) and 17β-oestradiol standards were purchased from Sigma Aldrich (Steinheim, Germany). Stock solution comprised of 100 ng/mL standard in methanol, blank was 80%, methanol. Both were stored at -20 °C until further used. Solvents methanol and acetonitrile (LCMS grade) were purchased from Fisher Scientific USA.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LCMS) MEASUREMENT

Standards and extracted feacal samples were injected into HPLC auto-sampler vials (Agilent Technologies, USA). The Agilent 6120 Series Quadrupole system with an electrospray ionisation (ESI) source (Agilent Corporation, Wilmington, DE, USA) was used for conducting the measurement. The sample cone voltage and the collision energy were set individually for each sample and standards were detected using Selected Ion Monitoring (SIM) in a positive ion mode with Agilent ChemStation Mass Spectrometer software.

The faecal extracts were first separated at 45 °C on a reversed-phase C18 column (Agilent ZORBAX Eclipse Plus, 2.1 mm × 50 mm × 1.8 μm, Agilent Technologies Incorporation, Wilmington, DE, USA). The composition of mobile phase A and B were water/acetonitrile (95/5, v/v) and water/acetonitrile (5/95, v/v), respectively, both contained 0.1% formic acid. The gradient was set at 30% A (0 - 4 min), 45% A (4 - 4.1 min), linearly increased to 60% for (4.1 - 6.1 min). Next, 3 μL of the extracted faecal

sample was injected, with the flow rate set at 0.3 mL/min. Information on the molecular mass of standards was used to distinguish both progesterone and 17β-oestradiol in the extracted faecal samples. The identifications of the compounds in the extract were based on retention time, m/z of progesterone and 17β-oestradiol standards (Sigma Aldrich, Steinheim, Germany).

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

All reagents were equilibrated at room temperature for 30 min and shaken gently before the assays. The antibody-coated well and Blank, Non-specific binding (NSB) total activity (TA), standards and the sample in duplicate were labelled carefully. All samples were arranged on the tube rack, with the arrangement recorded according to the layout sheet given in the assay ELISA kits.

PROGESTERONE ELISA

The concentrations of faecal progestogens were analysed using a Progesterone ELISA kit, with goat antibody specific to a mouse IgG plate (Enzo Life Sciences, Switzerland). Cross-reactivity for the primary antibody was 100% for progesterone and 5α-Pregnane-3, 20-dione, 3.46% for 17-OH-progesterone, and 1.43% for 5-pregnen-3β-o1-20-one. The standards for progesterone were prepared to 500, 250, 125, 62.5, 31.25, and 15.62 pg/mL, respectively, using the serial dilution technique. The plate was read immediately using a ChroMate® microplate ELISA reader for optical density (OD) at a wavelength of 405 nm.

17B-OESTRADIOL ELISA

The concentrations of faecal oestrogens were analysed using a 17β-oestradiol high sensitivity ELISA kit, with donkey antibody specific to a sheep IgG plate (Enzo Life Sciences, Switzerland). Cross-reactivity for the primary antibody was 100% for 17β-oestradiol, 17.8% for oestrone, and 0.9% for oestriol. The standards for 17β-oestradiol (Sint-7) were prepared and serially diluted to 3000 (initial), 1000, 500, 250, 125, 62.5, 31.3, and 15.6 pg/mL, respectively. The plate was immediate read using a ChroMate® microplate ELISA reader for OD at a wavelength of 405 nm.

DATA ANALYSIS

Identification of the compounds was presented in a total ion chromatogram with the respective retention time for both the progesterone and 17β-oestradiol standards

used as a reference. Agilent ChemStation software was used for data acquisition and analysis. All data recorded in ELISA were analysed with a repeated measurement using two-way ANOVA, IBM SPSS Statistics 21. The significant level of $p < 0.05$ was used to study the variation between the faecal hormone extraction methods, pregnancy status and the mean of the data was calculated and presented in the standard error of the mean ± SEM.

Results

CHROMATOGRAM ANALYSIS - LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LCMS)

From the chromatograms, progesterone was detected in samples prepared using Method A (Figure 1). However,

progesterone was unidentified in extracts from Methods B, C, and D. While for 17β-oestradiol was also not identified from in all faecal sample extracts (Figure 2).

