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Lab-based and on-field analysis of progesterone metabolites in faeces samples of different species

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Chapter 1: Introduction

The clinical significance of natural steroids has been acknowledged by both the academic and the veterinary community especially in the reproductive monitoring in animals. Steroid hormones which are metabolized in the liver are excreted in faeces, urine and the bile, and are also present in milk, dried blood spots, plasma and saliva. The studies of faecal steroid hormones which have primarily been conducted on the metabolism and excretion, have been further extended to areas such as puberty, oestrous cycle, pregnancy, abortion, reproductive behaviour, seasonality, and the monitoring of treatment therapies in the zoo and domestic animals [15,16]. Progesterone (P4), an important steroid hormone of the reproductive tract of female animals, regulates the oestrous cycle and plays a major role during implantation, ovulation and the maintaining of pregnancy [1]. Native progesterone in mammals is mainly synthesized from the cholesterol following its conversion to pregnenolone by cytochrome P450, which is a protein stored in the inner surface of the inner mitochondrial membrane of various cells in the body. Pregnenolone is further converted into progesterone through a reaction catalysed in the liver by 3β-hydroxysteroid dehydrogenase, an enzyme in the adrenal gland, the ovaries and the testis [2,22]. Progesterone is metabolized by the liver into several metabolites and is thereafter excreted in faeces [11]. The analysis of P4 metabolites is used for characterising ovarian cycles and diagnosing reproductive cycle abnormalities, for diagnosing pregnancy, for timing of placentation and abortion, for monitoring treatment therapies, for correlation of hormone levels and social behaviours, and for seasonal hormonal profiling [16,18]. Faeces contain measurable quantities of progesterone metabolites and the extraction of progesterone from faecal samples, as a non-invasive method for detecting reproductive status, is an efficient and reliable tool which has been used in multiple research fields such as wildlife endocrinology, animal welfare, ecology and reproduction [13]. This method, which considers the type of species being inspected, and the polarity and composition of the steroid metabolite in their faeces, is non-stressing on the animal, economical, animal-saving, and allows for long-term studies where frequent sampling can be done on the same animal [17]. Comparing the progesterone concentrations has a direct correlation to different stages of plasma progesterone and therefore can be collected, using non-invasive methods, from a huge variety of animals without blood sampling. Extracting progesterone from faeces avoids problems related to animal managing, restraints, fertilization, embryo transport and embryo survival which are common in the traditional methods of extraction of samples from plasma or milk. Under normal circumstances, stress hormones are

released in small amounts throughout the day due each animal's body reacting differently to such changes as they can increase by unusual levels in response to numerous factors such as physical stress or physiological stress [3]. The collection of progesterone and cortisol from faecal samples have proven to be less stressful and require less restraint compared to blood samples. Some species require proper handling during cortisol sampling and the process can influence the cortisol value due to physiological stress and restraint being used in the form of sedatives and tranquilizers. Those issues have pushed for the development and inclusion of non-invasive techniques to study reproductive and endocrinological processes in animals. While the extraction of progesterone from milk and urine sample are non-invasive, it requires certain techniques, the fixing of catheters, some animal restraints and a specific time-frame for the collection of milk and urine [11]. The extraction of progesterone from faecal samples offers a comparable advantage. As such, this non-invasive method provides a basis for reproductive monitoring and lays out information about the reproductive physiology of many endangered species and is vital for the success of assisted reproductive technologies such as artificial insemination, in vitro fertilisation and embryo transfer techniques [18].

Chapter 2: Literature review

2.1: Limitations to conventional progesterone analysis

Conventional methods and the most commonly used methods of progesterone extraction are from blood, urine, milk or hair samples. Urine samples require a mean of restraint such as sedatives in racehorses while saliva samples can be challenging considering some animals' anatomical features such as giraffes or giant anteaters which have a relatively narrow and small mouth. Taking such samples can trigger a lot of stress and subsequently cortisol release during the collection of these samples. Blood sample usually remain the optimum sample for evaluating the reproductive function in animals as this means of sample collection delivers the true concentration of circulating steroid hormones at any given time [4]. The collection process from which blood, milk and hair samples are derived for progesterone metabolite requires a substantial amount of animal restraint. In animals requiring milk extraction for progesterone analysis, there needs to be a specific time allowance during which the animal is lactating for the sample collection to take place and requires restraint as well. Faeces samples provide a great balance between time-efficiency

and accuracy, as it allows for reliable testing of multiple animals at the same time, as well as for long-term analysis of the same animal.

