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Optimizing captive short-beaked echidna (*Tachyglossus aculeatus*) fecal sample identification and hormonal analysis

Kate J. Dutton-Regester[1] | Alice Roser[2] | Haley Meer[2] | Marilyn B. Renfree[3] | Stephen D. Johnston[1] | Tamara Keeley[1]

[1]School of Agriculture and Food Sciences, The University of Queensland, Gatton, Queensland, Australia

[2]Currumbin Wildlife Sanctuary, Currumbin, Queensland, Australia

[3]School of BioSciences, The University of Melbourne, Melbourne, Victoria, Australia

Correspondence

Kate J. Dutton-Regester, School of Agriculture and Food Science; The University of Queensland, Gatton, QLD 4343, Australia.
Email: k.duttonregester@uq.edu.au

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Abstract

The objectives of this study were to develop a fecal marking protocol to distinguish male from female samples during the echidna breeding season and to determine if normalizing fecal progesterone metabolite data for inorganic content improves the detection of biologically relevant changes in metabolite concentrations. Over a period of 6 weeks, four echidnas were provided with green food coloring powder mixed into 20 g of their regular feed with the dose adjusted weekly by 0.05 g. The proportion of organic (feces) versus inorganic matter (sand) in the fecal samples of three echidnas was determined by combustion of organic matter. Hormonal data was then expressed as metabolite concentration per total dry mass (with sand) of extracted sample versus metabolite concentration per total mass of organic material (without sand). The optimal dose of food coloring powder was 0.30 g: this was excreted in the feces of all echidnas within 24 h of consumption with color present for two consecutive days. Correction for inorganic content (sand) did not significantly affect variability of fecal progesterone metabolite levels (mean CV ± SE with sand: 142.3 ± 13.3%; without sand: 127.0 ± 14.4%; $W = 6$, $p = .2500$), or the magnitude of change from basal to elevated fecal progesterone metabolite concentrations (mean ± SE with sand: 8.4 ± 1.7; without sand: 6.6 ± 0.5, $W = 10$, $p = .1250$). Furthermore, progesterone metabolite concentrations before and after correction for sand contamination correlated strongly ($r = .92$, $p = < .001$). These methods will facilitate future reproductive endocrinology studies of echidna and other myrmecophagous species.

KEYWORDS

fecal marker, progesterone metabolites, sand contamination, tachyglossidae

1 | INTRODUCTION

The short-beaked echidna is myrmecophagous, feeding almost exclusively on ants and termites; consequently, the digestive system has adapted to accommodate for this specialist diet (Griffiths, 1978). The echidna secures ants and termites using an elongated tongue that is moistened with sticky saliva; inadvertently inorganic matter such as soil and sand are ingested (Augee et al., 2006). The ingested soil and sand have an important role in the digestive process by assisting the tough cornified stomach in grinding invertebrate bodies (Augee et al., 2006) and has been shown to facilitate fecal formation in the numbat (*Myrmecobius fasciatus*; Power & Monaghan, 2003) and Sunda pangolin (*Manis javanica*; Cabana & Tay, 2020).

Fecal hormonal analysis has become a popular noninvasive alternative to the potentially stressful and difficult collection of blood samples for the reproductive management of many species in captivity (Schwarzenberger & Brown, 2013). However, for fecal hormonal analysis to be of value it is pertinent that fecal samples can be reliably collected from target individuals and that hormone metabolites are accurately measured.

When male and female animals are co-housed for reproductive purposes, a fecal marker is commonly incorporated into the diet of the target animal (Fuller et al., 2011). A fecal marker is a substance that is not or is minimally absorbed from the alimentary tract and as such, can be found in the feces (Kotb & Luckey, 1972). Examples include particulate (e.g., colored plastic beads, glitter, seeds, corn) and particle (food colorants and lake pigments) markers (Fuller et al., 2011); however, suitability of each marker may be species dependant. Food grade polyethylene pellets (1 × 1 × 3 mm) have been trialed in echidnas and were readily consumed by four female echidnas; however, not all pellets were recovered in feces (Higgins et al., 2004). Although 0.008 g/day of blue food dye mixed in food was also tried, limited information regarding its utility and reliability were documented (Higgins et al., 2004) suggesting the need to investigate this technique more rigorously.

