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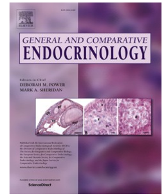

Research paper

Non-invasive assessment of fecal glucocorticoid, progesterone, and androgen metabolites and microbiome in free-ranging southern white rhinoceros (*Ceratotherium simum simum*) in South Africa

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ABSTRACT

Increased poaching in northern South Africa has necessitated relocation of large numbers of southern white rhinoceros (*Ceratotherium simum simum*) to the Eastern Cape Province. The climate and grassland ecology of this province differ from that of northern South Africa which may impact the health of this species. This assessment of fecal steroid levels and microbiome in 10 free-ranging southern white rhinoceros in the Eastern Cape will provide insights into white rhinoceros physiology in this biome. Fecal steroid metabolites were analyzed using enzyme immunoassay (EIA) and ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS). Fecal microbial composition was assessed via next generation sequencing. EIAs with antibodies raised against progesterone (P4; mouse monoclonal – CL425 clone), testosterone (T; rabbit polyclonal), corticosterone (B; sheep polyclonal) were utilized. Pregnant females had large quantities of fecal progesterone metabolites (FPMs) detected by CL425 EIA. Pregnant females also had native P4 and 11α-hydroxydihydroprogesterone (11αOHDHP4; 4-pregnen-11α-ol-3,20-dione) detected by UPC^2-MS/MS but these concentrations were 1000-fold less than the concentrations of FPMs detected by the CL425 EIA. By contrast, non-pregnant females had FPM concentrations detected by CL425 EIA which were similar to native P4 and 11αOHDHP4 concentrations detected by UPC^2-MS/MS. Mean fecal androgen metabolite (FAM) concentrations detected by the T EIA were similar between males and females. 11-ketoandrostenedione (11KA4) detected by UPC^2-MS/MS was higher in females than males. However, there was no difference between males and females in the concentration of fecal glucocorticoid metabolites (FGMs) detected by the B EIA. *Bacteroidia*, followed by *Clostridia*, was the most abundant classes of fecal microbes. The unfiltered microbiome of females was more diverse than that of males. The core fecal microbiome of young rhinoceros had a higher observed species richness (Shannon diversity index, and Simpson diversity index) than that of old rhinoceros. In the alpha male, immobilization was associated with an increase in FGMs detected by 11-deoxycortisol (S) detected by UPC^2-MS/MS coupled with decreased abundance of *Spirochaetia*. We detected substantially different FAM and FPM concentrations from those previously reported for both captive and wild white rhinoceros. Comparison of our UPC^2-MS/MS and EIA results underscores the fact that most EIAs are highly cross reactive for many steroid metabolites. Our data also demonstrates a distinct effect of stress not only on FGMs but also on the fecal microbiome. This is the first non-invasive assessment of fecal steroid metabolites by UPC^2-MS/MS and the fecal microbiome in wild white rhinoceros.

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1. Introduction

The International Union for Conservation of Nature's Red List of Threatened Species lists the southern white rhinoceros (*Ceratotherium simum simum*) as "near threatened" with an overall decreasing population trend (Emslie, 2020). In 2015, the South African government introduced a strategic Biodiversity Management Plan to combat poaching by relocating rhinoceros from areas with the highest poaching threat in the northern portion of the country (Africa, 2015; Ferreira et al., 2017). Under this management plan, the percentage of white rhinoceros managed on private game reserves in South Africa grew by 3 % from 2015 to 2018 (Africa, 2015, 2019).

The historical range of the southern white rhinoceros in South Africa extends from Kruger National Park to KwaZulu-Natal, a region that contains lowveld, mesic and dry highveld grassland, and sub-escarpment grassland ecoregions (Rutherford et al., 2006). With protected areas becoming scarce due to human encroachment and increased poaching, southern white rhinoceros are now found throughout South Africa (Balfour et al., 2015, Balfour et al., 2018). While the Eastern Cape has grassland habitat suitable for southern white rhinoceros, the grass species in this region, which is comprised of Albany thicket, differ from grass species in the north (Rutherford et al., 2006; Trytsman et al., 2020). The grass subfamilies Aristidoideae, Chloridoideae, and Panicoideae are found in both the north and the Eastern Cape (Trytsman et al., 2020). Yet, the subfamily Arundinoideae, which is high in cellulose and liginin (Codron et al., 2007), is found only in the north and the subfamilies Danthonioideae and Pooideae, which are high in cellulose and complex carbohydrates (Soreng et al., 2017), are found only in the Eastern Cape (Trytsman et al., 2020). Certainly, the different nutrient content and composition of the grasses between these ecoregions could certainly impact the gastrointestinal transit time, microbiome composition, and conjugation and excretion of biologic compounds. Furthermore, little is known about the long-term ecological effects of introducing rhinoceros subpopulations on reserves and parks outside of their historic natural range (Richard Emslie and Kock, 2009; Rookmaaker and Antoine, 2012; Sheil and Kirkby, 2018).

Conservation of the southern white rhinoceros depends on its reproductive success. However, translocation and introduction to captivity can affect both overall health and reproductive function (Carlstead and Brown, 2005; Yang et al., 2019). Monitoring stress and reproductive health is essential to assessing both short-term effects of capture and relocation and long-term adaptation of a sub-population to a new, non-native range (Turner et al., 2002). Characterization of the overall health of wild rhinoceros non-invasively can be done via correlation of fecal steroid hormones and microbes with behavior. Non-invasive methods for the analysis of glucocorticoid (GC) and reproductive steroid hormones have been established for both black and white rhinoceros (Edwards et al., 2020; Ganswindt et al., 2012; Metrione and Harder, 2011; Schwarzenberger et al., 1996). It has been reported that fecal steroid hormone assays show that fecal glucocorticoid metabolites (FGMs) reflect adrenal activity in captive rhinoceros (Brown et al., 2001; Carlstead and Brown, 2005; Metrione and Harder, 2011). Fecal progesterone metabolites (FPMs) fluctuate in response to changes in ovarian progesterone (P4) output throughout the cycle. Yet, variability in cycle length among captive female rhinoceros has limited our understanding of normal ovarian cycle duration in black and white rhinoceros (Brown et al., 2001; Kobus, 2012). Patton et. al. (1999) actually found that estrous cycles in white rhinoceros were either one or two months in duration with similar inter-luteal (follicular) phase lengths in each of these estrous cycle lengths. Those rhinoceros that had two-month-long estrous cycles had extended luteal phase length (Patton et al., 1999). Given that one-month-long estrous cycles were more prevalent than two-month-estrous cycles, Patton et. al. considered the one month long estrous cycles "normal". By contrast, Brown et. al. (2001) found that out of 29 characterizable estrous cycles across seven female white rhinoceros, only five were approximately-one-month-long estrous cycles whereas 24 were approximately-two-month-long estrous cycles (Brown et al., 2001). Additionally, in this study there was a large amount of variability in the follicular phase length (2–21 days)(Brown et al., 2001).

Whether radioimmunoassays (RIAs) and enzyme immunoassays (EIAs) commonly used to detect fecal steroid metabolites in free ranging wildlife have sufficient steroid specificity to ascertain normal endocrine physiology and pathophysiology is debatable. Many EIAs and RIAs use antibodies raised to specifically detect unmetabolized target steroids. However, cross reactivity of these antibodies with similar steroids and their metabolites often occurs (Graham et al., 2001; Touma and Palme, 2005). Furthermore, the dominant steroid hormone metabolites excreted vary between species (Palme, 2019). Cortisol (F) is the dominant GC in most mammalian species, but corticosterone (B) is the main GC in mice, rats, rabbits and avian species (Sheriff et al., 2011). Moreover, there are species differences regarding the route of excretion (feces vs urine) and the formed metabolites (Palme et al., 2005). In human medicine, liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography tandem mass spectrometry (GC–MS/MS), sensitive analytical techniques that can measure steroid hormones in small sample volumes, are routinely used (Bloem et al., 2015; Boggs et al., 2016; Koal et al., 2012). To date, these techniques have been applied to wildlife species in toxicology-based studies (Luzardo et al., 2014), studies on the blubber of cetaceans (Boggs et al., 2017; Dalle Luche et al., 2019), and, recently, a study of FGMs in koala (Santamaria et al., 2021).

Captivity significantly alters the rhinoceros microbiome and, therefore, may affect the immune status of captive black rhinoceros (Roth et al., 2019). The microbiome of wild rhinoceros is richer in observable taxonomic units (OTUs) and more diverse than the microbiome of captive rhinoceros (Gibson et al., 2019). Interestingly, intestinal microbiota indirectly regulate the sensitivity of GCs or directly convert conjugated estrogens and phytoestrogens into deconjugated counterparts (Baker et al., 2017; Vodicka et al., 2018). Phytoestrogen-rich diets are negatively correlated with fertility in captive rhinoceros (Tubbs et al., 2012; Tubbs et al., 2016). However, the species and diversity of fecal microbiota are specific to rhinoceros species. In captive white rhinoceros, the estrogens in their legume-based diet can be metabolized by the gut microbiome, indicating that vegetation and habitat may indirectly influence reproductive success via microbiota composition (Williams et al., 2019).

The aim of this study was to non-invasively characterize the gastrointestinal and endocrine health of wild southern white rhinoceros in the Eastern Cape of South Africa. We measured fecal steroid metabolites and compared EIAs with ultra-performance convergence chromatography (UPC^2-MS/MS), a highly sensitive and specific method for the accurate identification and quantitation of steroids. We performed metagenomic sequencing to ascertain fecal microbiome composition, and monitored rhinoceros behavior.