THE CONCENTRATIONS OF PROGESTERONE AND 17B-OESTRADIOL

From all the extraction methods, the fecal metabolites of progesterone were similar between methods and pregnancy status ($p > 0.05$) (Table 1). While for fecal metabolites of 17β-oestradiol, indicated the significant values within extraction methods and pregnancy status of Malayan tapirs ($p < 0.05$) (Table 1). Among the methods, highest detection of 17β-oestradiol fecal metabolite was observed in Method C.
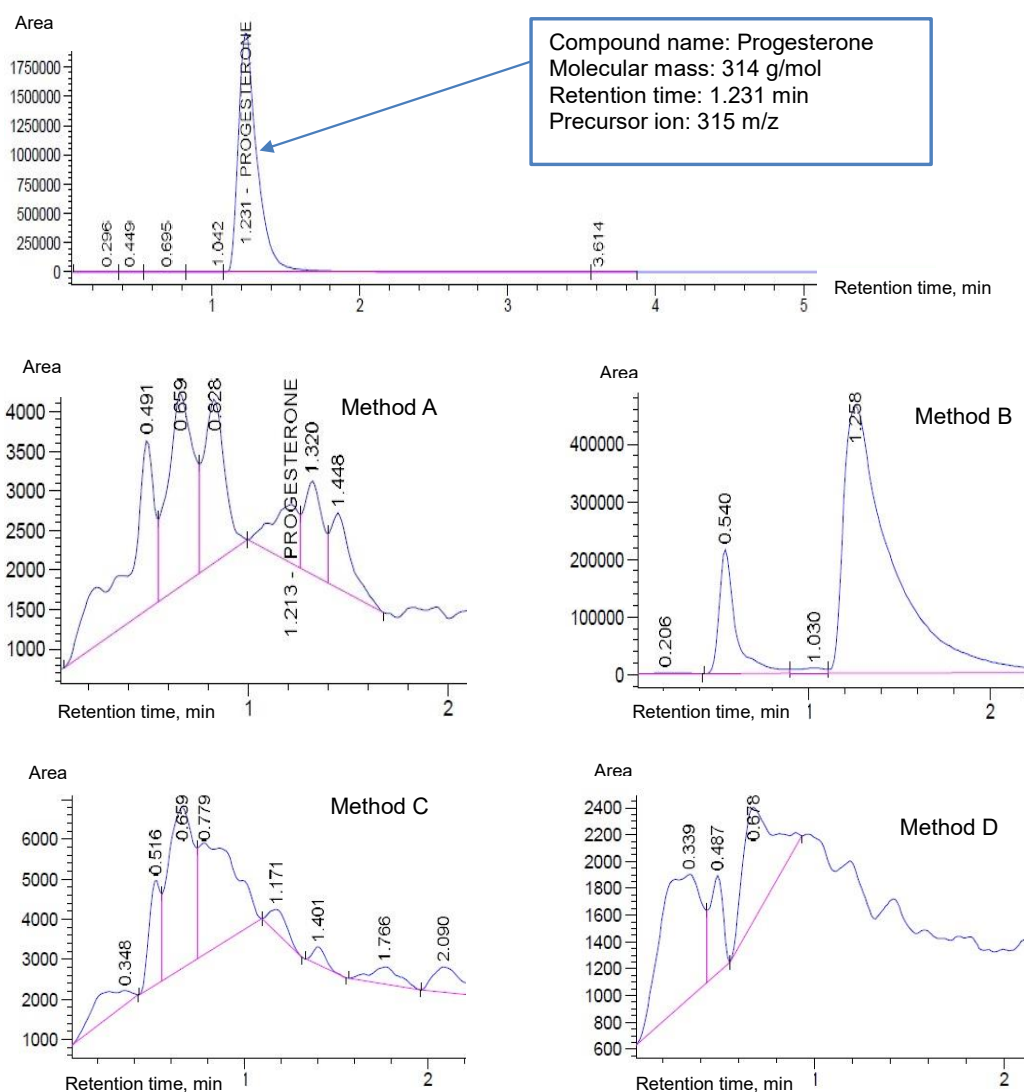