Once fecal samples have been collected, and before hormone extraction, it may be necessary to determine the optimal method to process the samples to control for effects such as moisture, bone, feathers, and inorganic materials which may mask, artificially inflate or add variability to hormone metabolite data (Palme, 2005). Thus, the presence of soil or sand in the feces of myrmecophagous mammals may introduce variation not related to endocrine activity, confounding the interpretation of hormone metabolite data. As considerable variability was observed in the initial analysis of echidna fecal samples in our laboratory, it became important to rule out possible influences of inorganic content.

The main objectives of this study were to: (1) develop a fecal marking protocol to distinguish male from female fecal samples while animals are paired for breeding purposes; and (2) determine if normalizing fecal progesterone metabolite (FPM) data for inorganic content (sand) can reduce the level of variability present, thereby improving the detection of biologically relevant changes in FPMs.

2 | METHODS

2.1 | Study animals and location

Five female and two male sexually mature echidnas (*Tachyglossus aculeatus*) from the echidna breeding center at Currumbin Wildlife Sanctuary (Gold Coast, Australia, 28.1360S, 153.4830E) were included in this study. These echidnas were long-term wild-caught echidnas, as such, the respective age of these animals was unknown. The average weight of female and male echidnas was 4.4± 0.04 and 5.2± 0.04 kg, respectively. The echidnas were housed and maintained as previously described by Dutton-Regester et al. (2021). The echidnas were obtained and maintained under the Queensland Government EPA scientific purposes permit WISP153546614 and the experiment was conducted with the approval of the University of Queensland Animal Ethics Committee (SAFS/334/17).

2.2 | Experiment 1—Fecal marker evaluation and optimization

This study was conducted over 6 weeks during April and May 2019. Two female (F1 and F2) and two male (M1 and M2) echidnas were randomly selected to determine the minimum amount of green, water-soluble food coloring powder (Chromacake, Baking Pleasures; ABN: 23 717 428 010) necessary to adequately be detected in their feces.

Food coloring powder was given to each echidna once per week during the trial to determine level and length of effectiveness. On Day 1 of the experiment, each individually housed echidna received a 20 g portion of their regular feed which had 0.20 g food coloring powder mixed in. Once each echidna had finished consuming the colored food mixture (either completely consumed or showing no further interest), the bowl was removed, and they were given their remaining portion of food. To quantify palatability, the quantity of colored food consumed by each echidna was recorded as A = all eaten (100%), M = most eaten (75%), H = half (50%), S = small portion (<50%). Palatability was considered acceptable if the echidna ate 75%–100% of the colored food mixture.

For the 6 days following fecal marker administration fecal samples were collected daily (10:00–11:00 h) from each echidna's enclosure and inspected in-hand by the same observer for the presence of color and graded as follows: 1 = entire fecal sample is distinctly colored, 2 = entire fecal sample faintly colored, 3 = fecal coloration patchy but distinct, 4 = no discernible color present. Fecal samples were also graded to assess for potential gastrointestinal upset as follows: 1 = firm but soft to touch (normal); 2 = firm and hard to touch (constipated); 3 = loose stool (diarrhea). The dosage was increased by 0.05 g each week until all four echidnas produced feces containing clearly visible color within 24–48 h of consuming the colored food mixture.

2.3 | Experiment 2—Normalizing fecal progesterone metabolite data for sand

This experiment utilized daily fecal samples collected from three female echidnas (F3, F4, and F5) between June and August 2020. Daily female fecal samples were placed into labeled plastic Ziplock bags and stored at −20°C. Before analysis, fecal samples were dried overnight at 60–65°C and external sand was removed after drying. Each sample was then pulverized using a rubber mallet and sieved using a 0.79 mm mesh strainer. Following this, 0.20 g (± 0.01 g) of each sample was weighed out in a glass scintillation vial; 5 ml of 80% methanol was added before the vials were placed on a rotating shaker overnight for hormone extraction. The next day, the samples were centrifuged for 10 min at 1000*g* and the supernatant decanted into a new scintillation vial (leaving the fecal pellet behind) and the extract stored at −20°C until further analysis.