2.2: Faecal progesterone analysis methods

An effective way of measuring progesterone, which is routinely carried out at the University of Veterinary of Medicine in Budapest is enzyme-linked immunosorbent assay (ELISA) by making use of anti-progesterone-antibodies. This test is used to determine substances such as antibodies, antigens, proteins, glycoproteins and hormones [35]. ELISA was developed from the modification done to the radioimmunoassay (RIA) [35], forgoing the use of radioactive materials and expensive equipment [36]. Thus, ELISA is a more effective, cheaper and quicker method than the latter to determine the ovulation or oestrus cycle by noting the differences in the values of progesterone programmed by software [9] and has been used to characterize ovarian cycles, screen open cows and assess reproduction function in wildlife [28,29,30].

To decide which method to use, several factors come into play such as which metabolites need to be analysed, how these metabolites are excreted and which is the safest method of transportation of samples to be used without having anomalies in the data collected [10]. High performance liquid chromatography (HPLC) is also efficient and used in laboratories to assess accurate results of progesterone metabolites [8]. Faecal progesterone analytical method in HPLC is frequently used in many laboratories for quantitative analysis of progesterone metabolites in biological samples [10,11,25,31,32]. However, since this technique is expensive, this kind of analysis is not part of daily routine.

2.3: Challenges in the analysis of faecal progesterone metabolite analysis

While progesterone extraction from faecal samples offers a safer and non-invasive method for reproduction monitoring in animals, there are challenges associated with this sample matrix. A main challenge is the transport and storing of the faecal sample. As it takes a long time for the sample to arrive at the laboratory for analysis, the handling of the sample needs to meet specific requirements for them to be reliable sample upon testing. Failure to properly store and transport the sample could lead to gut bacteria and their enzymes to further metabolize the progesterone metabolites and yield inaccurate data upon testing [10, 26, 27]. Faecal samples can be kept moist or dry with the latter providing a stabilization of progesterone metabolites of up to 180 days [15]. Dried faecal samples are considered advantageous for sampling in remote locations as is the matter when dealing with wild

animals. However, freezing is also a plausible option for preserving progesterone metabolites from wild animal [15] but requires electricity for refrigeration, a resource not readily available in those habitats.

When left in ambient environmental conditions without preservation, the concentration of progesterone metabolites in faeces decreases significantly [11]. For example, faecal samples from cows have been argued to have a significant decrease in net progesterone metabolite levels over short- and medium- term storage without preservatives [11,25]. However, some studies have evaluated this claim and Galama [11] reported no difference detection between day 0 and day 30 of storage without preservative in faecal progesterone concentration of black rhinoceroses. On the other hand, Wasser [33] asserts that the faecal progesterone levels in primates' faeces, 6 hours following excretions without any preservative element, were notably elevated. While currently there is not a widely accepted method of preserving faecal samples, the most suitable method for storage and preservation of faecal sample, which would yield more accurate data, is by storing the sample in methanol or ethanol before analysis. Caution should be placed on the amount of alcohol in the preservation of the samples as a 24% alcohol content would be labelled under explosive or flammable substance which would need to be handle with care when moving from remote locations [37].

