Gut microbiota and phytoestrogen-associated infertility in southern white rhinoceros

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Abstract

With recent poaching of southern white rhinoceros (*Ceratotherium simum simum*; SWR) reaching record levels, the need for a robust assurance population is urgent. However, the global captive SWR population is not currently self-sustaining due to the reproductive failure of captive-born females. Dietary phytoestrogens have been proposed to play a role in this phenomenon, and recent work has demonstrated a negative relationship between diet estrogenicity and fertility of captive-born female SWR. To further examine this relationship, we compared gut microbial communities, fecal phytoestrogens, and fertility of SWR to another rhinoceros species–the greater one-horned rhinoceros (*Rhinoceros unicornis*; GOHR), which consumes a similar diet but exhibits high levels of fertility in captivity. Using 16S rRNA amplicon sequencing and mass spectrometry, we identified a species-specific fecal microbiota and three dominant fecal phytoestrogen profiles. These profiles exhibited varying levels of estrogenicity when tested in an *in vitro* estrogen receptor activation assay for both rhinoceros species, with profiles dominated by the microbial metabolite, equol, stimulating the highest levels of receptor activation. Finally, we found that SWR fertility varies significantly with respect to phytoestrogen profile, but also with the abundance of several bacterial taxa and microbially-derived phytoestrogen metabolites. Taken together, these data suggest that in addition to species differences in estrogen receptor sensitivity to phytoestrogens, reproductive outcomes may be driven by gut microbiota's transformation of dietary phytoestrogens in captive SWR females.

Background

The southern white rhinoceros (SWR; *Ceratotherium simum simum*) has returned from the brink of extinction through extensive *in situ* and *ex situ* conservation efforts, with wild populations increasing from approximately 100 to 20,000 over the last century (1). However, wild SWR now face an uncertain future due to the recent dramatic increase in poaching (2). An additional challenge facing the species is the reproductive failure of the once robust *ex situ* assurance populations (3,4). Together, poaching, long gestational length (~16 months) and inter-calving interval (~ 2.5 years) (5), and captive infertility (3,4) have rendered both wild and captive populations no longer self-sustaining. Without any change in poaching rates, wild SWR populations will likely face the threat of extinction within the next two decades (6).

Previous work has implicated captive diets in the reproductive failure of captive SWR (4,7). In the wild, SWR are pure grazers, consuming up to ~40 kg/day of various grasses (8,9). In contrast, diets in managed settings typically contain phytoestrogen-rich legume hays and soy- and alfalfa-based concentrated feeds (4). A survey of nine SWR-breeding institutions demonstrated that diet estrogenicity was strongly associated with the amount of soy and/or alfalfa-based pellets fed. Moreover, female SWR born at institutions feeding highly estrogenic diets exhibit lower fertility than female SWR born at institutions feeding low phytoestrogen diets (4).

Due to their structural similarity to endogenous estrogens, phytoestrogens may interact with estrogen receptors (ERs) and disrupt normal endocrine function, reproduction, and development (10-13). Previously, we showed that SWR ERs exhibit higher maximal activation by phytoestrogens than ERs of the greater one-horned rhinoceros (*Rhinoceros unicornis*; GOHR) (5). Both species consume similar high-phytoestrogen diets in captivity, but GOHR do not

73 exhibit the decrease in fertility observed in SWRs. These data suggest that at the receptor level,
74 SWR are particularly vulnerable to the deleterious effects of phytoestrogen exposure. Whether
75 SWR possess additional species-specific characteristics that predispose them to phytoestrogen
76 sensitivity remains unclear.

77 Due to the limitations of collecting biological samples from a threatened megafaunal
78 species, little is understood about the specific physiological consequences of SWR consuming
79 estrogenic diets. Altered endocrine and reproductive function by phytoestrogen exposure has
80 been described in humans, rodents, and livestock species (11,13,14). Many of these effects,
81 including reproductive tract pathologies, erratic or absent luteal activity, and reduced fertility,
82 parallel findings in captive female SWR (15-17). However, the potential role of phytoestrogens
83 in the onset of these pathologies has not been investigated. In other species, the physiological
84 outcomes of phytoestrogen exposure are profoundly affected by transformation of parent
85 compounds following consumption. For example, in ewes, reproductive pathologies and
86 infertility develop following consumption of diets high in the isoflavone daidzein, but it is equol,
87 a daidzein metabolite, that is thought to be the driver of this effect (10). Equol production relies
88 exclusively on microbial transformation, and several other phytoestrogens are metabolized by
89 members of the gastrointestinal tract microbiota to produce metabolites that vary in estrogenicity
90 (18-21). Coumestrol, a compound from another class of phytoestrogens, the coumestans, also has
91 been associated with sheep infertility (9), but to date, the microbial metabolism of coumestans
92 has not been explored. Whether gut microbiota may play a similar role in SWR responses to
93 dietary phytoestrogens is unclear.

94 The relationship between animals and their associated microbes is important, as
95 microbiota are essential for many biological processes within their hosts (22). However, an

96 understanding of how interactions between phytoestrogens and resident gut microbiota may
97 affect fertility is lacking for any vertebrate species. Given what is known about bioactivation of
98 phytoestrogens by gut microbiota in other mammalian species (23) and the strong link between
99 dietary phytoestrogens and reproductive failures in rhinoceros (4), an investigation into
100 phytoestrogen metabolism by rhinoceros gut microbiota is warranted. To examine these
101 interactions, we characterized SWR and GOHR fecal microbiota as a proxy for gut microbiota.
102 In addition, we compared fecal phytoestrogen composition and metabolite profile estrogenicity,
103 using mass spectrometry and ER activation assays, respectively, between the two species. By
104 sampling separately housed, but similarly managed SWR and GOHR females from the same
105 institution, we sought to reduce variation by eliminating known drivers of gut microbiota
106 composition, such as diet and geographic location (24-26), to better identify species differences.
107 Finally, we used historical breeding records to examine the relationships between specific
108 microbial taxa, phytoestrogen metabolites, and SWR reproductive success. With these data, we
109 shed light on the role microbiota may play in captive SWR infertility with the aim to develop
110 techniques to support and increase this species' assurance population.
111
112 **Results**
113
114 ***Composition of fecal microbiota, but not phytoestrogens, differ by species***
115
116 Sequencing of 16S rRNA fecal samples (SWR: $n = 42$; GOHR: $n = 16$; Table S1) from
117 eight individual rhinoceros (SWR: $n = 6$; GOHR: $n = 2$; Table S1) revealed that GOHR samples
118 had significantly higher inter-sample diversity compared to SWR despite SWRs having a higher
119 number of unique, low (>1 %) relative abundance operational taxonomic units (OTUs) overall
120 (Table S2). Significant differences in fecal community structure and composition between rhino