2. Methods

2.1. Behavioral observation and fecal sample Collection:

This work was approved by the Rhodes University Animal Research Ethics Committee (# 2019–0920-045 T). Ten (3.7) southern white rhinoceros (*Ceratotherum simum simum*) aged 3–18 years old in a single crush were monitored daily on a private game reserve in the Eastern Cape of South Africa (Supplemental Table 1). The biological sample size was limited by availability of samples as well as the cost of the technologies utilized to interrogate the samples. Rhinoceros behavioral data were collected on the reserve by field monitors using CyberTracker software (Cape Town, South Africa). Fecal samples from 10 rhinoceros were collected from October 2018 to October 2019 only when the field monitor could: visually confirm the identity of the rhinoceros, verify that the rhinoceros did not urinate on the fecal pile, and collect the feces

within 10 min of defecation. Given that the samples were difficult to collect and opportunistically collected, they were not all collected at the same time each day. Glucocorticoids, as well as other steroid hormones, fluctuate in the blood with a circadian pattern (Parikh et al., 2018). Furthermore, fecal glucocorticoids in rodents have been shown to also fluctuate with a peak of these steroid hormones just before waking (Cavigelli et al., 2005). Taking this knowledge into consideration, collection of our samples during various points during the day could have affected measured steroid output in the samples, particularly with reference to glucocorticoids. Each fresh fecal pile had several large spoon-sized pieces of feces (~20 g) placed into a Whirl-Pak®. After collection, fecal samples were placed on ice packs in a cooler for up to two hours before freezing at −20 °C for 2–4 weeks. Samples were sequentially moved to −80 °C for storage prior to processing and analysis.

2.2. Enzyme immunoassays – corticosterone, progesterone, and testosterone

Wet fecal matter (~0.15 g) was taken from the middle of each ~20 g fecal sample and was weighed into 2.0 mL micro-centrifuge screw cap tubes (Fisher Scientific, Waltham, MA, USA). Fecal samples were extracted in 1.5 mL of 80 % methanol with glass beads using a bead-beating method (Fisher Scientific, Waltham, MA, USA). The suspended samples were vortexed at maximum speed for 60 s and centrifuged at 5,000 g at 4 °C for 20 min. After centrifugation two supernatant aliquots were collected in clean glass vials: 1.0 mL for assessment of sex steroid metabolites and native corticosterone (B), and 0.5 mL for the fecal B metabolite assay. The supernatants were dried under nitrogen in a 50 °C water bath for 90 min. All dried samples were capped, covered with Parafilm® (Bemis Company, Neenah, WI, USA), and stored at 4 °C for two weeks until further processing.

Prior to analysis, dried samples were resuspended in 0.25 mL 100 % methanol and sonicated for 15 min, capped, and vortexed for 10 s. This sonicate-vortex cycle was repeated three times, increasing the sonication time by 10 min and the vortexing time by 10 s with each replication of the procedure. Before the last sonicate-vortex cycle, another 0.25 mL methanol was added to each vial. After vortexing, vials were wrapped in Parafilm ® and stored at −80 °C until EIAs were performed.

FGMs, FPMs, and fecal androgen metabolites (FAM) were assayed using double antibody EIAs. The Corticosterone ISWE Mini Kit (Catalog#: ISWE007, Arbor Assays, Ann Arbor, MI, USA) was used to measure B levels, the Progesterone ISWE Mini-Kit (Catalog#: ISWE003, Arbor Assays; CL425 clone) was used to measure P4 levels, and DetectX Testosterone EIA Kit (Catalog#: K032-H5, Arbor Assays) was used to measure T levels. The B EIA utilized a polyclonal antibody raised in sheep to the 3-CMO derivative of corticosterone. The T EIA utilized a polyclonal antibody raised in rabbits to the 3-CMO derivative of testosterone.

The antibodies raised against B, P4, and T have been validated for assessment of FGM in rhinoceros and FPM for ovarian cycles in female rhinoceros and FAM for testicular function in male rhinoceros (Edwards et al., 2020; Kretzschmar et al., 2004). Each assay was biochemically validated for use with these samples by calculating parallelism of a dilution curve of a pooled sample to a standards curve for each assay. The EIA assessment of B used a sheep polyclonal antibody raised against B with the following known cross reactivities: B (100 %); F (0.22 %); cortisone (E) (0.08 %); P4, testosterone (T), and 17β-estradiol (<0.5 %). The EIA assessment of P4 used a mouse monoclonal antibody (Quidel CL425 clone) raised against 4-pregnen-11-ol-3, 20-dione hemisuccinate: bovine serum albumin (BSA)(Munro and Stabenfeldt, 1984) with the following known cross reactivities: P4 (100 %); 3β-hydroxyprogesterone (172 %); 3α-hydroxyprogesterone (188 %); 11β-hydroxyprogesterone (11βOHP4) (2.7 %); 5α-dihydroprogesterone (DHP4) (7.0 %); pregnenolone (P5) (5.9 %); B (<0.1 %); androstenedione (A4) (<0.1 %). The EIA assessment of T used a rabbit polyclonal antibody raised against T with the following known cross reactivities: T (100 %); 5α-dihydrotestosterone (DHT) (56.8 %); A4 (0.27 %); androsterone (AST) (0.04 %); dehydroepiandrosterone (DHEA) (0.04 %), cholesterol (0.03 %); 17β-estradiol (0.02 %); P4, P5, hydrocortisone, and cholic acid (<0.02 %). Table 1 contains all steroid hormones that cross reacted with the three EIAs as well as that were detected by UPC^2-MS/MS in this study or were previously detected in other published studies.

2.3. Ultra-Performance convergence chromatography -Tandem mass Spectrometry:

11-Keto-5α-androstanedione (11K5αdione), 11-ketoandrostenedione (11KA4), 11-ketoandrosterone (11KAST), 11-ketodihydrotestosterone (11KDHT), 11-ketotestosterone (11KT), 11β-hydroxyandrostanedione (11OH5αdione), 11β-hydroxyandrostenedione (11OHA4), 11β-hydroxyandrosterone (11OHAST), 11β-hydroxytestosterone (11OHT), 11-ketodihydroprogesterone (11KDHP4), 11-ketoprogesterone (11KP4), 11KPdione, 11βOHP4, 11αOHDHP4, 11α-hydroxyprogesterone (11αOHP4), 21-deoxycortisol (21dF), 21-deoxycortisone (21dE), and alfaxalone were purchased from Steraloids (Newport, USA). 11β-dihydrotestosterone (11OHDHT), 11KPdiol, 11OHPdiol, 11OHPdione, 11β-hydroxydihydroprogesterone (11βOHDHP4), and 3,11diOHDHP4 were purchased from IsoScience (Pennsylvania, USA). 3α-Androstanediol (3αdiol), 5α-androstanedione (5αdione), A4, AST, E, DHT, T, 11-deoxycortisol (S), 16α-hydroxyprogesterone (16 *α* OHP4), 17α-hydroxyprogesterone (17 *α* OHP4), 17α-hydroxypregnenolone (17αOHP5), 18-hydroxycorticosterone (18OHB), aldosterone (ALDO), B, F, DHEA, 11-deoxycorticosterone (DOC), estrone (E1), P4, and P5 were sourced from Sigma-Aldrich (St. Louis, MO, USA). All steroids used had a purity of 98 % or greater. Methyl *tert*-butyl ether (MTBE), analytical-grade methanol, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). FOODFRESH CO_2 was supplied by Afrox (Cape Town, South Africa). Deuterated steroids were acquired from Cambridge Isotope Laboratories (Tewksbury, MA, USA) and included testosterone 1,2-D2 (D2-T; 98 %), progesterone 2,2,4,6,6,17,21,21,21-D9 (D9-P4; 98 %), and 4-androsten-11β-ol-3,17-dione-2,2,4,6,6,16,16-D7 (D7-11OHA4; 98 %).

Fecal aliquots (±1.0 g wet weight) were removed from the middle of the ~ 20 g of fecal sample and were lyophilized and ground into a powder, after which samples (100 mg dry weight) were resuspended in 500 μL ddH_2O. Deuterated internal steroid standards, D7-11OHA4 (15 ng), D9-P4 (15 ng), and D2-T (1.5 ng) in 100 μL were added to the resuspended fecal sample followed by 3.0 mL MTBE. Samples were vortexed and incubated at −80 °C, after which the organic phase was collected and subsequently dried under nitrogen at 55 °C. The dried residue was resuspended in 50 % methanol (75 μL) and stored at −20 °C prior to analysis (Gent, pre-print (2022)).

Steroid standards, 1 mg/mL, were used to prepare a dilution range, 0–350 ng/mL, in fecal solution stripped of endogenous steroids with charcoal. The separation and quantification of steroids in the fecal samples were carried out using UPC^2-MS/MS as previously reported and briefly described herein. Briefly, steroids were separated in three chromatographic steps: C_{19}, C11- oxy C_{19} steroids, and E in 4 min; C_{21} and C11-oxy C_{21} steroids in 3.8 min; and adrenal steroids including mineralocorticoids, GCs, and androgens, in 3 min. Steroids were separated using an ACQUITY UPC^2 system (Waters Corporation, Milford, MA, USA) with a Viridus supercritical-fluid chromatography (SFC) ethylene-bridged hydrid (BEH) column (3 mm × 100 mm, 1.7 μm particle size) fitted with an ACQUITY UPC^2 BEH Van-Guard^{TM} pre-column (2.1 mm × 5 mm, 1.7 μm particle size) coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation) for quantitative mass spectrometric detection. The mobile phase consisted of CO_2 modified with methanol containing 1 % formic acid. Analysis was carried out in MRM (ESI +) mode with an injection volume of 2.0 μL and steroids separated at a flow rate of 1.5–1.8 mL/min. Instrument parameters used were as follows: source temperature of 150 °C, desolvation temperature of 350 °C, cone gas flow of 150 L/hour, desolvation gas flow of 900 L/hour.