FIGURE 1. Chromatographic separation of progesterone standard at 100 ng/mL with retention time of 1.231 min and Methods A, B, C and D (an example from pregnant animal/sample; n = 1) generated via Agilent Chemstation Mass Spectrometer Software
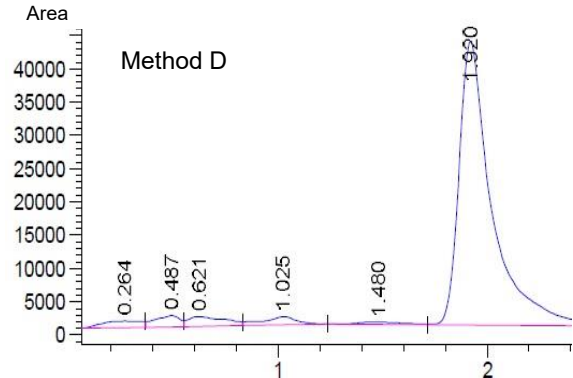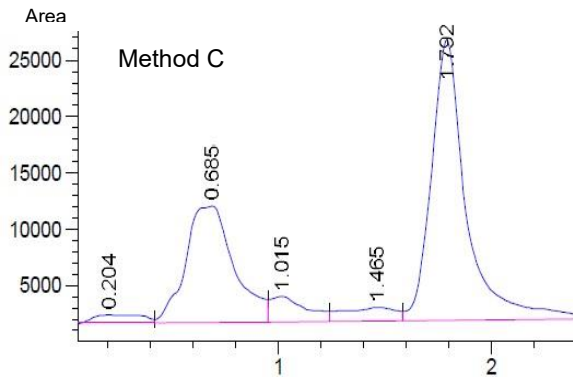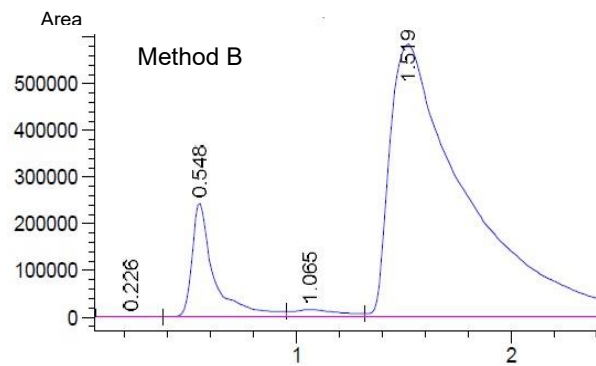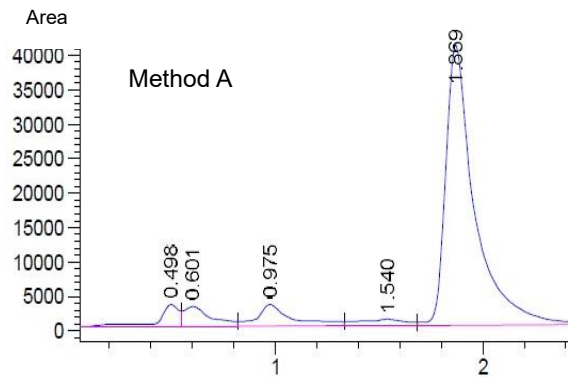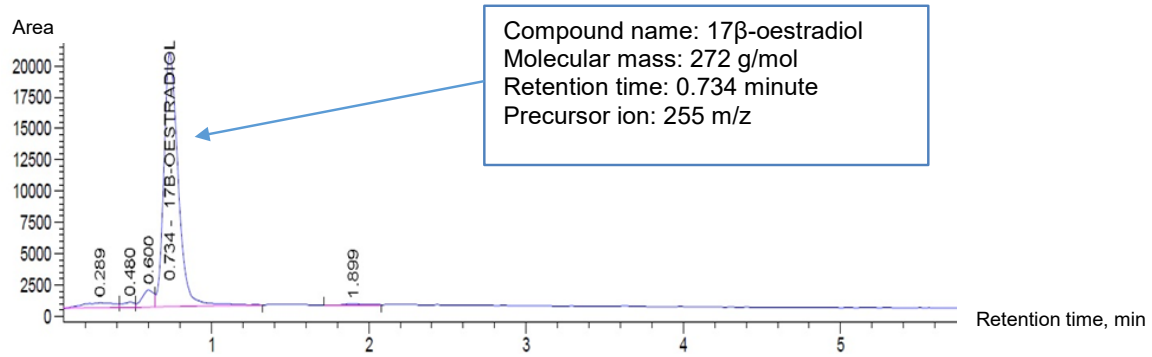
FIGURE 2. Chromatographic separation of 17β-oestradiol standard at 100 ng/mL with retention time of 0.734 min and Methods A, B, C and D (an example from pregnant animal/sample; n = 1) generated via Agilent Chemstation Mass Spectrometer Software

