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Short communication

Non-homogeneous distribution of steroids in fecal pellets: An example in brown brocket deer (*Mazama gouazoubira*) with progesterone metabolites

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ABSTRACT

Measuring reproductive hormones in feces has become an important tool in the endocrine characterization of wild animals' reproduction. However, several factors may influence its success, such as fecal collection and storage techniques, knowledge of steroid hormone metabolism, the extraction procedure, immunoassay selection, inherent factors, and the distribution of steroid hormones in the feces. It is known that the distribution of these hormones in the feces is not homogeneous, and prior to the extraction of the steroidal metabolites, homogenization of the feces is recommended. Hormonal analysis is based on only a small fraction of the feces, which in theory should be representative of the total. In the case of cervids and other ruminants, feces consist of pellets. Here, the concentration of the steroid metabolites of each pellet was measured in order to evaluate the distribution of the fecal progesterone metabolites concentration in 10 pellets/fecal mass from five female *Mazama gouazoubira*. There were large variations in fecal progesterone metabolites concentrations between the pellets of the same feces/female, showing the following amplitude variations: Animal A: 112%; Animal B: 164%; Animal C: 115%; Animal D: 62%; Animal E: 108%. These results show the importance of adequate homogenization prior to steroid metabolite extraction.

1. Introduction

The brown brocket deer (*Mazama gouazoubira*) is one of the most abundant deer species in the Neotropical region (Duarte, 1996) both in captivity and free range. It is one the most studied species regarding the endocrinology of the estrous cycle and pregnancy (Pereira et al., 2006) and the development of reproductive biotechnologies, such as estrous synchronization (Duarte and Garcia, 1995; Zanetti et al., 2010). This scientific efforts increased knowledge concerning the species, and favor its use as an experimental model to evaluate reproduction techniques as a tool for Neotropical deer conservation (Zanetti et al., 2010).

In wild species, a less invasive alternative to monitoring hormone concentration instead of blood collection is measuring them in the urine and feces, which animal handling is unnecessary (Graham, 2004). The fecal matrix, for example, has been shown to be a good method of non-invasive monitoring (Palme, 2005; Pereira and Polegato, 2010; Polegato et al., 2018).

However, in order to obtain accurate results, observations of these techniques should be performed, such as the collection method and fecal storage, knowledge of steroid hormone metabolism, the extraction procedure, immunoassay selection, biological relevance (physiological validation of hormones) and factors inherent to the individual (sex, age, reproductive cycle) (Palme, 2005; Palme et al., 1996).

Moreover, the distribution of steroid hormones in fecal samples is another aspect to be considered. Studies have shown that the steroid hormones distribution in feces can be unequal within an individual fecal sample regardless of the species (Brown et al., 1994; Wasser et al., 1996). Brown et al. (1994) related that fecal estrogens and progestins are not evenly distributed in cheetahs (*Acinonyx jubatus*), clouded leopards (*Neofelis nebulosa*) and snow leopards (*Panthera uncia*) within individual fecal samples collected from this species. Wasser et al. (1996) showed that the concentrations of radio-labeled estrogen and progesterone metabolites in African elephant (*Loxodanta Africana*) were higher on the outside of the sample compared to the inside.

In the case of cervids and other ruminants, the fecal mass is composed of pellets. As for any fecal analysis, a small amount of feces (in

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this case, a few pellets for ruminants, roughly 0.5–2 g) is collected for fecal mass analysis (Hodges and Heistermann, 2003). However, Millspaugh and Washburn (2003) examined the precision of fecal glucocorticoid measurements from small portion of pellets of white tailed deer and showed that glucocorticoid metabolites may not be evenly distributed in white-tailed deer feces. The authors also stated that this variation is due to a few selected pellets sampling, which could contain very low or high amounts of fecal glucocorticoid metabolites relative to the entire fecal mass.

Notwithstanding, this sampling technique assessed by the authors did not evaluate fecal glucocorticoids amount that can be found in each pellet of the fecal mass. The quantity of steroid storage can vary among the sampling pellets, which can be the main variability source of fecal hormone measurement. Regardless of the hormone evaluated in that study from Millspaugh and Washburn (2003), the pattern of this distribution could be the same for other steroid hormones (e.g. progesterone metabolites). In brown brocket deer, progesterone assay is a well-established protocol for progesterone metabolites measurement in *Mazama gouazoubira* species and it is used to determine ovarian activity, pregnancy status and diagnosis, estrous cycle (Pereira et al., 2006; Zanetti et al., 2010).

In order to clarify these sampling issues, this study aimed to evaluate the concentration of progesterone metabolites in each pellet from the same fecal mass and compare progesterone metabolites concentration within the ten random selected pellet of the same fecal mass in *Mazama gouazoubira*.