Fecal extracts were analyzed in duplicate by enzyme immunoassay procedures previously described for progesterone (Swinbourne et al., 2017). Progesterone antibody No. CL425 (C. Munro, UC Davis) was used at a dilution of 1:120,000. Details regarding cross-reactivity and sensitivity are reported elsewhere (Munro & Stabenfeldt, 1984).

To quantify the proportion of organic (feces) versus inorganic (sand) matter in fecal samples, the fecal pellet remaining after decanting the 80% methanol after extraction were air-dried at room temperature for 7 days. Once dry, each pellet was placed into a preweighed ceramic crucible and weighed to confirm initial weight (organic + inorganic weight). This was then placed in a muffle furnace (Modutemp, model WE630AM01) at 550°C for 8 h to combust the organic matter. Once cool, each crucible with the remaining inorganic matter was weighed to determine the weight of the inorganic component of the fecal sample. Organic content of the original fecal pellet was calculated as the initial weight minus the final weight. Fecal metabolites were then expressed as values per unit of the dry weight of extracted fecal material (i.e., the original mass, 0.20 ± 0.01 g) and as values per total mass of organic material (i.e., the newly established organic content) for analysis.

2.4 | Statistical analysis

An iterative process was used to calculate baseline hormone metabolite concentrations using the *hormLong* package (Fanson & Fanson, 2015) in "R" (version 3.6.2), as described by Dutton-Regester et al. (2021). Correlations between FPM levels expressed per mass extracted feces (organic + inorganic) and per "sand-free" fecal (organic only) mass were assessed using Spearman's rank correlation coefficient. To evaluate the influence of sand contamination on characteristics of FPM pattern, we compared the variability in FPM concentrations (calculated as coefficient of variation (CV)) between uncorrected and "sand-corrected" FPM profiles as well as the magnitude of change from basal to elevated concentrations under the two conditions; both comparisons were carried out using two-tailed Wilcoxon signed-rank tests with the statistical significance level set at .05. All statistical tests were conducted using GraphPad Prism (version 9.3.0 for Mac; GraphPad Software).

3 | RESULTS

3.1 | Experiment 1

All echidnas readily consumed the entire plate of colored food mixture (see Supporting Information: Video file "Eating") except for one instance where F2 only ate half of the food provided (Figure 1). All fecal samples inspected during this experiment were of normal consistency. The green food coloring powder typically imparted a blue coloration to fecal samples; however, bright green coloration was sometimes observed. This coloration was most apparent in the first fecal sample defecated after consumption of the colored food portion, which was within 24 h, with the entire sample distinctly colored; however, the color become fainter and progressively less uniformly distributed over the following days. The minimum quantity of food dye that provided clear, consistent color in feces was 0.30 g; this was present for 2 days for both female and male echidnas. When the fecal marker dose was increased to 0.50 g, the color was at least partially present in feces for 3–4 days.

3.2 | Experiment 2

The mean percentage of sand in the 0.20 g (± 0.01 g) fecal extraction samples for F3, F4, and F5 was 63% (range: 20%–88%; $n = 20$), 89% (range: 76%–94%; $n = 19$) and 83% (range: 32%–98%; $n = 24$), respectively; or 78.3% across all animals (Figure 1a,c). Correlations between FPM levels expressed per mass extracted feces (organic + inorganic) and per "sand-free" feces (organic only) for F3, F4, and F5 were 0.86, 0.96, and 0.91, respectively ($p \leq .001$) and the qualitative patterns of FPM profiles were similar (Figure 2). Correction for sand contamination did not significantly affect variability of FPM levels (mean CV ± SE with sand: 142.3 ± 13.3%; without sand: 127.0 ± 14.4%; $W = 6$, $p = .2500$), or the magnitude of change from basal to elevated FPM concentrations (mean ± SE with sand: 8.4 ± 1.7; without sand: 6.6 ± 0.5, $W = 10$, $p = .1250$). Mean baseline (mean ± SE (standard error) with sand: 1761.5 ± 1402.5; without sand: 6212.5 ± 2381.2 ng/g; $t = 3.9$, $p \leq .05$) and mean elevated (mean ± SE with sand: 10,573.8 ± 5672.1; without sand: 46,189.8 ± 13,013.4; $t = 4.8$, $p \leq .05$) FPM concentrations were significantly higher when calculated for organic (fecal) mass than the original mass (organic + inorganic). For F3 and F5, after mating FPM concentrations were first elevated above baseline by Days 14 and 10, respectively, irrespective of whether the data set was normalized for sand or not (Figure 2a,c), but for F4 FPM concentrations were elevated above basal values 2 days earlier when using the original mass (Figure 2b).