2.4: Faecal progesterone samples in different species

As animals such as giant anteaters and sloths do no exhibit external signs of mating or pregnancy, they require follow-ups to allow for breeding management and avoid mortality of new-borns, which can be observed through non-invasive methods of analysis of their reproductive cycles. Although observation of external signs such as heat detection or behavioural indicators can provide an estimate of the mating cycle or pregnancy, it must be complemented with other techniques to determine accurately the most suitable stage of the mating period or the most precise time for artificial intelligence, especially in wild and endangered species. The excretion of steroid hormones can differ greatly among different species. Although blood samples were the most common basis for progesterone extraction and monitoring of the reproductive activities in animals, when dealing with wild animals it requires restraint which induces stress that can compromise the health of the animal and affects the concentration of gonadal steroids [34]. Faecal samples provide a better alternative considering the virtually unlimited number of faeces which facilitates sample collection without putting constraints on the animal.

2.4.1: Progesterone extraction in sloths

A study in 2006 observed the reproductive cycles of 4 captive three-toed sloths by following their oestrogen and progesterone levels in their faeces. Blood samples were also taken to compare the values of progesterone in order to evaluate the efficacy. The evaluation of the radioimmunoassay of 10 frozen 0.3 g samples was carried out after being boiled in 5 mls of 90% ethanol for 20 minutes, centrifuged at 500 G (relative centrifugal force) for 15 minutes vortexed for 30 seconds and re-centrifuged [39]. To measure the oestrogen and progesterone levels in the faecal sample, radioimmunoassay (RIA) is used. Around day 59 in the study, a record of P4 metabolite found that the P4 level rose from 274.10 ng/g to 722.86 ng/g in wet faeces. This was followed by continuous rise in P4 reaching 3242.68 ng/g on the 74^{th} day suggesting ovulation took place [39]. The analysis on the high P4 was confirmed by the delivery of a healthy offspring from the sloth later.

In a study done in 2015, the faecal samples of 4 two-toed sloths in several German zoos were observed for early signs of pregnancy diagnosis [40]. We measured 0.5 g of faecal samples which is mixed with 0.7 mls of distilled water and 4.8 mls of methanol before being shaken for 30 minutes. The mixture was centrifuged at 1500 G for 15 minutes at 20 degrees celsius before the supernatant was extracted and placed into an Eppendorf tube at -20 degrees celsius for storage. For analysis both radioimmunoassay (RIA) and enzyme immunoassays (EIA) methods were used [40]. The results of the analysis on the progesterone concentration showed that P4 levels were on average 345.0±288.0 ng/g which increased to approximately 7588.0±6717.0 ng/g until birth [40]. The study took a baseline of P4>= 911 ng/g to confirm a pregnancy. The study also concluded that a sudden higher than normal progesterone during the luteal phase, for about three weeks which would continue to increase, would signal pregnancy.

2.4.2: Progesterone extraction in giant anteaters

The numbers of giant anteaters are significantly declining throughout the years and progesterone extracted from faeces samples became an absolute necessity to determine when the best time for ovulation for mating is.

A study made by Knott et al. at the Nashville Zoo in 2013 observed 7 giant anteaters to determine their reproductive cycles. We measured 0.503±0.001 g of wet faeces which was collected during 5 random days and stored at 4 degrees celsius overnight [12]. Thereafter the samples were mixed in enzyme immunoassay buffer with “0.1 M phosphate-buffered

saline containing 0.1% bovine serum albumin" [12]. The concentrations of faecal oestrogens and P4 were elevated through the pregnancy and these results correlate with a previous study done in 1998 by Patzl et al [38]. A point for concern in giant anteaters is that they experience obligate diapauses due to "lactation or metabolic stress" during pregnancy which is common in armadillos, minks and giant pants for example [12]. Knott et al. found that the gestation range for giant anteaters were 171-183 days. They also highlighted that the concentration of P4 was more prominent during the luteal phase and that the "pronounced elevation in P4, oestrogen and GC metabolites" would appear after the initial post-mating delay between 81 to 105 days [12] which would indicate a confirmation of a pregnancy diagnosis.

2.4.3: Progesterone extraction analysis in bovines.