TABLE 1. The fecal progesterone and 17β-oestradiol presence/concentrations ± SEM (ng/g wet faeces) in pregnant (n = 2) and non-pregnant (n = 2) of Malayan tapir across different faecal extraction methods

Faecal Extraction method	Solvent Polarity	Temperature	Time	Type of extraction	ELISA detection			
					P4 (ng/g) (Pregnant)	P4 (ng/g) (Non-pregnant)	E2 (ng/g) (Pregnant)	E2 (ng/g) (Non-pregnant)
A	80% methanol	Room temperature	Over night	Single phase	1613.1 ± 161.4	1184.6 ± 45.2	496.8 ± 29.1 [a,x]	139.3 ± 24.0 [a,y]
B	Petroleum ether	Room temperature	Over night	Single phase	1516.8 ± 210.5	1708.1 ± 313.6	276.4 ± 28.9 [a,x]	131.0 ± 19.9 [a,y]
C	90% ethanol + 80% methanol	90 ℃	20 min	Multiple phase	1202.2 ± 401.2	1887.3 ± 150.9	1153.4 ± 76.3 [b,x]	692.0 ± 25.1 [b,y]
D	90% ethanol	Room temperature	30-40 min	Simple phase	1658.8 ± 207.6	1272.2 ± 100.1	531.2 ± 45.9 [a,x]	254.2 ± 13.8 [a,y]

[a, b], Different letters within column indicate significant differences (P < 0.05) between fecal extraction method, [x, y], Different letters within row indicate significant differences (P < 0.05) between reproductive status (pregnant and non-pregnant)
Method A; Schwarzenberger et al. (1996) (80% methanol), Method B; Schwarzenberger et al. (1996) (Petroleum ether), Method C; Brown et al. (2001), Method D; Shutt et al. (2012)

DISCUSSION

The extraction method selected are different in parameters of extraction such solvent polarity (Methods A and B), temperature (Method C) and time (Method D). Polar solvents were used in all methods except Method B that used petroleum ether which is a non-polar solvent. The introduction of heat allows the sample to be extracted in 20 min (Method C), whereas Method D allowed the shortest time (30 - 40 min) of solvent to react with solvent in room temperature.

In Method A, 80% methanol which is a polar solvent was used, and the samples were allowed to homogenized overnight. This can be implied that method A was suitable for LCMS analysis of progesterone. Apparently, 80% methanol has been preferred as solvent of extraction compared to other solvent used in this study. The use of 80% methanol has been recommended to extract progesterone and glucocorticoids hormones for the majority of mammalian species (Palme et al. 2013). Consequently, increases the hormone recovery and chances for the hormone to be recognised in assays (Palme 1997). Furthermore, methanol has also been used to extract progestogen metabolites from the faecal samples of animals such as cows, rhinoceros and felids (Brown et al. 2001; Peter et al. 2018). Previously reported, oestrone and 17β-oestradiol were the main oestrogens found in mares (Celebi & Demirel 2003). A study reported

that 40% of oestrogens in felid species were excreted as conjugated oestrone and oestradiol in faeces (Brown et al. 1994), thus, it could likely be implied in the case of tapir. However, it is not conclusive as the reference standard for oestrone and oestriol were not included in this study. In this study the used of LCMS is for detection purposes only.

With respect of ELISA present results, this is the first study to directly compare progesterone and 17β-oestradiol concentrations via immunoassays technique of four different fecal extraction methods. Five months of fecal samples collection from 4 female Malayan tapir in this exploratory study, our results showed that fecal 17β-oestradiol extracted using any Methods in this study can be measured because the results reflected their reproductive status. The present results were similar with reported study by Bamberg et al. (1991) and Brown et al. (2001).