Table 1
Fecal steroid hormones assessed in white rhinoceros via enzyme immunoassay and ultraperformance convergence chromatography tandem mass spectrometry.

IUPAC Name	Trivial name/Abbreviation	EIA*	UPC[2]	Published[#]
Progesterone Metabolites				
4-pregnen-3,20-dione	Progesterone/P4	✓	✓	Hindle and Hodges 1990†; Schwarzenberger et. al. 1998 (100 %[1]; 100 %[2]); Patton et. al. 1999 (100 %[10]); Graham et. al. 2001 (100 %[3]); Metrione and Harder 2011 (2.7 %[4]); Van der Goot et. al. 2015 (100 %[1]); Pennington et. al. 2019 (100 %[3]); Penny et. al. 2020 (1.7 %[6];100 %[7]); Hermes et. al. 2021 (100 %[1]); Corticosterone ISWE Mini-kit (0.14 %)
4-pregnen-3α-ol-20-one	3α-hydroxyprogesterone/3OHP4	✓	ND	Schwarzenberger et. al. 1998 (390 %[1]; Graham et. al. 2001 (188 %[3]); Van der Goot et. al. 2015 (390 %[1]); Pennington et. al. 2019 (188 %[3]); Hermes et. al. 2021 (390 %[1])
4-pregnen-3β-ol-20-one	3α-hydroxyprogesterone/3αOHP4	✓	ND	Graham et. al. 2001 (172 %[3]); Pennington et. al. (171 %[3])
4-pregnen-11β-ol-3,20-dione	11β-hydroxyprogesterone/11βOHP4	✓	✓	Graham et. al. 2001 (2.7 %[3]); Pennington et. al. 2019 (172 %[3])
4-pregnen-11α-ol-3,20-dione	11α-hydroxyprogesterone/11αOHP4	✓	✓	Graham et. al. 2001 (147 %[3]); Pennington et. al. 2019 (172 %[3])
4-pregnen-3,11,20-trione	11-ketoprogesterone/11KP4	ND	✓	
5-pregnen-3β-ol-20-one	Pregnenolone/P5	✓(	✓	Progesterone ISWE Mini-kit (5.9 %[3]); Penny et. al. 2020 (1.43 %[7])
4-pregnen-16α-ol-3,20-dione	16α-hydroxyprogesterone/16αOHP4	ND	✓	
4-pregnen-17α-ol-3,20-dione	17α-hydroxyprogesterone/17αOHP4	✓	✓	Penny et. al. 2020 (3.5 %[7])
5-pregnen-3β,17α-diol-20-one	17α-hydroxypregnenolone/17αOHP5	ND	✓	
5α-pregnan-17α-ol-3,20-dione	Pregnanedione	ND	✓	
5α-pregnan-3,20-dione	Allopregnanedione/ dihydroprogesterone/DHP4	✓	✓	Schwarzenberger et. al. 1998 (168 %[1]; 75 %[2]); Graham et. al. 2001 (55 %[3]); Van der Goot et. al. 2015 (168 %[1]); Penny et. al. 2020 (100 %[7]); Hermes et. al. 2021 (168 %[1])
5β-pregnan-3,20-dione	5β-dihydrotestosterone	✓	ND	Schwarzenberger et. al. 1998 (151 %[2]); Graham et. al. 2001 (8 %[3]); Pennington et. al. 2019 (8 %[3])
5α-pregnan-3α,17α-diol-20-one	Pregnanediol	✓	✓	Patton et. al. 1999 (4.8 %[10])
5α-pregnan-3α-ol-20-one	Allopregnanolone	✓	ND	Schwarzenberger et. al. 1998 (89 %[1]; 8 %[2]); Patton et. al. 1999 (36.2 %[10]); Graham et. al. 2001 (64 %[3]); Van der Goot 2015 (89 %[1]); Pennington et. al. 2019 (64 %[3]); Hermes et. al. 2021 (89 %[1])
5α-pregnan-3β-ol-20-one	Allopregnan-3β-ol-20-one	✓	ND	Schwarzenberger et. al. 1998 (56 %[1]; 102 %[2]); Patton et. al. 1999 (96 %[10]); Graham et. al. 2001 (94 %[3]); Van der Goot et. al. 2015 (56 %[1]); Pennington et. al. 2019 (94 %[3]); Hermes et. al. 2021 (56 %[1])
5α-pregnan-3α,20α-diol		✓	ND	Patton et. al. 1999 (0.2 %[10])
5β-pregnan-3α-ol-20-one	Pregnanolone	✓	ND	Schwarzenberger et. al. 1998 (88 %[1]; 20 %[2]); Patton et. al. 1999 (7.4 %[10]); Graham et. al. 2001 (2.5 %[3]); Van der Goot et. al. 2015 (88 %[1]); Pennington et. al. 2019(2.5 %[3]); Hermes et. al. 2021 (88 %[1])
5β-pregnan-3β-ol-20-one	Epipregnanolone	✓	ND	Schwarzenberger et. al. 1998 (36 %[2]); Graham et. al. 2001 (12.5 %[3]); Pennington et. al. 2019 (12.5 %[3]); Hermes et. al. 2021 (36 %[1])
5α-pregnan-11β-ol-3,20-dione	11β-hydroxydihydroprogesterone/ 11βOHDHP4	ND	✓	
5α-pregnan-11α-ol-3,20-dione	11α-hydroxydihydroprogesterone/ 11αOHDHP4	ND	✓	
5α-pregnan-3,11,20-trione	11-ketodihydroprogesterone/ 11KDHP4	ND	✓	
5α-pregnan-3α,11β-diol-20-one	3,11diOHDHP4	ND	✓	
5α-pregnan-3α-ol-11,20-dione	Alfaxalone	ND	✓	
Glucocorticoid Metabolites				
4-pregnen-11β,17α-diol-3,20-dione	21-deoxycortisol/21dF	ND	✓	
4-pregnen-17α-ol-3,11,20-trione	21-deoxycortisone/21dE	ND	✓	
5α-pregnane-3β,11β,21-triol-20-one	3β,5α-tetrahydrocorticosterone	✓	ND	Touma et. al. 2003 (100 %[9]); Badenhorst et. al. 2016 (100 %[9])
5α-pregnane-3β,11β,20β,21-tetrol		✓	ND	Touma et. al. 2003 (110 %[9]); Badenhorst et. al. 2016 (110 %[9])
5α-pregnane-3β,11β,17α,21-tetrol-20-one	3β,5α-tetrahydrocortisol	✓	ND	Touma et. al. 2003 (45 %[9]); Badenhorst et. al. 2016 (45 %[9])
5α-pregnan-17α-ol-3,11,20-trione	11KPdione	ND	✓	
4-pregnen-17α,21-diol-3,20-dione	11-deoxycortisol/S	✓	✓	Metrione and Harder 2011 (0.03 %[4])
4-pregnen-11β,17, 21-triol-3,20-dione	cortisol/F	✓	ND	Metrione and Harder 2011 (0.23 %[4]); Penny et. al. 2020 (0.05 %[6]); Corticosterone ISWE Mini-kit (0.22 %)
Desoxycorticosterone		✓	ND	Metrione and Harder 2011 (14.3 %[4])
4-pregnen-21-ol-3,20-dione	11-deoxycorticosterone/DOC	✓	✓	Penny et. al. 2020 (28.6 %[6]; 0.06 %[7])
4-pregnene-11β,21-diol-3,20-dione	corticosterone/B	v	✓	Brown et. al. 2001†; Metrione and Harder 2011 (100 %[4]); Penny et. al. 2020 (100 %[6]; 0.77 %[7]); Corticosterone ISWE Mini-kit (100 %)
Tetrahydrocorticosterone	THCC	✓	ND	Metrione and Harder 2011 (0.9 %[4]); Penny et. al. 2020 (0.28 %[6])
4-pregnen-11β,17α,21-triol-3,20-dione	cortisol/F	✓	✓	Brown et. al. 2001†
4-pregnen-11β,18,21-triol-3,20-dione	18-hydroxycorticosterone/18OHB	ND	✓	
4-pregnen-11β,21-diol-3,18,20-trione	aldosterone/ALDO	✓	✓	Brown et. al. 2001†; Penny et. al. 2020 (0.18 %[6])
Androgen Metabolites				
5-androstan-3β-ol-17-one	dehydroepiandrosterone/DHEA	✓	✓	Penny et. al. 2020 (0.013 %[7]; 0.72 %[8]);); DetectX Testosterone EIA (0.04 %)
4-androsten-3,17-dione	androstenedione/A4	✓	✓	Brown et. al. 2001†; Penny et. al. 2020 (0.28 %[7]; 7.2 %[8]); DetectX Testosterone EIA (0.27 %)

(*continued on next page*)