121 species were also observed at the phylum, family, and OTU level using permutational analysis of
122 variance (PERMANOVA) and accounting for relative abundances using weighted UniFrac (all P
123 < 0.001). A difference in microbial communities was also observed by nonmetric
124 multidimensional scaling (nMDS; Fig. 1, inset). Members of four phyla were found to
125 significantly contribute to variation (Fig. 1), with the relative abundance of the Bacteroidetes
126 (SWR: 55 ± 1.1 %; GOHR: 30 ± 1.8 %) and the Firmicutes (SWR: 33 ± 1.2 %; GOHR: 55 ± 2.2
127 %) found to significantly differ with respect to rhino species (Welch's *t-test,* both $P < 0.001$; Fig.
128 1). Several members of these phyla were also found to be significantly different at both the
129 family and OTU level, with six families and eleven OTUs contributing to these significant
130 differences (Fig. 1).

131 The observed differences in microbial community are likely related to the different
132 foraging strategies exhibited by the two species. All individuals in this study live in large
133 exhibits where they are provided diet of soy and alfalfa based pellets supplemented with either
134 grasses and browse. SWR, which in the wild are grazers, consume additional hay and fresh
135 grasses (8,9). In contrast, GOHR, a predominantly browsing species, consume a more varied diet
136 that includes fruits and leaves (27). This difference in foraging may be driving species
137 differences in gut microbiota, as observed in other closely related species (28). Nevertheless,
138 both species are herbivorous and their gut microbiota are similar in that the dominant
139 microorganisms present in both species are those capable of fiber degradation and therefore
140 fulfill similar functional niches (29).

141 Despite species differences in microbial communities, neither overall structure nor
142 composition of detected phytoestrogen analytes varied significantly between SWR and GOHR
143 (Fig. 2A, PERMANOVA, $P > 0.05$). However, species differences were observed at the

144 individual analyte level. Concentrations of equol (EQ), enterolactone (EL), methoxycoumestrol
145 (MOC), and coumestrol (CO) were significantly higher in the GOHR (Fig. 2B-D, Table S3).
146 Several phytoestrogens were detected exclusively in the diet, the isoflavones, formononetin (FM)
147 and genistein (GN) (Fig. 2B), whereas microbially-derived metabolites EQ, 4'-ethylphenol
148 (PEP), EL, and enterodiol (ED) were detected only in feces (Fig. 2BC). Two other
149 phytoestrogens, biochanin-A and *o*-demethylangolesin, were not detected in any sample type
150 (both, < 65 ppb). In general, there was an overall trend for excreted quantities of phytoestrogens
151 and metabolites to be higher in GOHR compared to SWR (Fig. 2B-D).
152 The relative abundances of specific OTUs provide some insight into the observed
153 phytoestrogen and metabolite concentrations described above. Overall, 77 OTUs were found to
154 significantly correlate with phytoestrogen concentration (Fig. 3A), which were overall
155 significantly more abundant in SWR compared to GOHR (Welch's *t-test,* $P < 0.0001$; Fig. 3B).
156 Interestingly, the 27 OTUs negatively correlated with phytoestrogen metabolite concentration are
157 nearly four times more abundant in SWR, while the 41 positively correlated OTUs are
158 approximately 5-fold less abundant in SWR (both, Welch's *t-test,* $P < 0.0001$; Fig. 3CD). Taken
159 together, these findings provide a plausible explanation for why there was an overall trend of
160 lower concentrations of individual phytoestrogen analytes in SWR samples.
161 One possible explanation for similarity in phytoestrogen composition between SWR and
162 GOHR is that the OTUs positively associated with phytoestrogen concentrations belong to taxa
163 of known fiber degraders. Many of these taxa, like members of the Bacteroidetes (Rikenellaceae)
164 in SWR, and the Firmicutes (Ruminococcaceae and Lachnospiraceae) in GOHR accomplish this
165 via β-glucosidase activity, as this enzyme also catalyzes early steps of phytoestrogen

166 transformation (23). Thus, the lack of species differences in phytoestrogen composition may be
167 driven by the overall functional similarity of the two species' gut microbial communities.
168
169 ***Three distinct phytoestrogen profiles examined***
170 With no clear species difference in metabolite composition, hierarchical clustering was
171 used to group similar fecal samples from both species of rhinoceros according to their
172 phytoestrogen composition. This approach identified three distinct phytoestrogen profiles
173 representing the most commonly observed fecal metabolite profiles in individual samples from
174 both SWR and GOHR (Fig. 4A-C, Fig. S1, Fig. S1D). For the two most similar profiles, the
175 moderately estrogenic EQ was the dominant metabolite produced, followed by the weakly
176 estrogenic EL (Profile B & C; Fig. 4BC, Table S3, Fig. S1). However, total phytoestrogen
177 concentrations in Profile C were approximately twice the total concentration of phytoestrogens
178 detected Profile B (8,884 ± 970 ppb and 4,254 ± 315 ppb, respectively; Fig. 4BC, Fig. S1). A
179 third, less similar profile was also identified, in which the dominant metabolite was EL (Profile
180 A; Fig. 4A, Table S3). The total concentration of phytoestrogens in this profile was significantly
181 lower (1,510 ± 229 ppb) (Fig. 4A, Fig. S1). Despite no visual difference in the overall
182 communities using nMDS (Fig. S1E), several bacterial taxa were found to differ significantly
183 with respect to phytoestrogen profiles (OTU 03, YRC22; OTU 07, OTU 27 Ruminococcaceae).
184 However, no individual OTU contributed to variation > 8.5 %, indicating that a group of
185 microbiota, not individual OTUs, may be important in driving differences between
186 phytoestrogen profiles.
187 To quantify the relative estrogenicity of phytoestrogen profiles (Profiles A, B, C), each
188 observed mixture was formulated *in vitro* and tested in estrogen receptor (ER) activation assays