Among all methods, Method C showed to be more efficient to extract the fecal samples and preferable based on higher concentration of fecal 17β-oestradiol compared to other methods. This could be due to type of solvent used, boiling process and multiphase extraction (Torres-Pelayo et al. 2011). The used of temperature and solvent in Method C makes it more solubility and solvated more marked hormone in ELISA. According to Brown et al. (1994), Method C increased the extraction

efficiency for both oestradiol and progesterone up to 50% compared with boiling in pure ethanol. In addition, most of the fecal steroid extraction recommends usage of vortex, so that greater steroid recuperation can be achieved during extraction when ethanol and methanol are used as solvents (Beehner & Whitten 2004).

Bamberg et al. (1991) reported that the baseline concentration of faecal oestrogen metabolites for non-pregnant Malayan tapir was 13 ng/g, while elevated to 70 ng/g at early gestation and peaked to 170 ng/g at late gestation. In another study by Kuckelkom (1994) in mares, it showed that total oestrogen concentrations in extraction protocols using non-polar solvents (i.e. chloroform/n-hexane and diethyl ether) resulted in the concentration range for pregnant mares between 800 and 1800 ng/g in faeces, while the concentration below 500 ng/g was observed for non-pregnant mares. The similar pattern is observed at the present results.

The concentrations of progesterone extracted from four types of fecal extraction methods were similar between pregnant and non-pregnant tapirs. However, the progesterone concentrations did not reflect with their reproductive status. In pregnant tapir's samples, the range concentrations for progesterone metabolites presented (< 2000 ng/g) were below the baseline values compared to other pregnant wildlife animals as described by Brown et al. (1994). For instance, the basal progesterone metabolite concentrations for pregnant leopard cat was 16,200 ng/g, with average concentration of 872,200 ng/g throughout pregnancy. Meanwhile, as for cheetah and clouded leopard, the baseline values for pregnant individuals were ranged between 3500 to 5700 ng/g and 2900 to 6200 ng/g, respectively. In rhinoceros, the baseline range for non-pregnant was 2120 ng/g, while high concentration for pregnant individuals which 17,630 ng/g (Brown et al. 2001).

In addition, our present results of faecal progesterone concentration, the cross reactivity used for primary antibody was 100% for progesterone and 5α-Pregnane-3,20-dione (5 alpha-DHP) which is the amongst the main progestins biosynthesized by CL (Hodges et al. 1997). In addition, we suggest to use 100% specific antibody of 5α-P-3OH, progesterone metabolite as studied in pregnant African elephants (Ghosal et al. 2012) to measure faecal progesterone in Malayan tapir. It might be useful as an indicator for evaluating the pregnancy status of wildlife animals, including *Tapir* sp. since 5 alpha-DHP and 5α-P-3OH are principal tissue where it is conversion products of radiolabeled pregnenolone and as major contributors to immunoreactivity as determined by P4 assay. Thus, the selection of relevant antibodies for immunoassays of faecal progesterone metabolites is crucial (Graham et al. 2001; Palme et al. 2005; Schwarzenberger et al. 2007). Therefore, analysis on fecal extraction protocols for progesterone hormone in female Malayan tapir is not yet conclusive as data showed in this study were not able to validate the pregnancy status.

CONCLUSION

In this study, we successfully extracted hormones progesterone and 17β-oestradiol from faecal samples from four fecal extraction methods. From the LCMS results, progesterone was detected in samples extracted using Method A, whereas 17β-oestradiol was not detected in any samples. Nevertheless, the LCMS analysis results were satisfactory as the concentration of the extracted hormones from all methods were further determined by ELISA analysis. Method C show higher concentration 17β-oestradiol compared to other methods between pregnant and non-pregnant tapirs in ELISA, due to polarity solvent used. While for fecal progesterone analysis, need to be explored as it is important indicator in determination of pregnancy status, mainly in endangered species such tapirs. Although the results are unlikely to show the significant difference, this study has successfully demonstrated the results of different extraction method that are frequently reported in hormone analysis study. Incorporating sample clean up procedure prior to both LCMS and ELISA analysis will allow more accurate determination of the hormones in the samples. At the end, will facilitate the development of pregnancy diagnosis for this species, which has not yet been carried out before. This will be the focus of our future research.

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