Table 1 (*continued*)

IUPAC Name	Trivial name/Abbreviation	EIA*	UPC[2]	Published[#]
5α-androstan-3α-ol-17-one	androsterone/AST	✓	✓	Kretzschmar et. al. 2004 (0.1 %[5]); Jenikejew et. al. 2021 (0.1 %[5]); DetectX Testosterone EIA (0.04 %)
5α-androst-2-en-17β-ol		✓	✓	Kretzschmar et. al. 2004 (1.5 %[5]); Jenikejew et. al. 2021 (1.5 %[5])
5α-androst-2-en-17-one		✓	✓	Kretzschmar et. al. 2004 (0.1 %[5]); Jenikejew et. al. 2021 (0.1 %[5])
5α-androstan-3α,17β-diol	3α-adrostanediol/3αdiol	✓	✓	Brown et. al. 2001†
5α-androstan-3,17-dione	5α-androstanedione/5αdione	ND	✓	
4-androsten-11β-ol-3,17-dione	11β-hydroxyandrostenedione/ 11OHA4	ND	✓	
5α-androstan-11β-ol-3,17-dione	11β-hydroxy-5α-androstanedione 11OH5αdione	ND	✓	
5α-androstan-3α,11β-diol-17-one	11β-hydroxyandrosterone/11OHAST	ND	✓	
4-androsten-3,11,17-trione	11-ketoandrostenedione/11KA4	ND	✓	
5α-androstan-3,11,17-trione	11-keto-5α-androstanedione 11K5αdione	ND	✓	
5α-androstan-3α-ol-11,17-dione	11-ketoandrosterone/11KAST	ND	✓	
5α-androstan-3β-ol-17-one	epiandrosterone	✓	ND	Kretzschmar et. al. 2004 (0.1 %[5]); Jenikejew et. al. 2021 (0.1 %[5])
5α-androstane-3β,11β-diol-17-one	11β-hydroxyepiandrosterone	✓	ND	Touma et. al. 2003 (230 %[9]); Badenhorst et. al. 2016 (230 %[9])
4-androsten-17β-ol-3-one	testosterone/T	✓	✓	Brown et. al. 2001†; Kretzschmar et. al. 2004† (100 %[5]); Metrione and Harder 2011 (0.64 %[4]); Penny et. al. 2020 (0.13 %[6]; 100 %[8]); Jenikejew et. al. 2021 (100 %[5]); Corticosterone ISWE Mini-kit (0.09 %); DetectX Testosterone EIA (100 %)
4-androsten-17β, 19- diol-3-one	19-hydroxytestosterone	✓	ND	Penny et. al. 2020 (14.6 %[8])
5α-androstan-17β-ol-3-one	dihydrotestosterone/DHT	✓	✓	Brown et. al. 2001†; Kretzschmar et. al. 2004† (13.6 %[5]); Jenikejew et. al. 2021 (13.6 %[5]); DetectX Testosterone EIA (56.8 %)
4-androsten-11β,17β-diol-3-one	11β-hydroxytestosterone/11OHT	ND	✓	
5α-androstan-11β,17β-diol-3-one	11β-hydroxydihydrotestosterone/ 11OHDHT	ND	✓	
4-androsten-17β-ol-3,11-dione	11-ketotestosterone/11KT	ND	✓	DetectX Testosterone EIA (2.34 %)
5α-androstan-17β-ol-3,11-dione	11-ketodihydrotestosterone/11KDHT	ND	✓	

* This column shows steroid hormones that EIAs could have detected based on cross-reactivities known for those assays.
† Indicates the study used HPLC to identify the given hormone in feces of white rhinoceros.
[#] Percentage cross reactivities for each steroid hormone are listed in parentheses behind the publication in which they were determined.
[1] Cross reactivities for an EIA using a polyclonal antibody raised against 5 α-pregnane-3 β-ol-20-one 3HS:BSA.
[2] Cross reactivities for an EIA using a polyclonal antibody raised against 5 β-pregnane-3 α-ol-20-one.
[3] Cross reactivities for an EIA using the CL425 monoclonal antibody.
[4] Cross reactivities for an EIA using the CJM006 polyclonal antibody.
[5] Coss reactivities for an EIA using a polycolonal antibody raised against 17α-OH-testosterone-HS-BSA.
[6] Cross reactivities for the ENZO Life Sciences Corticosterone EIA (catalogue # ADI-900–097) with a polycolonal antibody raised against corticosterone.
[7] Cross reactivities for the ENZO Life Sciences Progesterone EIA (catalogue # ADI-900–011) with a monoclonal antibody raised against progesterone.
[8] Cross reactivities for the ENZO Life Sciences Testosterone EIA (catalogue # ADI-900–065) with a monoclonal; antibody raised against testosterone.
[9] Cross reactivities for the group specific corticoid metabolite EIA that used a polyclonal antibody raised against 5α-pregnane-3β,11β,21-triol-20-one.
[10] Cross reactivities for an EIA using a monoclonal antibody raised against 4-pregnen-11-o1-3,20-dione hemisuccinate:BSA.
ND: not detected by the given assessment method. This means the method was not set up to detect a given steroid hormone rather than that the steroid hormone was assessed for but was not found in the fecal specimen.

Potential cross-reactivity was avoided as previously reported (du Toit et al., 2020) and different combinations of standards were injected to allow optimum chromatographic separation and elimination of cross-talk. In addition, quantifier and qualifier ions for each steroid metabolite were optimised for best selectivity and reduced cross-talk within other channels (du Toit et al., 2020; Gent, pre-print (2022)). All data were collected, analyzed, and quantified using the Masslynx 4.1 Software package (Waters Corporation).

2.4. Microbiome Assessment:

Eight fecal samples were thawed on ice for approximately 30 min, after which 150–160 mg of feces had DNA extracted using a *Quick*-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research Corporation, Irvine, CA, USA). A NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to quantify the extracted DNA (Supplemental Table 2). Agarose gel electrophoresis (1 % agarose gel; 40 mM Tris-acetate, 1 mM EDTA and ethidium bromide at 0.5 µg/ml) was carried out at 90 V × 1 hr. The 16S rRNA gene was amplified using the following tagged primers:

16SF 5′ -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGCAG CCGCGGTAA – 3′.

16SR 5′ -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTAAG GTTCYTCGCGT – 3′. PCR was performed with AccuPOL DNA Polymerase (Ampliqon, Denmark). Amplification products were visualized under UV light after 1 % agarose gel electrophoresis (40 mM Tris-acetate, 1 mM EDTA). The DNA amplification products (±500 bp) were excised using a Zymoclean (D4001) Gel DNA Recovery kit (Zymo Research Corporation). Excised amplification products were sequenced on the Illumina MiSeq platform (MiSeq V3, 600 cycle chemistry).

A total of 553,638 reads from 8 random fecal samples were processed and analyzed using RStudio v1.3.959[1]. Reads were pre-processed, filtered, dereplicated, and error corrected using the dada2 R package v1.0.3[2]. Forward and reverse sequences were trimmed at 290 and 230 nucleotides, respectively, based on initial quality inspection using the *plotQualityProfile* function. Data were filtered to remove reads with a quality score of less than or equal to 2, a maximum expected error rate of >2, or any reads with ambiguous bases. The dada function was used to infer true biological sequences on each sample individually, and forward and reverse reads were merged to reconstruct full amplicons. A sequence

table was generated using merged reads, which were further trimmed to a length between 450 and 475 nucleotides. Chimeras were removed with the function *removeBimeraDenovo*. Taxonomy was assigned using the Silva reference database v138[11][12] with the *assignTaxonomy* function in dada2, which uses the naïve Bayesian classifier method to assign taxonomy from Kingdom to Genus and the function *assignSpecies* to assign taxonomy at the Species level. The dada2 count table, taxonomy table, and sample metadata were combined using the phyloseq package v1.16.2[3] for further analysis.

Amplicon sequence variants (ASV) were filtered to remove 1) ASVs that were not present in at least 50 % of the samples and 2) ASVs with an abundance <10. An original 2,801 ASVs were filtered to a "core microbiome" of 406 ASVs used in further analysis. The ggpubr v0.3.0[7] function *stat_compare_means* was used to perform a Kruskal–Wallis test for differences in alpha diversity measures (observed species richness, Shannon diversity index, and Simpson diversity index) between the sexes and by maturity class. Plots were generated using the phyloseq function *plot_richness*. Differences in beta diversity between both sex (male vs female) and age (≤6 years old vs >6 years old) were tested by PERMANOVA using the Adonis function and group dispersion with the betadisper function from the vegan package v2.4.2[9]. The DEseq2 package v1.12.3[8] was used for generating PCoA plots after variance stabilizing transformation on raw counts of the core microbiome ASvs.

2.5. Statistical analysis for steroid hormone data

All steroid hormone variables were assessed for normality using PROC MIXED in SAS 9.2 (SAS, Inc., Cary, NC, USA). A Shapiro-Wilke test statistic was utilized to determine whether variables were normally distributed. Non-normal datawas square root transformed prior to analysis with a Student's *t*-test with alpha set at 0.05 and with a posthoc Tukey's multiple comparison test. Steroid hormone concentrations were analyzed by sex. Statistical significance was set at alpha = 0.05.