189 using ERα or ERβ from SWR and GOHR (Fig. 4D-F, Fig. S1F-G), as described previously (5).
190 All three phytoestrogen profiles activated SWR and GOHR ERs (Fig. 4D-F, Fig. S1F-G), with
191 Profile C, the most potent agonist for both SWR ERs reaching maximal activation relative to
192 17β-estradiol (E_2) (Fig. 4F, Fig. Fig. S1F-G, Table S4). Similarly, Profile B stimulated maximal
193 activation of SWR ERα, and near maximal activation of SWR ERβ relative to E_2, despite having
194 less than half the total concentration of analytes of Profile C (Fig. 4E, Fig. Fig. S1F-G, Table
195 S4). We attribute this high activation primarily to EQ, which is a dominant metabolite in both
196 profiles and a known potent agonist to rhino ERs (5). However, it is interesting that activation of
197 SWR ERα by Profiles B & C was significantly greater than that of GOHR ERα (Table S4), as
198 previous work has shown GOHR ERα to be more sensitive to EQ than its SWR homologue (5).
199 In contrast, the least potent Profile A stimulated significantly greater activation of GOHR ERβ
200 relative to SWR ERβ (Fig. 4D, Fig. Fig. S1F-G, Table S4). This is also noteworthy, as no
201 phytoestrogen tested in previous studies has ever been shown to be a more potent agonist of
202 GOHR ERβ than SWRβ. What is driving these differences is unclear, as the dominant
203 metabolites in Profile A, EL and PEP, do not appreciably bind or activate ERβs from either
204 species, and the known agonists present in Profile A (DZ, EQ and CO) are more potent activators
205 of SWR ERβs than those from GOHR (5). Nevertheless, this observation highlights the
206 importance of evaluating the effects of mixtures of suspected endocrine disrupting chemicals on
207 receptors, in addition to individual chemicals, as this method better mimics *in vivo* conditions.
208
209 ***Interactions with SWR fertility explored***
210 To assess fertility of our SWR population, the number of pregnancies achieved and/or
211 calves born were determined for both the period of sample collection as well as for the lifetime

of each of the SWR included in this study (Table S1). Pregnancies achieved ($Pregnancy_{study}$, PS; $Pregnancy_{life}$, PL) were confirmed via elevations in fecal progestagen levels and were included in the analysis since rhino gestation length (~16 months) exceeded the duration of sample collection (4 months). Fertility ($Calf_{study}$, CS; $Calf_{life}$, CL) represents calves born per reproductive year using calculations described previously (4). When comparing phytoestrogen profiles using CS, we did not find any significant difference in mean fertility (Fig. 4G-I; Table S5). Using the PS calculation, however, we showed that individuals exhibiting Profile A had the lowest mean pregnancy rate, and those producing Profile C had the highest (Fig. 4G & I). For lifetime measures, we found a similar relationship, with PL and CL for Profile C producers being significantly greater than Profile A producers (Fig. 4G & I). Although not significantly different, SWR producing Profile B profiles tended to have higher mean fertility than individuals belonging to Profile A across all measures (Fig. 4GH).

Mean reproductive success, in terms of pregnancies achieved and calves born, was highest in individuals with the greatest concentrations of fecal metabolites (Fig. 4I; Profile C). For some of the metabolites produced, these findings parallel observations by others. For example, all measures of SWR fertility were positively correlated with production of EL (Table S5). This finding is consistent with studies in humans that have demonstrated a link between high levels of EL and increased reproductive success (31). Our previous work shows EL does not appreciably bind or activate SWR ERs and therefore possesses little endocrine disrupting potential as a xenoestrogen (7). However, the positive relationship between EQ and calf-based fertility measures is unexpected (Table S5). *In vitro*, EQ is a relatively potent agonist of both SWR ERα and ERβ (7), and in other vertebrate species EQ is cleared from the circulation less quickly than other isoflavones, increasing its bioavailability (32). This suggests that high levels

of EQ production should negatively affect SWR fertility, as is well documented in other grazing species (11,12).

Also unexpected was the finding that individual SWR producing the most estrogenic profiles (Profile B & C) exhibited the highest fertility (Fig. 4HI), while SWR fertility was lowest in individuals producing profiles with the lowest overall estrogenicity (Profile A). These observations lead to several new questions. Do SWR belonging to Profile A produce novel phytoestrogen metabolites that are more estrogenic? We observe high levels of certain compounds, such as MOC and CO in feeds, but low levels are detected in feces. CO is a potent SWR ER agonist (7) and has been associated with infertility in sheep (9), but little is known about possible microbial metabolites and their relative estrogenicity. It is possible that these coumestans are converted into a novel metabolite that could be highly estrogenic to SWR. Another possible explanation for the positive association between profile estrogenicity and fertility is that the varying degrees of fecal profile estrogenicity result from differences in phytoestrogen absorption or excretion between individuals. Specifically, it could be hypothesized that elevated excretion of phytoestrogens and metabolites would reduce circulating levels, thus limiting the potential for these chemicals to cause reproductive harm. This is supported not only by our findings in individual animals, but also by our species-level observations where the more fertile GOHR generally excrete higher levels of phytoestrogens than the less fertile SWR. This does not appear to be case in in sheep and cattle, where concentrations of phytoestrogens and metabolites in excreta (i.e., urine) generally correlate to plasma levels (33-35). However, detailed studies examining the generation and clearance of phytoestrogen metabolites, and their subsequent endocrine disrupting effects on target tissues, are lacking even for relatively well-studied species. Addressing such relationships in SWR will be challenging, if not impossible.

Nevertheless, the findings presented here do provide the opportunity to apply potentially innovative approaches, like using non-targeted mass spectrometry to identify novel metabolites, or using fecal EL or EQ concentrations to identify individual SWR with high reproductive potential.