In 2007, a study was made by the Mahidol University and Chiang Mai University which analysed faecal and serum samples on 24 Holstein-Friesian cows to test for the concentration of progesterone extraction. While the blood samples were gathered by jugular venipuncture [41], the faecal samples were collected directly from the rectum of the cows and kept frozen at -20 degrees Celsius. The 0.5 g of faecal samples were mixed with 2.5 mls of water and 3 mls of methanol before being vortexed for 30 minutes and "centrifuged at 1000 G for 15 minutes and the supernatant was harvested and stored at -20 degrees celsius until assay" [41]. Direct enzyme immunoassay (EIA) method was used for the progesterone extraction and the study determined that the level of P4 metabolites from the faecal samples which were above 100 ng/g during the days 19-22 post-insemination, would correlate to the diagnosis of a pregnant cow. The conclusion after the analysis of the progesterone extraction from serum and faecal samples against ultrasonography showed accuracies of pregnancy and non-pregnancy determination with a positive correlation of r=0.78 between the levels of serum P4 and faecal P4 metabolites in cows. The progesterone levels during days 19-22 post-insemination during the study yielded 100% accuracy in distinguishing non-pregnant cows while the percentage of accuracy to determine pregnant cows was 67%. [41].

Chapter 3: Goals

This study was conducted to determine the efficiency and accuracy of the extraction of progesterone metabolites from faecal samples and its reliability in three species: giant anteater, Holstein-Friesian cattle and two-toed sloths. Physiological stress from pregnancy and other environmental factors can influence the level of P4 in animals which have been a downturn in progesterone quality with conventional progesterone extractions methods. However, for the purpose of this study, the use of faecal samples as a source of progesterone extraction yields a reliable extraction of P4 without any alteration in its values through an extraction process done using two methods: centrifugation at the laboratory and shaking the test tubes on-field. The objective of this study is to assess whether the laboratory-based hormonal extraction protocol, which requires centrifugation can be avoided and replaced with hand-shaking.

Chapter 4: Materials and methods

4.1: General remarks

Determination of progesterone concentrations can be crucial to evaluate whether the animal is ovulating, hence establishing the appropriate time to mate with a partner. As the metabolism and excretion of steroids differ widely between species and even between sexes, it is crucial that the non-invasive method be extensively corroborated for each specific species. The extraction technique should be chosen based not only on the species, but also on the varying polarity and composition of the steroid metabolites present in the faecal material [14]. Although biological data gathered in an isolated species obtained in different laboratories is comparable, the challenge for comparison comes from the values of faecal progesterone metabolites among laboratories, as they use different extraction procedures and anti-bodies with different cross-reactivities to the progesterone evaluation in faeces [11]. The methodology used for this study is ELISA to determine progesterone concentration in faecal samples from ant eaters, bovines and sloths.

4.2: Faecal sample collection and preparation

Faecal samples collected from sloths (4 samples), giant anteaters (3 samples) and bovine (from a pregnant Holstein-Friesian cattle) and were immediately frozen at -20 degrees. Sloth and giant anteater samples were collected in the Jászberény zoo while bovine samples were collected in a dairy farm.

Each sample was thawed and refrozen on a daily basis to analyse whether repeated freezing-thawing cycles cause degradation in the progesterone metabolite content.

Figure 1. Frozen-thawed faeces samples of giant anteater.

4.3: Faecal progesterone extraction and hormone measurement

We compared and calculated the efficacy and accuracy of determining the progesterone value using hand shaking or centrifugation. Hormone measurement was carried out with a home-made competitive progesterone enzyme-linked immunoassay (ELISA) in the Laboratory for Endocrinology of Department of Obstetrics and Food Animal Medicine Clinic. The extraction and measurement protocols were the same in all of the samples and species.

ELISA works by using sensitized plate which is washed with 0.05% Tween20 solution. The sensitized plate which is washed consists of anti-P4-Ab conjugated to the plate. Using the lab-based method with the centrifugation machine, 250 mg of faeces samples were measured in a glass tube place in a beaker on a weighting scale. 2.0 mls of pure ethanol (80%) were pipetted in the glass tube. The glass tube containing the faeces samples dissolved in ethanol was tightly closed with a rubber cork and shaken for 3 minutes. Thereafter, it was placed in a centrifuge at 3000 rounds per minute for 10 minutes. Once centrifugation was completed, 280 micro litres of the supernatant containing P4 was pipetted and transferred to an empty Eppendorf tube. The same procedure was performed for the on-field measurement of P4 except for the centrifugation step. Hand shaking of the glass tube was performed for 2-3 minutes, after which, the glass tube was placed in a

plastic rack to allow the faeces to settle down for collection of the supernatant at the top with a micropipette.