3. Results

3.1. Behavioral Observations:

Female rhinoceroses TMB and NFZ were pregnant at the time of their fecal sample collections. Based on their subsequent parturition dates, TMB's sample was collected at 6.75 months of gestation and NFZ's sample was collected at 9.25 months of gestation both of which would be time period of gestation. Male rhinoceroses IKL and PND fought on June 12, 2019, which was two weeks prior to their fecal sample collection dates. PND suffered multiple injuries during this fight and was immobilized for medical treatment on June 28, 2019.

3.2. Fecal steroid Metabolites:

The concentration of FGM levels were analyzed by the B EIA alongside S, 18OHB, F, DOC, and 21dF, which were quantified by UPC^2-MS/MS (Table 2). Despite sample interrogation for a large diversity of steroids (Table 1), only the steroids shown in the tables in the results section were detected by this method. FGMs concentrations detected by the B antibody tended to be higher in females (17.72 ± 2.33 ng/g wet feces) than males (11.47 ± 1.72 ng/g wet feces; $p = 0.055$). Interestingly, for all rhinoceros samples, B was below the limit of detection of UPC^2-MS/MS analysis, but the B antibody nevertheless elicited an antibody/antigen signal, indicating that the antibody detected GC or other metabolites or cross-reactants. S was the only glucocorticoid metabolite that was detected in all samples by UPC^2-MS/MS. However, S did not differ significantly between sexes ($p = 0.570$). Although other glucocorticoid metabolites were detected by UPC^2-MS/MS, there were not enough samples containing these metabolites to make a statistical comparison (labeled "NA" in Table 2).

According to EIA assessment, fecal samples from pregnant females tended to have higher P4 levels (4832.5 ± 728.96 ng/g wet feces) than those from non-pregnant females (136.41 ± 29.37 ng/g wet feces; $p = 0.097$) and males (73.51 ± 8.95 ng/g wet feces; $p = 0.097$). FPMs concentrations detected by EIA tended to be higher in non-pregnant females than males ($p = 0.087$). However, P4 quantified by UPC^2-MS/MS for pregnant females (43.68 ± 27.76 ng/g) was much lower than that measured for pregnant females by EIA. None of the other P4 metabolites were consistently detectable by UPC^2-MS/MS across all individuals in a given sex. As this crush of rhinoceros are monitored around the clock every day, the mating patterns of the crush are well-characterized and are seasonal. Only P4 was detectable in a sufficient number of samples to make a comparison by season and no significant differences were found in this steroid hormone by season.

FAMs quantified by EIA were analyzed alongside 11KA4, 11KT, 11OHT, A4, and T quantified by UPC^2-MS/MS (Table 4). The UPC^2-MS/MS panel did not detect 11OHT, 11KT, A4, and T in rhino feces in concentrations above limits of detection in the majority of samples. FAMs assessed by EIA were 16.18 ± 1.61 ng/g wet feces for pregnant

Table 2
Fecal glucocorticoid metabolite concentrations (ng/g) assessed by EIA and UPC^2-MS/MS.

Method of Detection			EIA (B antibody)	UPC^2-MS/MS					
Rhino	Collection Date	Age (yr)	FGM	B	S	18OHB	F	DOC	21dF
Female:									
KVS	10/2/19	3	13.82	ND	81.68	ND	ND	ND	ND
KVS	10/5/19	3	8.66	ND	336.47	ND	ND	ND	ND
KTH	7/12/19	4	24.13	ND	27.09	ND	ND	ND	ND
KON	8/10/19	5	15.34	ND	53.73	ND	ND	ND	ND
NMF	8/10/19	5	17.41	ND	87.75	ND	ND	ND	ND
NFZ	10/2/19	7	23.75	ND	16.73	ND	ND	ND	ND
TMB	7/5/19	8	27.04	ND	363.04	ND	206.25	ND	24.75
LDW	10/2/19	11	11.62	ND	17.36	ND	ND	ND	ND
Female mean ± SEM:	17.72 ± 2.33[a]	NA	122.98 ± 50.46	NA	NA	NA	NA		
SIP	10/30/18	4	4.71	ND	67.33	ND	ND	7.58	ND
SIP	8/10/19	5	13.44	ND	20.74	ND	ND	ND	ND
IKL	6/27/19	11	13.23	ND	252.43	ND	ND	ND	ND
PND	6/26/19	18	8.45	ND	9.19	403.86	ND	ND	ND
PND	6/30/19	18	16.54	ND	123.64	ND	ND	ND	ND
PND	9/26/19	18	12.43	ND	45.09	ND	8.45	14.62	ND
Male mean ± SEM:	11.47 ± 1.72[b]	NA	86.41 ± 37.10	NA		NA	11.10 ± 2.03	NA	

ND: not detectable; NA: not applicable (Although other glucocorticoid metabolites were detected by UPC^2-MS/MS, there were not enough samples containing these metabolites to make a statistical comparison); B: Corticosterone; S: 11-deoxycortisol; 18OHB: 18-hydroxycorticosterone; F: Cortisol; DOC: 11-deoxycorticosterone; 21dF: 21-deoxycortisol [a,b] $0.05 < p < 0.10$ between sexes for the corticosterone EIA.
FPMs quantified by EIA was analyzed alongside P4, 11KP4, 11αOHDHP4, and 16αOHP4 quantified by UPC^2-MS/MS (Table 3).

Table 3
Fecal progesterone metabolite concentrations (ng/g) assessed by EIA and UPC^2-MS/MS.

Method of Detection			EIA(P4 antibody)	UPC^2-MS/MS			
Rhino	Collection Date	Age	FPM	P4	11KP4	11αOHDHP4	16αOHP4
Female:							
KVS	10/2/19	3	74.64	ND	ND	ND	ND
KVS	10/5/19	3	58.10	ND	ND	ND	ND
KTH	7/12/19	4	102.91	ND	ND	ND	ND
KON	8/10/19	5	174.21	ND	ND	167.99	ND
NMF	8/10/19	5	249.92	280.70	ND	64.62	ND
NFZ*	10/2/19	7	4103.54	15.92	ND	16.74	ND
TMB*	7/5/19	8	5561.45	71.45	ND	ND	ND
LDW	10/2/19	11	158.66	ND	4.32	ND	ND
Female mean ± SEM:			1310.43 ± 781.12	122.69 ± 49.37	NA	83.12 ± 27.33	NA
Male:							
SIP	10/30/18	4	63.28	90.37	ND	ND	36.07
SIP	8/10/19	5	55.81	24.26	ND	ND	ND
IKL	6/27/19	11	80.50	ND	ND	140.26	ND
PND	6/26/19	18	54.98	ND	ND	ND	ND
PND	6/30/19	18	73.02	ND	ND	ND	ND
PND	9/26/19	18	113.43	80.57	ND	ND	ND
Male mean ± SEM:			73.51 ± 8.95	65.07 ± 14.56	NA	NA	NA

ND: not detectable; NA: not applicable (Although other progesterone metabolites were detected by UPC^2-MS/MS, there were not enough samples containing these metabolites to make a statistical comparison); P4: Progesterone; 11KP4: 11-ketoprogesterone; 11αOHDHP4: 11α-hydroxydihydroprogesterone; 16αOHP4: 16α-hydroxyprogesterone. * indicates pregnant at the time of sample collection.

Table 4
Fecal androgen metabolite concentrations (ng/g) assessed by EIA and UPC^2-MS/MS.

Method of Detection			EIA (T antibody)		UPC^2-MS/MS			
Rhino	Collection Date	Age	FAM	11KA4	11KT	11OHT	A4	T
Female:								
KVS	10/2/19	3	12.33	ND	ND	ND	ND	ND
KVS	10/5/19	3	7.05	ND	ND	ND	ND	ND
KTH	7/12/19	4	12.49	19.57	ND	ND	ND	ND
KON	8/10/19	5	7.44	9.88	ND	ND	ND	ND
NMF	8/10/19	5	11.06	15.25	ND	ND	ND	ND
NFZ*	10/2/19	7	17.79	5.83	ND	49.74	ND	ND
TMB*	7/5/19	8	14.57	34.83	ND	25.92	ND	ND
LDW	10/2/19	11	10.36	8.58	ND	ND	ND	ND
Female mean ± SEM:			11.64 ± 1.26	15.66 ± 3.75^a	NA	37.83 ± 5.95	NA	NA
Male:								
SIP	10/30/18	4	3.89	3.32	ND	ND	25.57	ND
SIP	8/10/19	5	8.57	ND	ND	ND	ND	ND
IKL	6/27/19	11	16.46	7.38	ND	ND	ND	ND
PND	6/26/19	18	9.78	3.42	ND	ND	ND	ND
PND	6/30/19	18	13.17	ND	ND	ND	ND	ND
PND	9/26/19	18	14.46	3.37	ND	ND	38.55	9.17
Male mean ± SEM:			11.05 ± 1.85	4.37 ± 0.82^b	NA	NA	32.06 ± 3.74	NA

ND: not detectable; NA: not applicable (Although other androgen metabolites were detected by UPC^2-MS/MS, there were not enough samples containing these metabolites to make a statistical comparison); T: Testosterone; 11KA4: 11-ketoandrostenedione; 11KT: 11-ketotestosterone; 11OHT: 11β-hydroxytestosterone; A4: Androstenedione ^{a,b} $p < 0.05$ between sexes for 11KA4.

females, 10.12 ± 0.97 ng/g wet feces for non-pregnant females, and 11.05 ± 1.86 ng/g wet feces for males. 11KA4 was detected in the majority of samples and significantly higher in females (15.66 ± 3.75 ng/g wet feces) than in males (4.37 ± 0.82 ng/g wet feces) when comparing all female samples ($p = 0.048$) and when comparing non-pregnant female samples (13.32 ± 2.07 ng/g wet feces; $p = 0.031$). 11OHT was detected only in pregnant females.