2. Materials and methods

2.1. Animals

This study was carried out at the Deer Research and Conservation Center (NUPECCE) facilities at São Paulo State University (UNESP/Jaboticabal Campus). Five females (aged 0.7–4 years old) were housed at NUPECCE. All deer were housed individually in stalls (4 × 2 m) and exposed to normal photoperiod fluctuations. They were fed *ad libitum* with a diet consisting of a pellet feed (12% crude protein, 2% crude fat, 10% crude fiber; Purina Co., Paulínia, São Paulo, Brazil) and approximately 1 kg/deer/day of perennial soybean (*Neonotonia wightii*) or mulberry branches (*Morus alba*). Water was also provided *ad libitum*.

2.2. Fecal sample collection

We collected individual fecal masses (n = 5), 1 fecal sample of each of the five captive adult (n = 5) (aged 0.7–4 years old) female brown brocket deer. Fecal collection was performed between 8:00 and 10:00 a.m. Each female was allocated to a male's stall to induce defecation. The product of one defecation per female was collected and placed in an individually labeled and identified plastic bag. All the fecal samples were collected, stored at −20 °C until we had all the (n = 5) samples. After we had all the samples, they were oven dried. The samples were initially oven dried (Mod. 320-SE®Fanem® Ltd.; São Paulo, Brazil) at 56 °C for approximately 72 h (Hamasaki et al., 2001; Yamauchi et al., 1997). Ten subsamples (pellets) per defecation/female were randomly selected after dried. Each dried pellet sample was pulverized with a hammer wrapped in a procedure glove, which was carefully cleaned with a towel paper after each pellet pulverization.

2.3. Sample extraction

For steroid extraction 5 ml of 80% methanol can be added to 0,5 g of triturated fecal mass as proposed by Graham et al. (2001). However, in order to analyze the progesterone metabolites concentration in each pellet, each entire pellet was weighed and added the quantity of 80% methanol calculated by the weight obtained from the pellet, to maintain the proportion feces:methanol (100 mg/ml). After vortexing for 30 s at high speed, the sample were shaken for 12 h on a mechanical shaker. After centrifugation at 400 × g for 20 min, the supernatant was transferred to a clean tube. Aliquots of supernatant were diluted at a ratio of 1:128 for a progesterone assay for enzyme immunoassay (EIA) analysis (Multiskan MS, Labsystem, Helsinki, Finland).

2.4. Enzyme immunoassay

The progesterone metabolites concentration were determined using CL425 (California University; Davis, CA, USA) for progestogens (P). This antibody was chosen due to its high cross reactivity with the metabolites excreted in *M. gouazoubira* feces—5 α- and 5 β-pregnanes (Polegato, 2004). Validation of hormone concentration was conducted according to Brown et al. (2004) by observation of parallel disposition between the standard curve and that formed by the pool of fecal extracts prepared by serial dilution ($y = -0.0228x + 2.4767$, $R^2 = 0.9695$). Inter-assay coefficients of variation were 5.3% (71.3% binding) for the control and 5.8% (26.8% binding) for P. Intra-assay coefficients of variation were < 10%. All results are expressed in ng/g per pellet/fecal mass/female.

2.5. Data analysis

Data analysis was performed by calculating the variance and coefficient of variation, which, together with the evaluation of the difference between mean and median, showed a random profile distribution of the hormonal concentration within the same fecal mass. The variation amplitudes of fecal progesterone metabolites among pellets/female were calculated using the highest and lowest values in the fecal mass to find the percentile of the variation amplitude.

3. Results

Progesterone metabolites concentrations are shown in Table 1. In the same animal, the values varied between the pellets, with the following amplitudes for the five different females: Animal A: 263–559 ng/g; Animal B: 478–1263 ng/g; Animal C: 309–664 ng/g; Animal D: 187–303 ng/g; Animal E: 479–995 ng/g. The percentage variation between the lowest and highest level in females A, B, C, D and E were 112, 164, 115, 62 and 108%, respectively.

Table 1
Maximum (ng/g), minimum (ng/g), and median (ng/g) values, standard deviation, coefficient of variation (%) and percentile of the amplitude (%) of fecal progesterone metabolites concentration.

Animal	Minimum (ng/g)	Maximum (ng/g)	Mean (ng/g)	Median (ng/g)	Standard Deviation	Coefficient of Variation (%)	Percentile of the variation amplitude (%)
A	263.71	559.79	348.95	320.16	92.88	26.50	112
B	478.24	1263.62	881.79	994.06	327.81	37.00	164
C	309.24	664.72	457.66	394.68	136.65	28.90	115
D	187.58	303.93	257.09	267.55	34.81	13.54	62
E	467.69	995.26	751.12	751.12	189.63	50.70	108
Mean					156.36	31.32	

4. Discussion

This is the first report to measure fecal progesterone metabolites in pellet of captive brown brocket deer. Our objective was to demonstrate how much steroid hormone concentration could vary among the pellets within the same individual fecal sample.