Few studies have examined the interaction between mammalian fertility and gut microbiota, and defining this link is difficult. Here, we found the abundance of six OTUs to correlate to fertility measures (Table S6). Of the four OTUs that negatively correlate to fertility, three also have significant negative correlations to EL and EQ. However, OTUs positively correlated to fertility did not display significant correlations to any phytoestrogen measured in our study (Table S6), and it is unknown what role these microbiota may play. Using correlations between microbial abundance and phytoestrogen metabolites to determine microbial activity is biased, as compositional data, like presented here, do not directly correlate to microbial activity (36). Therefore, it is possible that less abundant taxa may significantly contribute to the transformation of phytoestrogens. For example, members of the Coriobacteriaceae, which have been shown to convert DZ to EQ (30), the Eubacteriaceae, which are capable of dehydroxylation of lignans to produce ED and EL (37), and the *Blautia* spp., which have displayed in both lignan and isoflavone metabolism (20, 30) are found in samples collected from both SWR and GOHR in low abundances (all, < 1.0 %). However, further work is needed to determine their contributions to phytoestrogen metabolism within the rhinoceros, including *in vitro* culture experiments to measure their microbial activity.

Discussion

Working with threatened species, such as the two species studied here, presents its own unique set of challenges. Despite these challenges, however, our combining of parallel sequencing, mass spectrometry, and estrogen receptor activation assays, provides insight into the host-microbe relationship with fertility that, to our knowledge, is novel for any vertebrate species. Such an approach is needed to understand and apply novel application of techniques within non-traditional systems.

Our work sheds light on how microbiota may drive reproductive outcomes in SWR, but they are not the only species that may benefit from the work presented here. Among its broader application to other vertebrates, our findings may be critical for the management of SWR's closest relative, the northern white rhinoceros (*Ceratotherium simum cottoni*, NWR), a subspecies with only two living members (38). Like SWR, NWR experience low fertility and a prevalence of reproductive pathologies in managed settings (17). As a closely related, grazing subspecies, it is likely sensitive to phytoestrogens as well. Currently, several rescue attempts are underway to prevent NWR extinction (39,40). Should these attempts to save the NWR be successful and with SWR facing a similar uncertain fate, any novel approaches to promote high fertility, such as managing microbial phytoestrogen transformation by altering microbiota through diet modifications and other therapeutic approaches, will be needed. With the information presented here, we plan to direct future work aimed at developing strategies to improve captive SWR reproduction, with the ultimate goal of alleviating their threat of extinction.

304

305 **Methods**

306 **Study animals.** Female greater one-horned rhinoceros (*n* = 2) and southern white rhinoceros (*n*
307 = 6) used in this study were housed at the San Diego Zoo Safari Park, Escondido, CA, USA in
308 two separate 24 ha mixed species exhibits (Table S1). All procedures were approved by San
309 Diego Zoo Global's Institutional Animal Care and Use Committee (#15-013).

310

311 **Sample collection**. Fresh fecal samples (SWR, *n* = 42; GOHR, *n* = 16, Table S1) were collected
312 weekly beginning September 3, 2015 through January 1, 2016, alternating weeks between SWR
313 and GOHR. Samples were collected from animals at the same time of day using binoculars to
314 identify individuals based on their unique horn structure. Following defecation, collection
315 occurred between one to twenty minutes, and samples were transported on dry ice and stored at -
316 80 °C prior to processing.

317

318 **DNA extraction**. Total genomic DNA from fecal samples and negative control was extracted via
319 mechanical disruption and hot/cold phenol extraction following Stevenson *et al.*'s protocol
320 (2007) with the exception that 25:24:1 phenol:chloroform:isoamyl alcohol was used in place of
321 phenol:chloroform at all steps. DNA was quantified using a Qubit Fluorometer (Invitrogen,
322 Carlsbad, CA, USA) and stored at −20 °C following extraction.

323

324 **Library Preparation & Sequencing**. Sequencing library preparation was carried out following
325 manufacturer's recommendations (Illumina, 2013) with some modifications. In brief, amplicon
326 PCR targeted the V4 region of the 16S rRNA gene using a forward (V4f:

TATGGTAATTGTGTGCCAGCMGCCGCGGTAA) and reverse (V4r: AGTCAGTCAGC CGGACTACHVGGGTWTCTAAT) primers in a 25-µL reaction with 1X KAPA HiFi Hot Start Ready Mix (Kapa Biosystems), 0.2 mM each primer, and 1.0 - 5.0 ng DNA (31). Amplification conditions were as follows: 95 °C for 2 min, 25 cycles of 95 °C for 20 s, 55 °C for 15 s, 72 °C for 30 s, and a final 10 min extension at 72 °C. PCR products were purified via gel extraction (Zymo Gel DNA Recovery Kit; Zymo, Irvine, CA) using a 1.0 % low melt agarose gel (National Diagnostics, Atlanta, GA) and quantified with a Qubit Fluorometer (Invitrogen). With the negative control producing no band, the expected area was excised. All samples were combined to yield an equimolar 4 nM pool. Following manufacturer's protocol, sequencing was conducted on an Illumina MiSeq using reagent kit V2 (2 x 250 bp cycles), as described previously (Illumina, 2013). All sequences were deposited into the National Center for Biotechnological Information's Short Read Archive under Accession Number SRP136468.

16S rRNA sequence analyses. Sequence analysis was carried out using mothur v.1.39.5 (43) following the MiSeq SOP (42). In brief, contigs were formed from 16S rRNA reads, and poor quality sequences were removed. Sequences were trimmed and filtered based on quality (maxambig = 0, minlength = 250, maxlength = 500). Unique sequences were aligned against the SILVA 16S rRNA gene alignment database (44) and classified with a bootstrap value cutoff of 80, and operational taxonomic units (OTUs) found with < 2 sequences in the total dataset were removed. Chimeras (chimera.uchime) and sequences identified as members of Eukaryota, Archaea, Cyanobacteria lineages, and mitochondria were also removed. Sequences were clustered into OTUs at a 97 % similarity cutoff using OptiClust (OTU table, Table S7). Negative

control yielded 273 sequences, comprised of low-level cross-sample contaminants; therefore, OTUs were not removed from dataset.