3.3. Fecal Microbiome:

The dominant microbe classes found in all fecal samples were *Bacilli, Bacteroides, Clostridia, Desulfovibrionia, Fibrobacteria, Gammaproteobacteria, Kiritimatiellae, Lentisphaeria, MVP-15, Negativicutes, Spirochaetia,* and *Verrucomicrobiae* (Fig. 1). In all animals assessed, *Bacteroidia* was the most abundant class, followed by *Clostridia*. Male PND had a noticeable change in *Spirochaetia* abundance between his June 2019 and September 2019 samples. *Spirochaetia* abundance in his fecal sample from June 30, 2019 sample, which was collected after the fight and immobilization, was lower than the *Spirochaetia* abundance in his fecal sample from September 26, 2019 and also lower than the *Spirochaetia* abundance in his fecal sample from for all other fecal samples (Fig. 1).

Alpha diversity was characterized by observed species richness, Shannon diversity index, and Simpson diversity index for the unfiltered and core microbiome. Sex and age classes were compared for each of these measures of alpha diversity (Fig. 2). Although the difference was not significant, female fecal samples displayed higher alpha diversity than male fecal samples after filtering for the core microbiome (Fig. 2**A**). The unfiltered microbiome for females had a significantly higher diversity than that for males (Shannon and Simpson indices, $p = 0.043$). The core fecal microbiome from young rhinoceros had a significantly higher observed species richness ($p = 0.043$), Shannon diversity index ($p = 0.021$), and Simpson diversity index ($p = 0.021$) than that from old

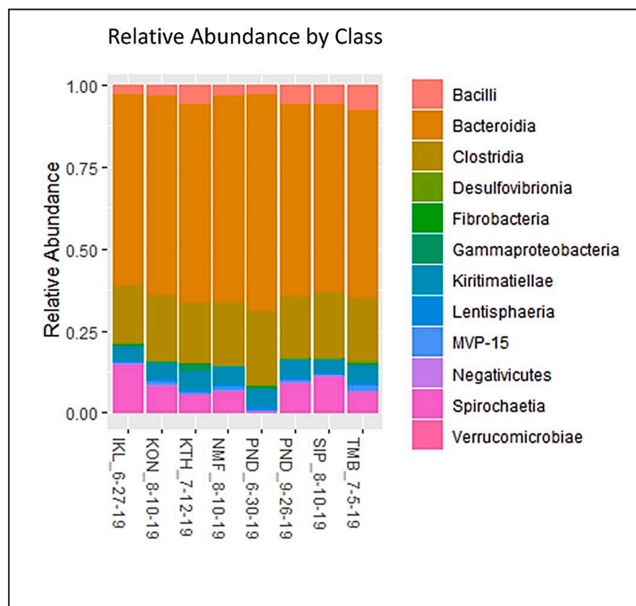

Fig. 1. Relative abundance of microbe classes in fecal samples from free-ranging southern white rhinoceros.

rhinoceros (Fig. 2**B**).

Principal Component Analysis (PCA) showed a clustering of fecal samples into 4 distinct groups: 1) KTH 7–12-19; KON 8–10-19; NMF 8–10-19; 2) SIP 8–10-19; 3) TMB 7–5-19; 4) IKL 6–27-19; PND 9–26-19; PND 6–30-19 (Fig. 3**A**). These groups are clustered by sex and age which suggests an effect of both biological variables. Cluster 1 is young females and 2 is a young male. Cluster 3 is an old female and 4 is old males.

Fecal sample microbiome samples from female rhinoceros were more tightly clustered than those from male rhinoceros (Fig. 3**B**). Fecal sample microbiome samples from young rhinoceros clustered tightly, and their clustering mimicked that of the females with the addition of SIP (Fig. 3**C**). Beta diversity differences tested by PERMANOVA indicated significant effects of sex and age (sex: $p = 0.026$; age: $p = 0.038$). The June 30, 2019 fecal sample microbiome from PND did not cluster by either age or sex and was totally different from his September 26, 2019 sample.

4. Discussion

Wildlife biologists have traditionally monitored the health and reproduction of free-ranging animals with non-invasive methods such as fecal analysis using EIAs to detect glucocorticoid, progesterone, and androgen metabolites. We quantified steroid hormone metabolites using both EIAs and UPC^2-MS/MS and characterized the microbial composition in fecal samples of wild southern white rhinoceros using next generation sequencing.

UPC^2-MS/MS analysis of steroid metabolites is an ideal technique for the simultaneous sensitive, accurate identification and quantification of many metabolites. Previous studies in wildlife species have detected steroid hormone metabolites and bile acids using LC-MS and LC-MS/MS in feces from canids, felids, and ruminants; and urine from primates (Dalle Luche et al., 2019; Habumuremyi et al., 2014; Molina-García et al., 2018; Nasini et al., 2013). This is the first reported use of UPC^2-MS/MS to detect and quantify fecal steroid metabolites in wild southern white rhinoceros. Although UPC^2-MS/MS may be costly, it is possible to eliminate the pitfalls of antibody cross reactivity due to steroid structure similarity which are characteristic of EIAs. UPC^2-MS/MS differentiates between numerous steroids in very short run-times resulting in accurate quantification and cost-effective analysis (du Toit et al., 2020).

UPC^2-MS/MS analysis did not detect B, yet the antibody used in the B EIA indicated the presence of B and/or metabolites in all samples. Unfortunately, the B antibody has not been tested for cross reactivity with all 5α/5β-reduced metabolites or 3α5α/5β metabolites nor does the antibody distinguish between C_{19}, C-11-oxy C_{19} and C-11oxy C_{21} steroids, suggesting it may detect numerous FGMs and potentially also androgens and progestins. Interestingly, a previous study on captive white rhinoceros in North America detected B and F in feces via high performance liquid chromatography (Brown et al., 2001). This same study detected B and metabolites with a double antibody ^{125}I RIA for B validated for white rhinoceros with parallelism. This same RIA was further biologically validated for FGM detection by demonstration of a strong correlation between increased mortality and large fluctuations in FGMs over time (i.e. increased exposure to stressful incidents)(Carlstead and Brown, 2005). An EIA used to measure FGMs in white rhinoceros by Metrione and Harder (2011) used the polyclonal antibody CJM006 which was made against corticosterone-3-carboxymethyloxime and cross reacted with corticosterone (100 %), desoxycorticosterone (14.25 %), tetrahydrocorticosterone (0.9 %), cortisol (0.23 %), 11-deoxycortisol (0.03 %) and cortisone (<0.01 %) (Metrione and Harder, 2011). Using this EIA captive white rhinoceros were found to have 200–600 ng/

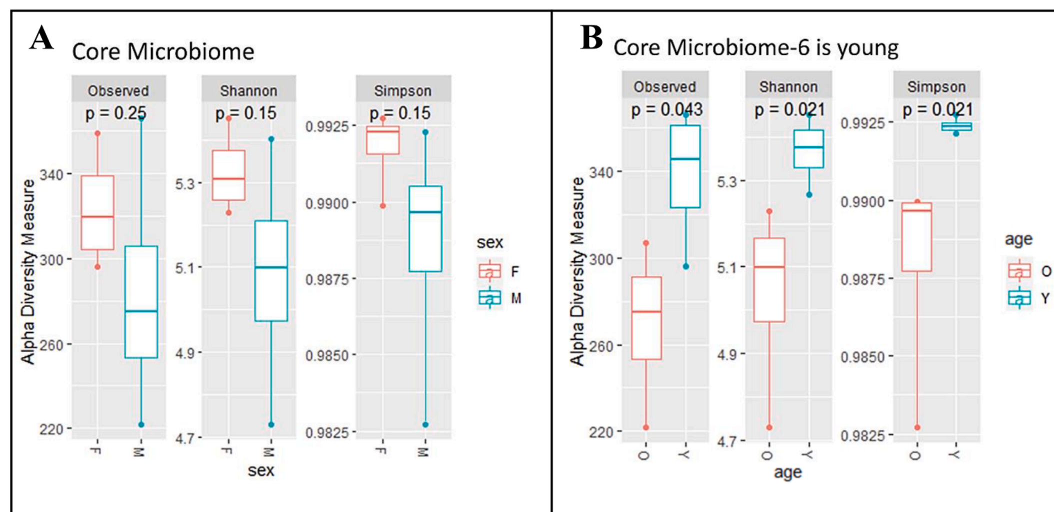

Fig. 2. Alpha diversity of the core microbiome by sex and age class. A) sex (male versus female); B) age (young ≤ 6 years old; old > 6 years old). F: female. M: male. Y: young. O: old.