Our results show that progesterone metabolites distribution among the ten pellets was unequally distributed. We noted a great difference in the values of fecal progesterone metabolites concentrations among the ten pellets of the same fecal mass, which varied from 62 to 164%.

We showed that one single fecal mass as we can observe by minimum and maximum values from all pellets can reach progesterone metabolites values related to the luteal phase (357.3 ± 22.0 ng/g) or pregnancy (middle or late pregnancy range of 620 and 630 ng/g respectively) as stated by Pereira et al. (2006) for brown brocket deer, considering that individual differences need to be considered when inferring on the reproductive status from a single sample.

One of the hypothesizes of this high inter assay variation among the pellets analyzed from the same fecal mass could be related to steroid metabolism thought the ruminant digestive system. The hormone metabolism and biliary excretion can be changed by fiber intake, which can also affects total bulk of excreta, diluting or concentrating the hormones accordingly (Wasser et al., 2002).

In addition to that, in the case of animals that excretes dry pellets, two neural mechanism are involved in the pellet formation by the spiral colon, giving the final shape of the boluses that become natural pellets and once formed, the pellets are propelled individually, or in groups, in a discontinuous but semiregular manner (Costa et al., 2015). Probably, that difference of pellets propulsion rate associated with the flow of the steroids in the gastrointestinal tract (Borisenkov, 2000), can cause variation of hormone concentration among the pellets.

This fecal progesterone metabolites concentration analyses or for any steroid measurement, such as glucocorticoids, contain several sources of variation such as measurement error associated with laboratory practices (e.g. pipetting) (Millspaugh and Washburn, 2003). In this present study, all hormone measurement procedure for progesterone metabolites concentration was validated by parallelism test, inter-assay coefficients of variation (< 10%) and intra-assay coefficients of variation (< 10%), which guarantee reliable results from the assays.

If we compare our results to other species, aspects with diet and fecal volume should also be taken into account. Brown et al. (1994) reported that large proportions of hair residue increases fecal estrogen and progesterone metabolite concentrations. Wasser et al. (1996) pointed out some variability in fecal progestin compared to serum progesterone, due to unequal hormone distribution in large fecal mass of African Elephant (*Loxodonta africana*). The absence of homogeneity is also observed in horses and pigs, which variation within one sample can be higher when compared to sheep (Palme et al., 1996).

According to Millspaugh and Washburn (2003) the variability in hormone metabolite distribution among the pellets is no so high in deer compared to other species, due to the small size of feces and their diet based on bulk likely can produce a more homogenous feces. Although this consideration, there is an uneven hormone distribution pattern described for progesterone metabolites in *Mazama gouazoubira*, phenomenon that is similar to the glucocorticoid distribution throughout the entire fecal mass of white tailed deer feces (Millspaugh and Washburn, 2003).

In that study, they compared three pellet groups (one from each end of the fecal mass and one from the center) versus sampling three small portions of the thoroughly mixed fecal mass. They observed that glucocorticoid metabolites measured from the pellet groups were higher than the fecal glucocorticoid measures from the mixed samples. In this regard, our findings corroborate their statement, that a few pellets from the fecal mass may bias assay interpretation.

However, our results contrast with the findings of Morrow and Monfort (1998), who showed a relatively homogeneous steroid distribution for oestrogens and progestins among pellets in scimitar-horned oryx (*Oryx dammah*), indicating that any portion of the sample should adequately represent the entire fecal mass. This consistent distribution was similar to that described for sheep (Palme et al., 1996) and sable antelope (*Hippotragus niger*; Thompson et al., 1998).

These considerations suggest that sample homogeneity can vary between species, individual, the type of hormone analyzed and the type of biological event related to this hormone (Wielebnowski and Watters, 2007).

Our findings demonstrate that fecal sampling based on a small portion of pellets in *Mazama gouazoubira* may lead to erroneous interpretations. We suggest that all defecation products should be mixed at the time of collection, before sampling for hormonal measurements (Wasser et al., 1996). Moreover, before conducting studies involving steroid hormone measurements, a preliminary test should be adopted to guarantee the homogeneity of the sample (Wielebnowski and Watters, 2007) in order to obtain reliable results that correspond to the physiological and endocrine processes of the studied species.

5. Conclusion

This study suggests that all defecation products should be used for hormone analysis, and that adequate homogenization of the fecal mass is required before extraction. This would ensure that the fraction sample for hormonal analysis more accurately represents the mean concentration of fecal progesterone metabolites, and consequently, the expected condition based on the animal's endocrine physiology.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.06.010.

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