Sequence coverage was assessed in mothur by rarefaction curves (Fig. S2) and Good's coverage (45). Samples were then iteratively subsampled 10 times to 6,825 sequences per sample, and OTU abundances were calculated as whole number means across iterations. Additionally, richness and diversity were calculated for each sample. All other calculations were carried out in R using both *vegan* and *phyloseq* packages (46,47). The similarity indices Bray-Curtis (48), Jaccard (49), and weighted UniFrac (50) were used to assess differences in bacterial community, and these differences were visualized by nonmetric multidimensional scaling plots (nMDS, iters=10,000) (51). Permutational analysis of multivariate dispersions (PERMDISP2) was used to test for heterogeneity of community structure and composition between rhino species, and with unequal variances observed, data were down-sampled to create even sample sizes using the *caret* package (52) prior to permutational analysis of variance (PERMANOVA, *vegan*::adonis, SWR, $n = 16$; GOHR, $n = 16$) to determine species differences. Similarity percentages (SIMPER, *vegan*) analyses then determined the contributions from each taxonomic group to PERMANOVA reported differences. Species-related differences in individual OTUs were examined by Welch's *t*-test (two-sided, SWR, $n = 16$; GOHR, $n = 16$). All data are expressed as the mean ± SE and considered significant if $P < 0.05$ unless otherwise stated.

Phytoestrogen extraction and quantification. Samples collected were batched into groups of ten and accompanied by quality control samples. Phytoestrogens were extracted from fecal samples by a two-phase extraction as described previously by Palme *et al*. (2013) with few modifications. In the first phase, fecal samples were diluted ten-fold using 80 % methanol in

372 water (Fisher Scientific), homogenized for 20 min using a Geno/Grinder® at 1,000 rpm,
373 centrifuged for 10 min at 4,000 x *g*, and the supernatant was recovered. In the second-phase, 1.0
374 mL of methanol extract was added to 4.0 mL diethyl ether (Fisher Scientific), 0.5 mL of 5.0%
375 NaH_2CO_3 (Sigma), and 4.0 mL of water (54), inverted four times, and centrifuged for 10 min at
376 4000 x *g*. The ether phase was removed, evaporated at 45 °C by a nitrogen flow of 0.4 psi and re-
377 suspended in methanol. Extracts were further filtered (0.22 μm) and analyzed by liquid
378 chromatography-coupled tandem mass spectrometry (LC/MS/MS) for all analytes with the
379 exception of 4'-ethylphenol (PEP) which was analyzed by gas chromatography-mass selective
380 detector (GC/MSD). Quality control samples included a blank matrix sample (grass) that was
381 absent of phytoestrogens to assess contamination during the extraction and a matrix spiked
382 sample which was fortified with a known concentration of phytoestrogens. The spiked matrix
383 sample was used to determine the efficiency of the extraction for every batch; recoveries ranged
384 between 50-150 %.
385
386 **LC/MS/MS method**. Analysis was performed using an Agilent 1260 liquid chromatograph
387 coupled to an Agilent 6430 Triple Mass Spectrometer. Chromatographic separation was
388 performed using an Agilent Zorbax Eclipse Plus (2.1 x 50 mm id, 1.8 μm) Rapid Resolution
389 column maintained at 40 °C. The mobile phases consisted of 5 mm ammonium formate and 0.1
390 % formic acid in water for the aqueous phase (A), with 5 mm ammonium formate and 0.1 %
391 formic acid in methanol as the organic phase (B). The flow rate was held at 0.4 mL/min and
392 gradient program was as follows: 0-0.5 min 10 % B, 0.5-3.0 min increasing to 90 % B. The
393 ionization of phytoestrogens was performed using electrospray ionization (ESI) in positive mode
394 with an auxiliary gas (N_2), source temperature of 300 °C, and a gas flow rate of 12 L/min, with

the exception of enterodiol which was run in negative mode. Optimized MRM conditions are listed in Table S8.

GC/MSD method. The analysis of PEP (Indofine, CAS: 123-07-09) was performed on an Agilent 7890B gas chromatograph (GC) coupled to an Agilent 5977A mass selective detector (MSD). The GC inlet temperature was set to 280 °C run in pulsed splitless mode with an injection volume of 1 µL. The GC oven temperature was set to 80 °C and increased to 200 °C between 1 and 13 minutes at a rate of 10 °C/min. The oven temperature was then increased to 300 °C between 13 and 22 minutes at a rate of 25 °C/min for a total run time of 22 minutes. Ultra high purity helium (carrier gas) was used at a constant flow rate of 1.5 mL/min with an Agilent DB-5MS UI (30 m x 0.250 mm) 0.25 µm analytical column. PEP was analyzed using an electron ionization (EI) source with a source temperature of 230 °C. Selected ion monitoring (SIM) mode was to monitor 77, 107, and 122 (m/z) ions with a gain factor of 10 and a scan speed of 1,562 (u/s).

Phytoestrogen analyses. Similar methods to those used for 16S rRNA analyses were used in determining species differences in phytoestrogen analyte composition. Differences were visualized following nMDS of Bray-Curtis and Jaccard similarity indices, and following normalization, normality testing, and down-sampling, PERMANOVA was used to determine if species differences were observed (SWR, n = 16; GOHR, n = 16). Welch's t-test was again used to measure significant difference between rhino species for individual analytes (SWR, n = 16; GOHR, n = 16). Since we did not observe a species-related difference using PERMANOVA, and no apparent clustering was observed with nMDS, hierarchical clustering (Bray-Curtis) was used to group phytoestrogen data into three profiles (A, B, C) based on their compositional

418 similarity for further analyses. SIMPER analysis was used to determine contributions of each
419 analyte to differences observed, and significant differences between groups were tested using
420 analysis of variance (ANOVA; Profiles A, n = 23, B, n = 26 , C, n= 9) with FDR correction.
421
422 **Receptor Activation**. The ability of phytoestrogens and metabolites to activate SWR ERs was
423 assessed using a SWR and GOHR estrogen receptor (ER) activation assay described previously
424 by Tubbs *et al*. (2012; 2016) with minor modification. For each species, ERα or ERβ sub-cloned
425 into pcDNA3.1(+) expression plasmid (Invitrogen) was co-transfected into human embryonic
426 kidney (HEK293) cells along with pCMX-β-galactosidase (β-gal), pGL2–3xERE luciferase
427 reporter plasmid. After 24 hr cells were treated with phytoestrogens or metabolites, alone or in
428 combination, and incubated for an additional 24 hr. For single test compounds, cells were treated
429 with 100 pM-10 μM of each compound or vehicle (DMSO) alone. To assess the estrogenicity of
430 phytoestrogen/metabolite profiles produced by SWR and GOHR microbial communities, cells
431 were treated with serial dilutions of mixtures created *in vitro* to reflect those generated *in vivo*.
432 Within each assay, a series of cells was treated with the endogenous estrogen, 17-estradiol (E_2;
433 0.001-100 nM), to determine maximal E_2 activation. Following incubation, cells were lysed and
434 luciferase and β-Gal activity was measured as described previously (Tubbs *et al*., 2012). All data
435 are presented as mean ± SE fold activation over vehicle treatment for each metabolite or mixture
436 relative to the maximal activation E_2. Differences in mean activation for both ERs were
437 determined by ANOVA with FDR correction (each treatment, n = 9 (inter-assay, n = 3, intra-
438 assay, n = 3)), and significant interactions were observed between rhinoceros species and
439 phytoestrogen profile. Data were sliced according the main effects, and differences within factor
440 were observed.