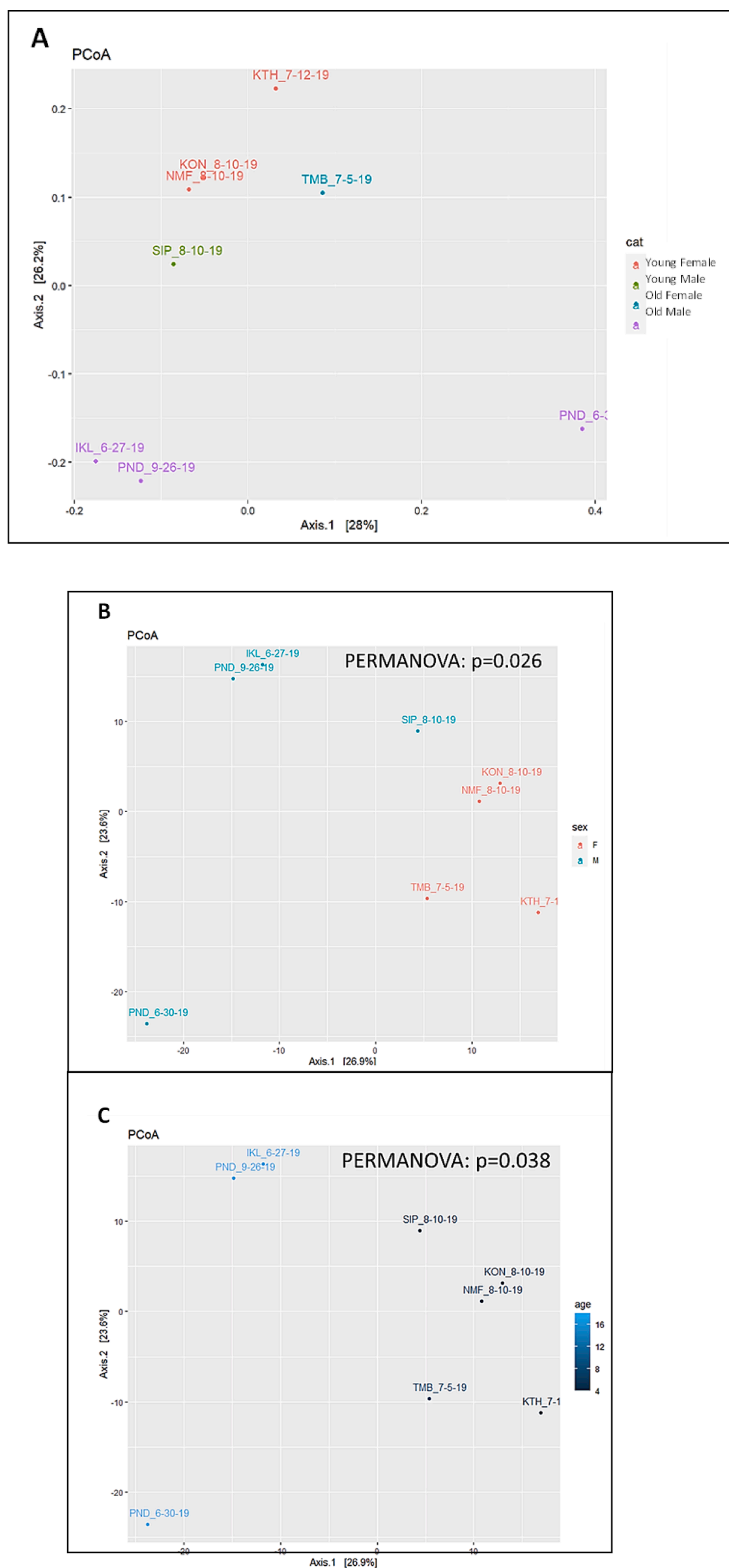

Fig. 3. Principal component analysis of fecal microbiome by sex and age. A) individual; B) sex (male versus female); C) age (young ≤ 6 years old; old > 6 years old). F: female. M: male. Y: young. O: old. Fecal microbiome significantly clusters by sex ($p = 0.026$) and age ($p = 0.038$). In panel 3A: cluster 1 is young females; cluster two is a young male; cluster three is an old female and cluster 4 is an old male.

g FGMs per sample depending on the time of sample collection. Our finding that 11-deoxycortisol was the predominant FGM detected by UPC^2-MS/MS is contrary to what has been potentially detected in captive white rhinoceros in the past. Additionally the average amount of 11-deoxycortisol detected by UPC^2-MS/MS is seven fold higher than the average amount of FGMs detected by the B EIA. Despite the HPLC data from Brown et. al. (2001) indicating that corticosterone is excreted in feces from white rhinoceros, our UPC^2-MS/MS data suggests that the B EIA (Arbor Assays), the CJM006 antibody (Metrione and Harder, 2011), and the corticosterone kit from ENZO Life Sciences (Penny et al., 2020) do not cross react with meaningful FGMs in the white rhinoceros species. 11-deoxycortisol and 5α-pregnane-3β,11β,21-triol-20-one to which other EIAs do cross react (Badenhorst et al., 2016; Touma et al., 2003) appear to be some of the main FGMs in this species.

It is also possible the different diets between wild and captive white rhinoceros in North America versus South Africa contributes to different steroid metabolism in the gastrointestinal tract. UPC^2-MS/MS analysis of fecal samples from captive white rhinoceros in North America would help to elucidate the cause behind our findings. Given the high levels of S detected for each animal by UPC^2-MS/MS, it is possible that the B antibody detected this GC metabolite as well but at lower concentrations because of decreased cross-reactivity for 11-deoxycortisol. The protocol booklet for this the B EIA does not indicate that the cross reactivity of the antibody was tested against 11-deoxycortisol. The two steroids are almost identical in structure except for B having a hydroxl moiety at C11 and S, a precursor of F, having a hydroxl moiety at C17. We have detected F in serum of white rhinoceros, however at far lower levels (Gent, pre-print (2022)). DOC, a precursor of B was detected only in feces from two males whose samples were collected during the spring months. It is interesting to note that GC levels are directly influenced by aggressive behavior which is reflected in UPC^2-MS/MS analysis but not in EIAs. A considerable increase was detected in S levels of PND after his fight with IKL, while minimal changes in FGMs were found with the B antibody-based EIA. The fight between PND and IKL was a physiologic stressor for PND, only indicated by increased S levels and not cortisol. Therefore, the EIA which assessed FGMs did not biologically validate for use in this species.

Interestingly, F and 21dF were detected by UPC^2-MS/MS in one of the pregnant females, TMB. Both of these hormones are products of 17αOHP4 which is produced in the adrenal cortex (Barnard et al., 2017; van Rooyen et al., 2020). TMB was 6.75 months of gestation at the time of collection in which both F and 21dF were detected in her feces, whereas NFZ was 9.25 months of gestation at the time of collection and lacked these two steroids in her feces. P4 and P5 are found in high concentrations in equine fetuses in the first third of gestation (Legacki et al., 2017). Given that rhinoceros are most closely related to equid species (Price and Bininda-Emonds, 2009), it is possible that rhinoceros in the first third of gestation (0–6 months), such as TMB, would have high levels of circulating P4 metabolites due to their production by the fetal adrenal and gonads. This possibility is supported by the fact that no other rhinoceros had 21dF detected in their sample. Our UPC^2-MS/MS analysis shows a high flux through the glucocorticoid pathway in TMB with notably high levels of S, F and 21dF. P4, which was significantly higher than that of NFZ is converted to 17OHP4 by cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) with cytochrome P450 21-hydroxylase (CYP21A2) converting the intermediate to S by leading to the production of F catalyzed by cytochrome P450 11β-hydroxylase (CYP11B1). High concentrations of 17OHP4 could also be a substrate for CYP11B isoforms to make 21dF (Barnard et al., 2017; van Rooyen et al., 2020). Our findings require further exploration with collection of multiple samples throughout gestation for GC, P4 and 21dF assessment by UPC^2-MS/MS. Given our data regarding the differences in S, F, 21dF, and P4 levels detected in the two pregnant females and their gestational stages, EIAs using antibodies raised against B and P4 would not contribute in the accurate assessment of relevant steroids in such investigations.