In order to determine the progesterone value, five progesterone samples with different concentrations were measured with known values marked, referred to as standard series: - 0 ng/ml, 2 ng/ml, 10 ng/ml, 50 ng/ml and 100 ng/ml. Furthermore, 2 internal controls (stored canine blood samples with low and high progesterone levels from previous measurements) were used to control the reliability of each measurement. 20 micro litres of standard series and each sample were added to the plate. Then 180 microlitres of conjugate solution (horse-radish peroxidase conjugated to progesterone) was added and incubated on shake for 1 hour. The same process applies for both the hand-shaked and centrifuged samples. After one hour of incubation, the plate is washed 3 times with Tween-20 based washing solution.

Then, 200 micro litres of 3,3′,5,5′-Tetramethylbenzidine (TMB) were added as a substrate in each well. The plate was then incubated with shaking for 30 minutes. In this step, a colour reaction took place. The intensity of the blue colour would determine the progesterone concentrations, with a darker colour representing fewer amounts of P4 and lighter colour representing higher amount of P4 as shown in figure 2. To terminate the reaction, 50 micro litres of sulphuric acid were added to each well after the incubation period making each of the progesterone samples in the wells turn yellow as shown in figure 3 and P4 level was measured in a microplate reader.

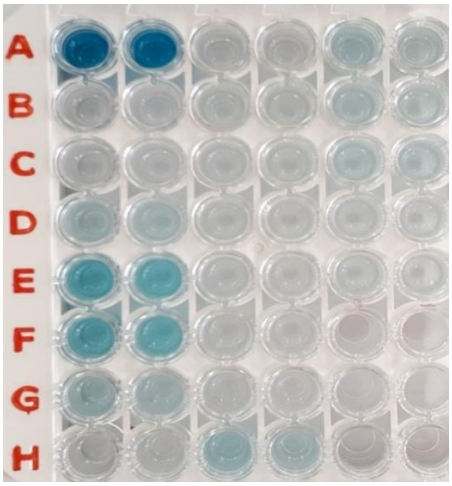

Figure 2. The intensity of the blue colour determines the progesterone concentrations with a darker colour representing fewer amounts of progesterone and lighter colour representing higher amounts of progesterone.

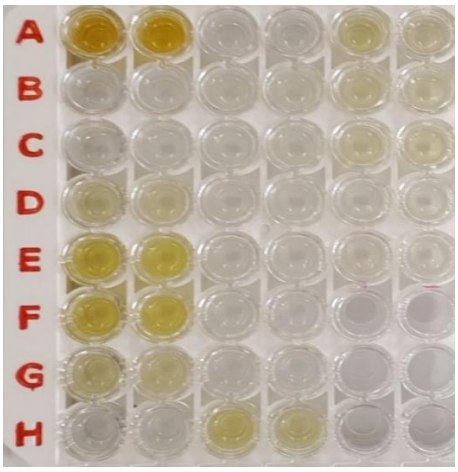

Figure 3. 50 micro litres of sulphuric acid were added to each well after the incubation period making each of the progesterone samples in the wells change colour from blue to yellow.

4.4: Statistical analysis

Intraclass correlation coefficient of centrifuged and shaked samples were calculated to assess the agreement of data. Interassay coefficients of repeatedly frozen-thawed samples were calculated to assess the reproducibility of measurement. Differences of interassay coefficients of variability percentages (CV%) between hand-shaked and centrifuged samples were analysed with Wilcoxon signes rank sum test. Data analysis was performed with version 3.6.2 of R (R Core Team. (2019)). R: The Language and Environment for Statistical Computing. R Foundation for Statistical Computing (https://www.R-project.org/). Since the aim of this study is to analyse the prospect of on-field extraction of the hormones and on-field concentration analysis, the calculation is based on the optical density (OD) values as these values can be read even without microplate reader.