4 | DISCUSSION

This study reports the successful development and application of a fecal marker technique to enable the accurate identification of individual samples while echidnas are paired, and that normalizing

FIGURE 1 This panel demonstrates: (a) food coloring powder in original form; (b) food coloring powder mixed into 20g of echidna food; (c) fecal sample collected within 24 h of consumption; (d) fecal sample collected within 24 h of consumption broken to view color and sand inside; (e) fecal sample collected within 48 h of consumption; (f) fecal sample collected within 72 h of consumption; (g) fecal sample collected within 96h of consumption, (h) fecal sample collected within 96h of consumption broken to view color and sand inside. [Color figure can be viewed at wileyonlinelibrary.com]

FPM data for sand content is unnecessary as it does not significantly improve the interpretation of progesterone metabolite secretion.

Our results show that the minimum dose of food coloring powder required to reliably impart color in echidna feces was 0.30 g; a much higher dose than the 0.008 g of a food colorant used in a previous study for which limited details were provided (Higgins et al., 2004). While higher doses of food coloring powder sometimes resulted in colored fecal output for 3–4 days, we recommend the

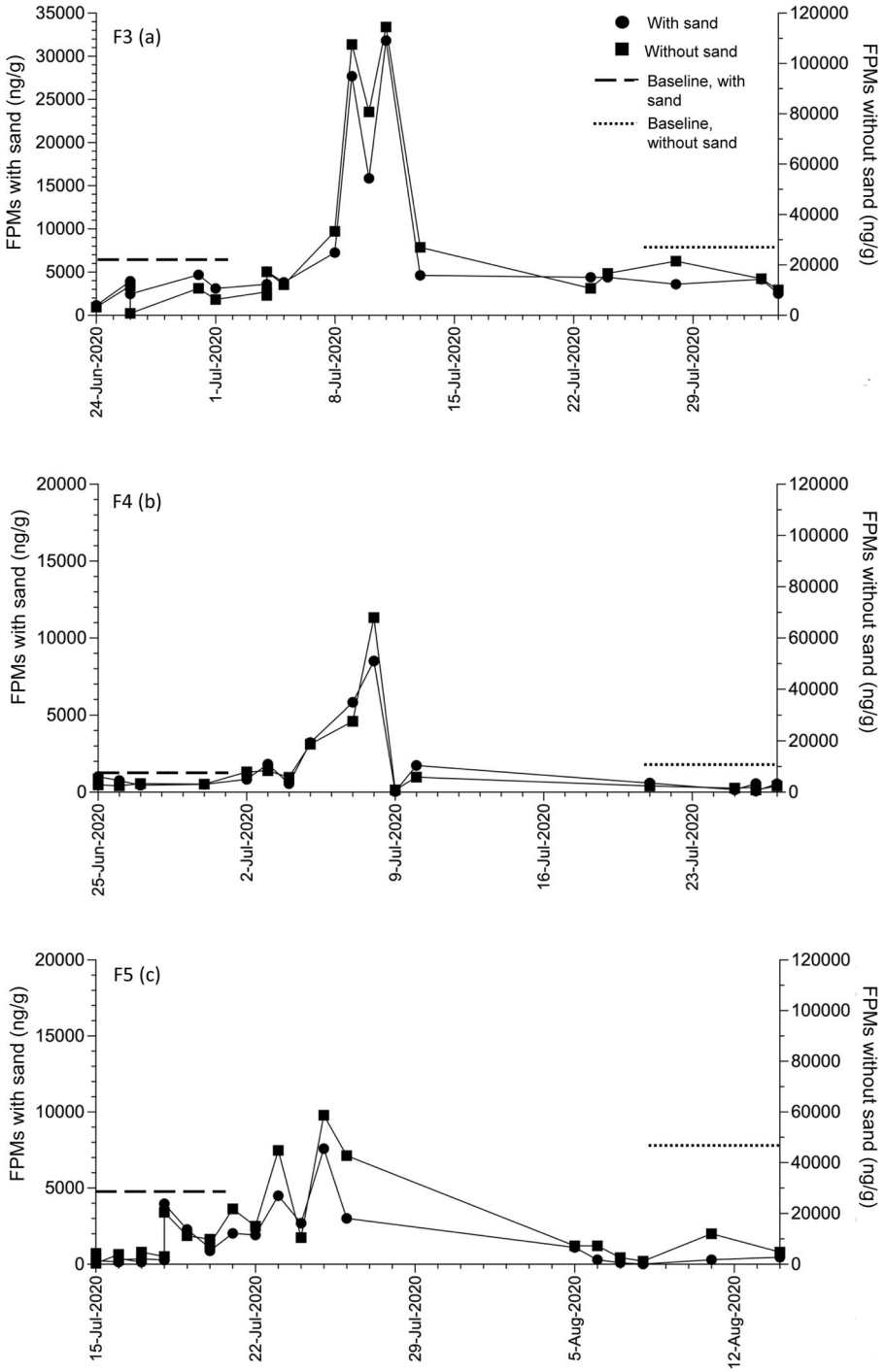

FIGURE 2 Fecal progesterone metabolite profiles (FPM) of female echidnas F3 (a), F4 (b), and F5 (c) uncorrected (with sand) and corrected for sand (without sand) content. Circles indicate FPM concentrations "with sand," squares indicate FPM concentrations "without sand," broken line indicates baseline FPM concentrations "with sand," dotted line indicates baseline FPM concentrations "without sand."

minimum dose (i.e., 0.30 g) be provided daily or every second day to ensure feces are reliably marked.