441

442 **Fertility.** Four calculations of SWR fertility were conducted for this study. Similar to Tubbs *et*
443 *al*., (2016), two calculations are based on the number of offspring produced by the female per
444 reproductive year through the completion of the study ($Calf_{study}$; CS) and current levels ($Calf_{life}$;
445 CL). With pregnancies also considered a success, we conducted two additional calculations
446 based on the number of pregnancies per reproductive year ending with the completion of the
447 study ($Pregnancy_{study}$; PS) through current ($Pregnancy_{life}$; PL). GOHR samples were removed
448 from the dataset so that data were not skewed by species differences.

449

450 **Correlations.** Using the *microbiome* package in R (55), we examined significant correlations
451 between OTUs (≥ 1.0 % relative abundance) and phytoestrogen analytes, OTUs and fertility
452 measures, and those between phytoestrogen analytes and fertility measures. These correlations
453 were carried out using the Spearman correlation method with multiple testing correction by FDR.
454 OTUs found to correlate to fertility were further examined using a linear model (lm, *stats*
455 package) (56).

456

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Author contributions

CW and CT designed the experiment, and with AY and AM, CW conducted experimentation. Data analysis was carried out by CW. The manuscript was written by CW, CT, AM, and AY, with editorial assistance by BD.

Competing interests

The authors declare that they have no competing interests.

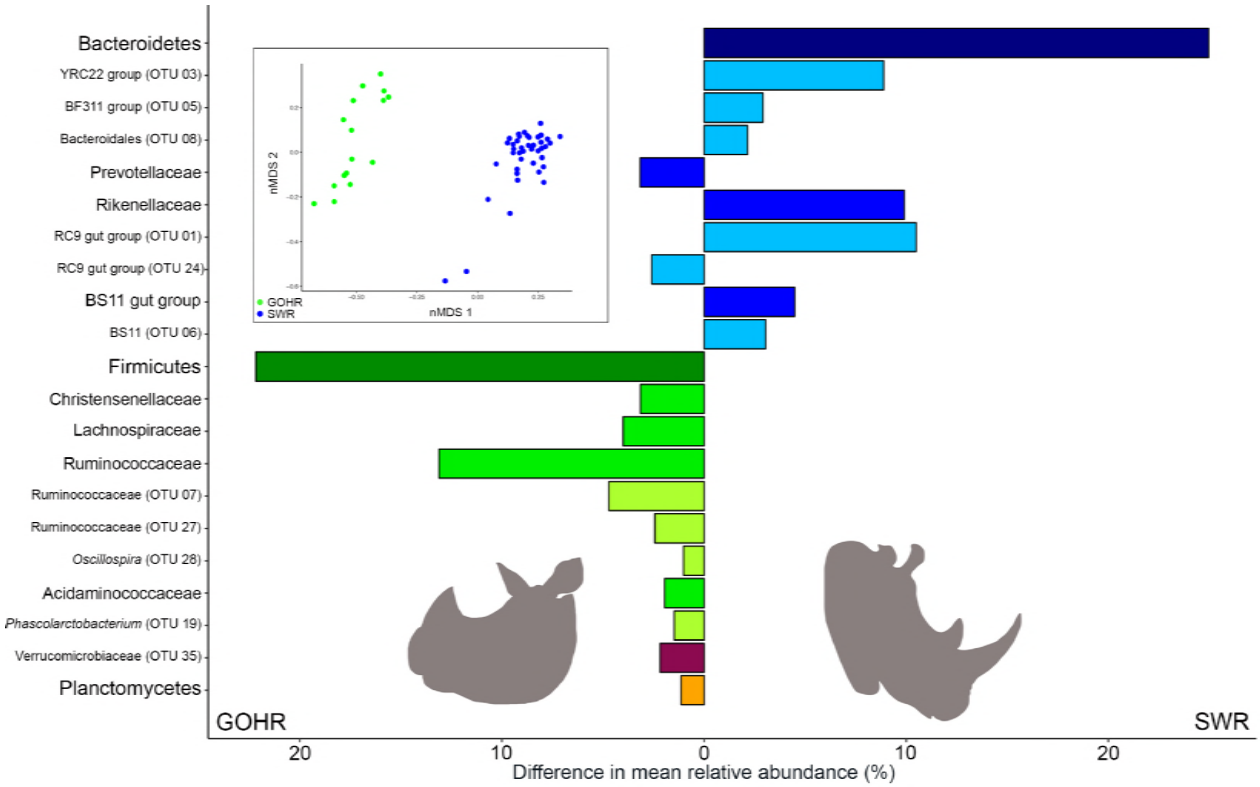

Figure 1. Differences in fecal microbiota between southern white rhinoceros (SWR) and greater one-horned rhinoceros (GOHR). Nonmetric multidimensional scaling (nMDS, inset) analysis displaying differences in microbiota observed by 16S rRNA amplicon sequencing based on Bray-Curtis distances (PERMANOVA, $P < 0.001$; stress: 0.13). Differences in mean relative abundance of bacterial taxa found to significantly contribute to variation between rhinoceros species (SIMPER ≥ 2.0 %; Welch's *t-test,* $P < 0.05$) are organized by color, with all members of a particular phylum sharing a similar color, with intensity decreasing from phylum to family to OTU level