Chapter 5: Results/conclusion

5.1: Effect of repeated freezing-thawing on reproducibility

In order to analyse whether repeated freezing-thawing of the samples affect the progesterone metabolite content, inter-assay variability ("reproducibility") was calculated in each species. Mean interassay cv% of hand-shaked samples of cattle, anteater and sloth were 7.76 ±5.02, 20.8±12 and 12.1±11, respectively. These data did not differ from the CV% of centrifuged samples.

	Hand-shaking		Centrifuge		No. of freeze-thaw cycles
	Mean	**SD**	**Mean**	**SD**	
OD values	0.11	0.005	0.12	0.009	24
CV%	7.76	5.02	10.45	7.04	

Table 1. Means of OD values and CV% of cattle samples, calculated from 24 freeze-thaw cycles.

	Sample	**Hand-shaking**	**Centrifuge**	**No. of freeze-thaw cycles**
Mean OD values	1	0.64	0.69	9
	6	0.46	0.40	9
	12	0.20	0.19	9
SD	1	0.22	0.08	
	6	0.05	0.07	
	12	0.03	0.03	
CV	Sample 1	34.41	11.76	
	Sample 2	11.97	16.77	
	Sample 3	16.02	16.05	
Mean CV%		20.8±12	14.86±2.7	p=1

Table 2. Means of OD values and CV% of giant anteater samples, calculated from 9 freeze-thaw cycles of 3 samples. P value indicates the result of Wilcoxon signed rank test (Mean cv% of hand-shaked VS centrifuged samples).

	Sample	Hand-shaking	Centrifuge	No. Of freeze-thaw cycles
Mean OD values	1	0.06	0.06	5
	2	0.12	0.30	5
	3	0.09	0.09	5
	4	0.06	0.06	
SD	1	0.002	0.01	
	2	0.02	0.45	
	3	0.02	0.01	
	4	0.001	0.002	
CV	Sample 1	3.32	19.60	
	Sample 2	17.39	149.27	
	Sample 3	25.85	13.23	
	Sample 4	2.01	3.02	
Mean CV%		12.1±11.5	46.3±69	p=0.38

Table 3. Means of OD values and CV% of two-toed sloth samples, calculated from 5 freeze-thaw cycles of 4 samples. P value indicates the result of Wilcoxon signed rank test (Mean cv% of hand-shaked VS centrifuged samples).

5.2: Agreement of data between different extraction protocols

Results of data agreement analyses are shown in Table 4.

SPECIES	INTRACLASS COEFFICIENT	RELIABILITY (AGREEMENT)
CATTLE	0.97	excellent
GIANT ANTEATER	0.86	good
TWO-TOED SLOTH	0.07	poor

Table 4. Results of intraclass correlation coefficient (ICC) calculation of cattle, giant anteater and two-toed sloth faecal samples. Reliability classification based on Koo & Li, 2016.

Chapter 6: Discussion

We evaluated the feasibility and suitability of field-friendly extraction of progesterone metabolites. We demonstrated that in two of the three examined species (bovine and giant anteater) this on-site extraction method generates data which is similar to the laboratory-based protocol which requires centrifugation.

Furthermore, we proved that a simple 80% ethanol-based extraction method is enough to obtain the progesterone metabolite content of the faeces samples. Despite the high toxicity of methanol, most of the laboratory-based methods apply methanol-based extraction, described by Palme et al., with some modifications [47]. However, methanol is not safe to be used on the field, and the replacement with ethanol is a cheap and feasible opportunity. Our data is in agreement with the findings of Nugraha et al. [48] where suitability of ethanol-based extraction in frozen-thawed and room temperature-stored samples were analysed in wild orangutans. These authors found high correlation and similarity of metabolite concentrations between the samples stored in different conditions.