None of the echidnas demonstrated clear aversion to the colored food mixture indicating no issue with palatability. It is unclear why F2 only consumed half of the mixture at the second smallest dose as this animal readily consumed the mixture at all other doses. However, this experiment was conducted over a relatively short duration of time with each echidna only receiving six portions of colored food, interspaced by 6 days.

Although we attempted to remove sand contamination from fecal samples before extraction, samples were still composed of around 78% sand. Correcting FPM data for sand resulted in significantly higher FPM concentrations compared to the original data set but this did not significantly change FPM patterns, nor did it affect magnitude of change from basal to elevated FPM concentrations. These findings are consistent with those of Ganswindt et al. (2012) and Braga Goncalves et al. (2016) who each investigated the influence of fecal soil or sand contamination on glucocorticoid

patterns in Ground-Feeding aardwolves and meerkats, respectively; the latter study reporting around 60% fecal sand contamination. While correcting FPM data for sand did not affect variability of FPM levels overall, FPM concentrations increased above baseline 2 days earlier for one female when using the original data set. For the other two females, FPM concentrations increased above baseline at the same rate for both data sets. Although our study had low sample sizes and is preliminary in nature, it suggests that normalizing echidna FPM data for sand contamination is unnecessary as it is unlikely to improve the detection of biologically relevant changes in FPMs.

In this study we were able to exemplify the utility of using green food coloring powder as a fecal marker and show that future studies investigating FPM excretion in echidnas do not need to apply extensive methods (such as fecal combustion) to determine inorganic matter content of samples before hormone analyses. The methods and findings described here will facilitate future studies of reproductive physiology that utilize fecal samples from the echidna or other myrmecophagous species.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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