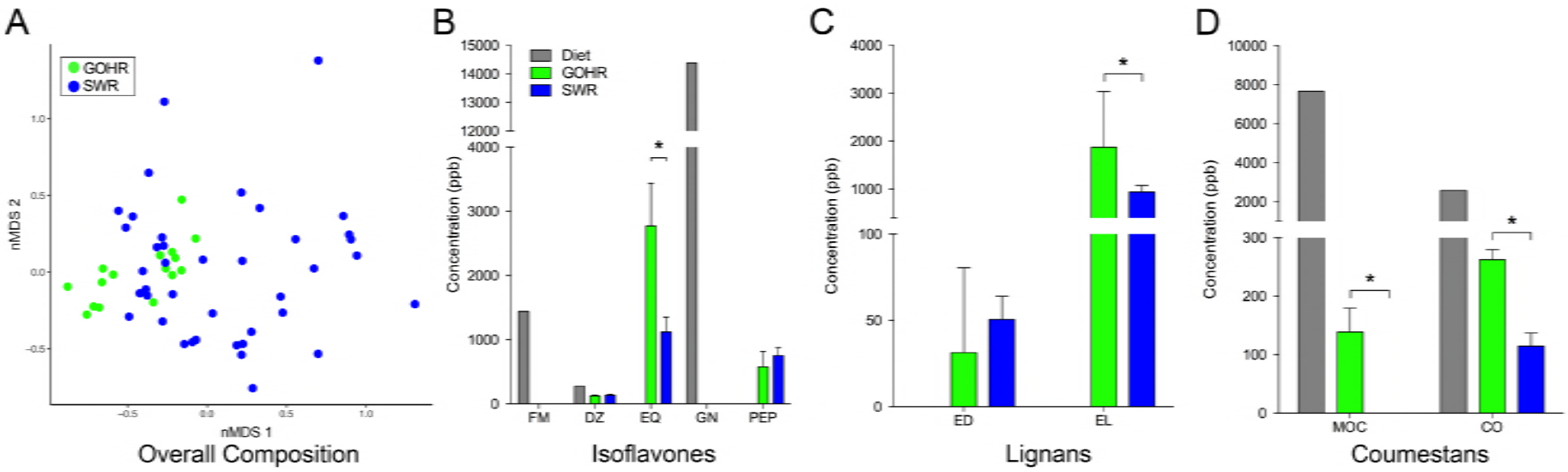

Figure 2. Comparison of fecal phytoestrogen composition between southern white rhinoceros (SWR) and greater one-horned rhinoceros (GOHR) A) Nonmetric multidimensional scaling (nMDS) analysis displaying overall composition of fecal phytoestrogens detected by mass spectrometry based on Bray-Curtis distances (PERMANOVA, $P > 0.05$; stress: 0.13). Mean ± SE analyte concentrations in parts per billion (ppb) of B) isoflavones, C) lignans, and D) coumestans for both SWR and GOHR and their diet. *Significantly different concentrations of fecal analytes (Welch's *t*-test, $P < 0.05$). FM: formononetin, DZ: daidzein, EQ: equol, GN: genistein, PEP: 4'ethylphenol, ED: enterodiol, EL: enterolactone, MOC: methoxycoumestrol, CO: coumestrol.

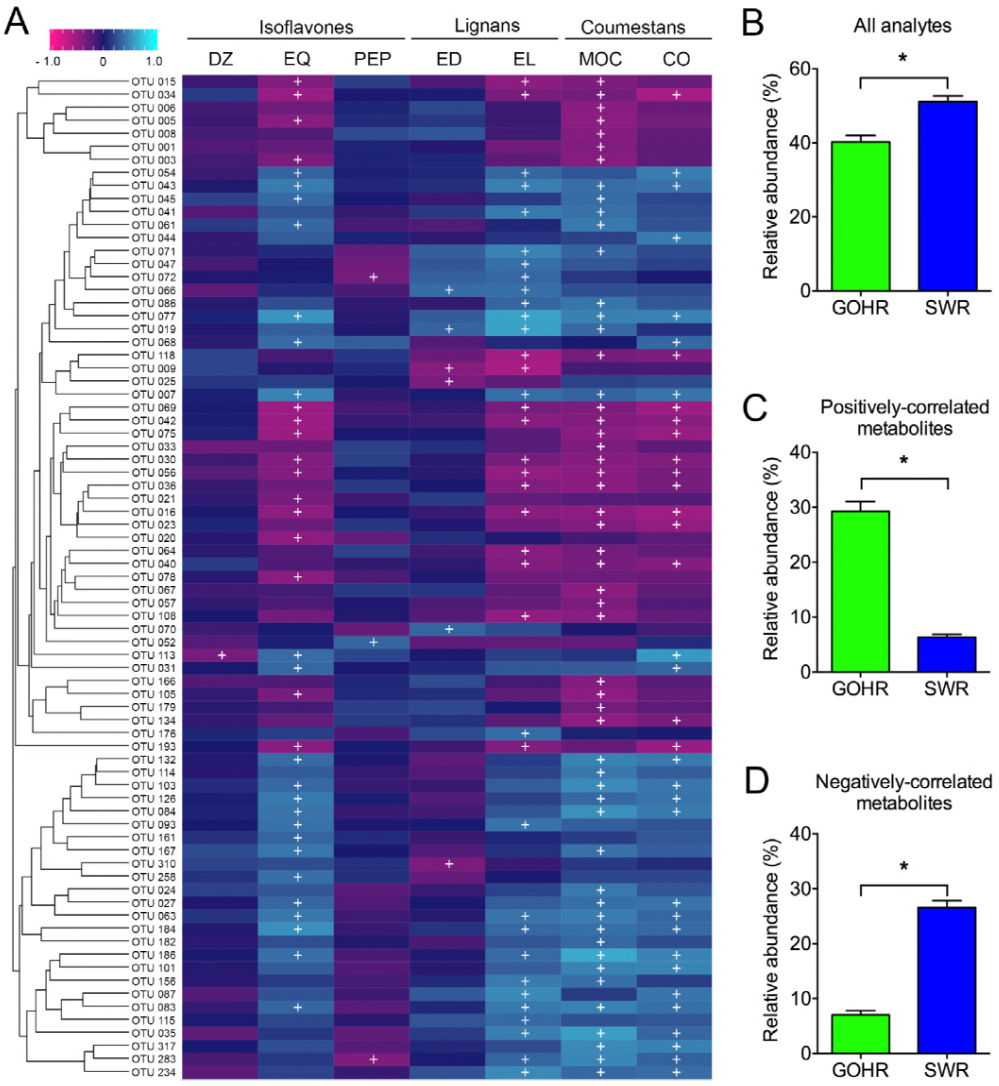

Figure 3. Relative abundance of OTUs and phytoestrogen concentrations significantly correlate. A) Heatmap depicting significant correlations between phytoestrogen analytes and microbiota (≥ 1.0 % relative abundance) using Spearman correlation method with FDR correction ($^{+}$Significance at $P < 0.05$). Dendogram displays OTUs that commonly co-occur by hierarchical clustering (Bray-Curtis). Species differences in mean ± SE relative abundance of observed OTUs correlating to B) phytoestrogen analytes, C) positively correlated metabolites, and for D) negatively correlated metabolites. * Welch's *t-test*, $P < 0.05$. DZ: daidzein, EQ: equol, PEP: 4'ethylphenol, ED: enterodiol, EL: enterolactone, MOC: methoxycoumestrol, CO: coumestrol, GOHR: greater one-horned rhinoceros, SWR: southern white rhinoceros.