The non-invasive method of measuring progesterone is time-efficient and cost-effective, as it allows for numerous faecal samples to be collected at once [6] by even a non-skilled person. However, the excretion time of progesterone metabolites is an important factor, which has to be taken into account when the fecal sample analysis is planned. There are notable differences among species: in non-ruminants, the lag time is approximately 48 hours, while in ruminants it is approximately 12-24 hours [5,11]. Considering this condition, fecal samples should be collected in a daily or a two-days manner. However, the intensive microbial metabolism and the changes in humidity and temperature can influence the steroid metabolite concentration in faecal samples [49]. Due to this fact, in some cases, repeated freezing-thawing is needed when a sample is analysed. Our results show that the field-friendly hand shaking method does not affect the repeatability of the measurements, which suggests that the hormone metabolite content remains the same even after 24 (bovine), 9 (anteater) and 5 (sloth) freezing-thawing cycles.

In order to analyse the reliability of the on-field method, the intraclass correlation coefficient was used. Before a well-established and widely used laboratory method is changed or modified for field application, the reliability must be established. Reliability reflects not only degree of correlation but also agreement between measurements [42]. Historically, Pearson correlation coefficient, paired *t* test, and Bland-

Altman plot [43] have been used to evaluate reliability [44,45]. However, paired *t* test and Bland-Altman plot are methods for analysing agreement, and Pearson correlation coefficient is only a measure of correlation, and hence, they are nonideal measures of reliability. A more desirable measure of reliability should reflect both degree of correlation and agreement between measurements. Intraclass correlation coefficient (ICC) encompasses those as an index. Based on the 95% confident interval of the ICC estimate, values less than 0.5, between 0.5 and 0.75, between 0.75 and 0.9, and greater than 0.90 are indicative of poor, moderate, good, and excellent reliability, respectively [46].

Our results show that extraction of progesterone metabolites from faecal sample can be carried out on the field. However, there are species-specific differences in the agreement of data, which have to be taken into account, when this extraction method is applied on the practice. The repeated freeze-thaw cycle did not affect the intraclass correlation coefficient and reproducibility, which suggests that the collected samples can be stored and later processed on diverse occasions.

Chapter 7: Abstract

English Abstract

In the topic of the reproductive cycle assessment and pregnancy diagnostics, there is an emerging need to develop immunoassays which are suitable for measuring faecal progesterone metabolite level. Faeces samples can be collected very easily and are alternatives for blood sampling in zoo and wild animals. However, the extraction of hormonal content is a labour-intensive process, which requires relatively long time. This step can be shortened, and centrifugation can be avoided, however, the agreement of the results from different extraction protocol is a key for the proper analysis. The aim of this study was to analyse the agreement of data from different extraction protocols (a laboratory based and an on-field alternative) using faeces samples of different species.

Faecal samples were collected from different species; cattle, giant anteater and sloth. Ethanol-based ethanol method were used in all the samples. Before the extraction, each sample was divided into two treatments, that is, centrifugation and manual shaking. Furthermore, after extraction, all samples were refrozen and rethawed to analyse the possible degradation of hormonal content. Progesterone metabolite analysis was carried out with ELISA. Intraclass coefficient of centrifuged and shaked samples were calculated to assess the agreement of data. Interassay coefficients of repeatedly frozen-thawed samples are calculated to assess the reproducibility of measurement.

Mean interassay cv% of hand-shaked samples of cattle, anteater and sloth were 7.76 ±5.02, 20.8±12 and 12.1±11, respectively. These results did not differ from the interassay cv% of centrifuged samples. Intraclass coefficients of the results of the two treatments were 0.966, 0.856 and under 0.5 in cattle, anteater and sloth, respectively.

Our results show that the extraction of progesterone metabolites from faeces samples can be carried out on the field. However, there are species-specific differences in the agreement of data, which have to be taken into account, when this extraction method is applied on the practice. The repeated freeze-thaw cycle did not effect the intraclass coefficient and reproducibility, which suggest that the collected samples can be stored and later processed on diverse occasions.