Comparing FPMs detected by the P4 antibody and UPC^2-MS/MS in sexually mature female rhinos (six years or older), pregnant females had higher levels of FPMs detected by EIA. Interestingly, none of the UPC^2-MS/MS P4 metabolites detected in pregnant females matched the high concentration of FPMs detected by the P4 antibody in the EIA. This finding suggests that P4 or its metabolites measured with this P4 antibody and reported to be associated with pregnancy in white rhinoceros, are not the metabolites detected by UPC^2-MS/MS. This is not surprising as the main FPM in white rhinoceros is allopregnanolone (5 α-pregnan-3 α-ol-20-one) (Graham et al., 2001; Patton et al., 1999). Therefore, it is possible the elevations in FPMs in pregnant females are due to this allopregnanolone as well as allopregnandione which is also known to be in high concentrations in the feces of female white rhinoceros. It should be noted that although allopregnanolone (5α-pregnan-3α-ol-20-one) is the primary fecal progestin in white rhinoceros (Schwarzenberger et al., 1998), this steroid hormone was not a part of the routinely run UPC^2-MS/MS panel and, unfortunately, was not assessed. However, the antibody used in the Progesterone ISWE Mini-Kit, the CL425 clone, is known to cross-react with allopregnanolone (Graham et al., 2001). Therefore, the FPM levels reported in Table 3 for the P4 EIA could be, in part, allopregnanolone or other cross-reactive progestins as outlined below. Interestingly, high performance liquid chromatography (HPLC) and antibodies specific to 5α and 5β pregnanes have been used to confirm that 5α-pregnane-3,20-dione (allopregnanedione), 5α-pregnane3α-ol-20-one (allopregnanolone), and 5α-pregnane-3β-ol-20-one (allopregnan-3 β-ol-20-one) are the dominant fecal pregnanes in black rhinoceros (Schwarzenberger et al., 1996). Another study used gas chromatography-mass spectrometry (GC–MS) and identified 5α-pregnan-3β, 20α-diol (allopregnan-3 β-20 α-diol) as the principal progesterone metabolite in black rhinoceros (Lance et al., 2001). The P4 CL425 antibody used in our study has the following cross reactivities: 172 % with 4-pregnen-3 β-ol-20-one (3 β-hydroxyprogesterone), 188 % with 4-pregnen-3 α-ol-20-one, 147 % with 11 α OHDHP4 (11 α-hydroxydihydroprogesterone)(Arbor Assays Progesterone ISWE Mini-kit Protocol Booklet), 94 % with 5 α-pregnan-3 β-ol-20-one (allopregnan-3 β-ol-20-one), 64 % with 5 α-pregnan-3 α-ol-20-one (allopregnanolone), 55 % with 5 α pregnan-3,20-dione (allopregnanedione), 12.5 % with 5 β-pregnan-3 β-ol-20-one (epipregnanolone), 8 % with 5 β-pregnan-3,20-dione (5 β-dihydroprogesterone), 2.7 % with 4-pregnan-11 β-ol-3,20-dione (11 β-hydroxyprogesterone) and 2.5 % 5 β-pregnan-3 α-ol-20-one (pregnanolone) (Graham et al., 2001) as well as 5.9 % with pregnenolone (Arbor Assays Progesterone ISWE Mini-kit Protocol Booklet). It is possible that one or more of these progesterone metabolites contributed to the high P4 measured in the EIA in pregnant white rhinoceros (Table 1). Furthermore, the antibody used in this P4 EIA has been utilized successfully to monitor ovulation induction in white rhinoceros previously (Pennington et al., 2019). Other investigators have used a progestin-based EIAs with antibodies raised against 5 α-pregnane-3 β-ol-20-one 3HS:BSA(Hermes et al., 2021; Schwarzenberger et al., 1998; Van Der Goot et al., 2013; van der Goot et al., 2015) or 5 β-pregnane-3 α-ol-20-one (Schwarzenberger et al., 1998) with great success in white rhinoceros, because the antibodies in these assays are highly specific with very high cross reactivites to the predominant progesterone metabolites known to be excreted in the feces in this species – 4-pregnen-3,20-dione, 4-pregnen-3 α-ol-20-one, 5 α-pregnan-3,20-dione, 5 β-pregnan-3,20-dione, 5 α-pregnan-3 α-ol-20-one (allopregnanolone), 5 α-pregnan-3 β-ol-20-one, 5 β-pregnan-3 α-ol-20-one, 5 β-pregnan-3 β-ol-20-one. However, a previous study in southern white rhinoceros found that injection of females with ^{14}C labeled P4 yielded a small amount of labeled native P4 but no labeled P4 metabolites in feces (Hindle and Hodges, 1990). Of note, while P4 was detected in all of the males by the P4 antibody EIA, UPC^2-MS/MS identified the steroid in only half of the male samples, possibly due to differences in the biosynthesis and metabolism of P4.

The androgen most commonly detected by UPC^2-MS/MS was 11KA4, and levels were higher in females than males. Additionally, 11KA4 was

the only androgen metabolite detected by UPC^2-MS/MS that was similar in concentration to the levels of FAMs detected by the T antibody EIA. In ruminants, 11KT is produced mainly by the adrenal upon stimulation with adrenocorticotropin hormone (Sid-Ahmed et al., 2013). The adrenal produces 11KA4 in either of two main oxidation pathways: 11βOHA4 to 11KA4 catalyzed by 11β-hydroxysteroid dehydrogenase type 2 or 11KT to 11KA4 catalyzed by 17β-hydroxysteroid dehydrogenase type 2 (Bloem et al., 2015; Gent et al., 2019; Rege et al., 2013; Swart et al., 2013). Our data suggests that the FAMs detected by the T antibody represent FAMs other than T as this steroid was detected in a single male by UPC^2-MS/MS. However, given the small number of males in the study and the numbers of samples collected from those males as well as the variable time of day in which the samples were collected, it is also possible that our samples are not truly representative of what are normal levels of FAMs in white rhinoceros. In fact, it is common in many species for FAMs to vary between successive days which means this sparse, stochastic set of samples is limited in its ability to represent normal FAM concentrations. The LOD for T was 0.03 nmol/L and, therefore, T may represent a negligible fraction of the measured EIA levels of FAMs. In addition, EIA levels of FAMs were similar between females and males and FAMs in males did not reflect accurate age associated levels such that FAMs were higher in older males. These findings suggest this particular T EIA is not biologically sound for use in this species. This finding is, of course surprising as Brown et. al. (2001) found a large peak on HPLC corresponding to T in feces from white rhinoceros. Another EIA utilized to successfully measure FAMs in white rhinoceros utilized an antibody raised against 17α-OH-testosterone-HS-BSA that had cross reactivity with testosterone (100 %) and also 5 α-androstan-17 β-ol-3-one (DHT; 13.6 %) (Jenikejew et al., 2021; Kretzschmar et al., 2004). It was surprising that T was found in only one male sample and even 11KT and 11OHT (metabolites of T) were not detected in any male samples by UPC^2-MS/MS. A previous study in male white rhinoceros from Limpopo Province in South Africa used an EIA with 100 % cross-reactivity for T to detect androgen metabolites and biologically validated this assay based on GnRH agonist stimulation of gonadal androgen production (Kretzschmar et al., 2004). The limit of detection for the T antibody in that study was 10 pg/mL, the same as our UPC^2-MS/MS method. Kretzschmar et al. (2004) found that FAMs levels were highest in September and October (80–100 ng/g feces) as compared to the rest of the year. Interestingly, the only time A4 and T were detected by UPC^2-MS/MS in the feces of males in our study was also during September and October. A study done on captive male white rhinoceros in North American zoos found that the highest immunoreactive androgen peak detected using HPLC was T, followed by DHT, and then A4 (Brown et al., 2001). On another note, we have detected 11OHA4 only in serum, not fecal, samples of white rhinoceros, at levels markedly lower than its 11KA4 products (Gent, pre-print (2022)). However, the physiologic reason for 11KA4 detected in fecal samples and 11OHA4 in serum requires further research. In fact, FAMs in white rhinoceros have not been examined frequently and is an area ripe for opportunity.

Perturbations to homeostatic immune status, such as infections, initiate a cascade of responses beginning with glucocorticoid production from the hypothalamic–pituitaryadrenal (HPA) axis (Wingfield et al., 1998). Wildlife endocrinologists typically correlate adrenal steroidogenesis, and therefore physiological stress, with FGM levels determined by EIA. However, the intestinal microbiome metabolizes steroid hormones thus altering both circulating steroid hormone profiles and excreted fecal steroid hormones (Bokkenheuser et al., 1977; Honour, 1982). Furthermore, increased FGM levels have been associated with decreased microbial diversity (Stothart et al., 2016), and intestinal microbial diversity has life-long impacts on immune health and resistance to infection (Cahenzli et al., 2013; Schuijt et al., 2016). Previous studies in captive white rhinoceros have found *Firmicutes* and *Bacteroides* to be the predominant fecal microbe classes (Bian et al., 2013; Roth et al., 2019). However, in our free-ranging white rhinoceros population, the predominant fecal microbes were *Bacteroides, Clostridia,* and *Spirochaetia.* Although *Clostridia* are not a dominant class in the fecal microbiome of captive white rhinoceros, *Clostridia* are found in great abundance in the feces of both captive and wild black rhinoceros (Gibson et al., 2019). Additionally, diet and environment or husbandry play a major role in modulating the fecal microbiome, so it is not surprising that the fecal microbiome in free-ranging white rhinoceros differs from that in captive white rhinoceros. GCs have a significant impact on the intestinal microbiome (Huang et al., 2015). PND fought with IKL in early June 2019 and was immobilized for medical treatment on June 28, 2019. PND's microbiome composition and clustering on June 30, 2019, differed significantly not only from his September 2019 microbiome but also from all other microbiome samples. PND had high levels of FGMs on June 30, 2019, and also an increased abundance of the *Spirochaetia* class of microbes. By comparison, a previous study in eastern black rhinoceros found no correlation between FGM levels and *Spirochaetia* (Antwis et al., 2019). Yet, *Spirochaetia* are not a dominant class of fecal microbes in this rhinoceros species, whereas this class was the third most prevalent in our study population. As this appears to be the first report of fecal microbiome in free-ranging white rhinoceros, more studies with larger sample sizes are warranted to appropriately define normal fecal microbial communities.

5. Conclusions

Fecal androgen and progesterone metabolite classes in our samples differed significantly from those classes previously reported in both captive and wild southern white rhinoceros. According to the panel of glucocorticoids we analyzed via UPC^2-MS/MS, we found S to be the major glucocorticoid in fecal samples from this species in this particular free-ranging population. However, a limitation of the study is that our panel did not include the common glucocorticoid metabolites containing 3 α-11 β hydroxy and 3 α-11oxo structures which have been increasingly measured in a variety of wildlife species including elephants and equids. Only one of the top three classes of fecal microbiota were similar between our wild population and those classes previously published for captive white rhinoceros. This is the first study in wild white rhinoceros to quantify fecal steroid metabolites by UPC^2-MS/MS and the fecal microbiome. Future studies with larger sample numbers of wild southern white rhinoceros in both the northern regions of South Africa and the Eastern Cape are recommended.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.114099.

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