Összefoglalás

A szaporodásbiológiai monitorozás területén az utóbbi években egyre nagyobb igény merül fel az olyan immunoassay-k alkalmazására, amelyek bélsár minták gyűjtését teszik lehetővé. Ez a mintatípus rendkívül könnyen gyűjthető, és az állatkerti, valamint vadonélő állatok esetében a vérvétel megfelelő alternatívája lehet. Jelenleg azonban a hormon mintából történő kivonása rendkívül munkaigényes, és nehezen elválasztható a laboratóriumi munkától. Van lehetőség azonban a kivonás során alkalmazott centrifugálási lépés kihagyására, az így kapott mérési adatok egyezősége az eredeti eljárással azonban vita tárgyát képezi, és kevéssé feltárt terület. Vizsgálatunk célja volt, hogy megállapítsuk a progeszteron-metabolit mérési adatok egyezőségét két féle bélsár extrahálási protokoll esetén, különböző fajokban.

A bélsár mintákat a következő fajokból gyűjtöttük: szarvasmarha, sörényes hangyász és lajhár. A kivonás ethanol alkalmazásával történt. Az extrahálás előtt a mintákat két csoportra osztottuk: centrifugált és kézzel rázott. Ezen kívül, minden mintát többször olvasztottunk ki és fagyasztottuk vissza, annak érdekében, hogy a hormon tartalom esetleges degradálódását megvizsgálhassuk. A minták progeszteron-metabolit koncentrációját ELISA módszerrel ellenőriztük. A centrifugált és kézzel rázott minták eredményeinek egyezőségét interclass coefficienssel fejeztük ki. A hormon tartalom változását az interassay hibaszázalék (CV%) mérésével vizsgáltuk.

A rázással feldolgozott szarvasmarha, hangyász és lajhár minták interassay hibaszázaléka 7.76 ±5.02, 20.8±12 illetve 12.1±11 %volt. Az eredmények nem különböztek a centrifugált minták azonos paraméterétől. Az intraclass koefficiens értékek (a kétféle mintafeldolgozásból származó eredmények egyezősége) 0.966, 0.856 és kevesebb, mint 0.5 volt szarvasmarha, hangyász, illetve lajhár mintákban.

Eredményeink azt mutatják, hogy a progeszteron metabolitok bélsárból történő kivonása terepi körülmények is lehetséges – fajonként eltérő hatásfokkal. Az ismételt fagyasztás-felolvasztás azonban nem volt hatással a kinyerhető progeszteron-metabolit értékekre.

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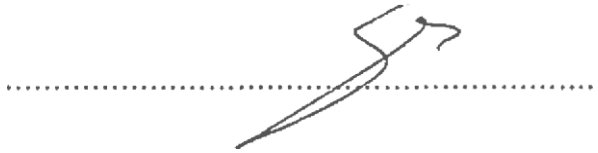
Declaration

I hereby declare that the thesis entitled „**Lab-based and on-field analysis of progesterone metabolites in faeces samples of different species**" is identical in terms of content and formal requirements to the TDK research paper submitted in 2022.

Budapest, 2023. 10. 16.



Dr. Bence Somoskői

Thesis progress report for veterinary students

Name of student: Neel Akash Bolaky

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Name and title of the supervisor: Dr. Bence Somoskői, senior research fellow

Department: Department of Obstetrics and Farm Animal Medicine Clinic

Thesis title: Lab-based and on-field analysis of progesterone metabolites in faeces samples of different species

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	'21	9	10	Principles and practical specs of ELISA	
2.	'21	10	10	Non-invasive hormonal measurement methods	
3.					
4.					
5.					

Grade achieved at the end of the first semester: ……5……...

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	'22	2	10	Sample preparation and data analysis	
2.	'22	3	5	How to write thesis	
3.					
4.					
5.					

Grade achieved at the end of the second semester: ……5……...

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and find suitable to defence,

Signature of the student: 

Signature of the secretary of the department 

Date of handing the thesis on 17th October 2022