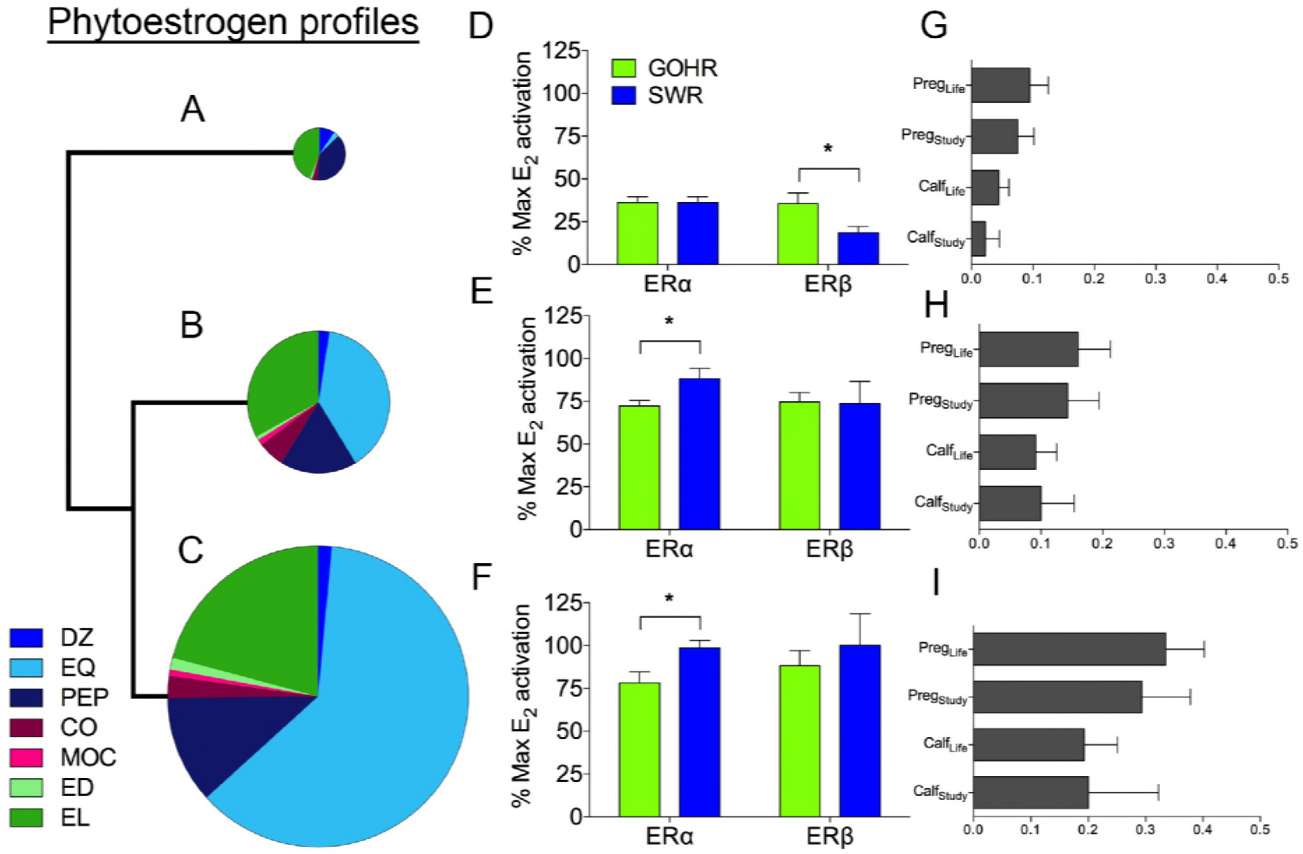

Figure 4. Relative estrogenicity and fertility of phytoestrogen profiles identified by hierarchical clustering. Phytoestrogen composition, as depicted by hierarchical clustering, with each profile's size relative to total concentration detected by mass spectrometry, A) Profile A, B) Profile B, and C) Profile C. The mean ± SE activation of ERα and ERβ of both southern white rhinoceros (SWR) and greater one-horned rhinoceros (GOHR), relative to maximal activation by 17β-E_2 by respective phytoestrogen profiles D) Profile A, E) Profile B, and F) Profile C, when tested at concentrations found *in vivo*. Differences in mean ± SE fertility measurements with respect to phytoestrogen profiles, G) Profile A, H) Profile B, and I) Profile C, *Significantly different activation (ANOVA, $P < 0.05$). DZ: daidzein, EQ: equol, PEP: 4'ethylphenol, ED: enterodiol, EL: enterolactone, MOC: methoxycoumestrol, CO: coumestrol.

521 **References:**

522 1. Emslie R (2012) The IUCN Red List of Threatened Species. Version 2014.2.
523
524 2. South African Dept. of Environmental Affairs, 2018, Rhino poaching statistics update.
525
526 3. Swaisgood R, Dickman DM, White AM (2006) A captive population in crisis: Testing
527 hypotheses for reproductive failure in captive-born southern white rhinoceros females.
528 *Biol Conserv*, **129**: 468-476.
529
530 4. Tubbs CW, Moley LA, Ivy JA, Metrione LC, LaClaire S, Felton RG, Durrant BS, Milnes
531 MR (2016) Estrogenicity of captive southern white rhinoceros diets and their association
532 with fertility. *Gen Comp Endocrinol*, **238**, 32-38.
533
534 5. Owen-Smith RN (1988) Megaherbivores: the influence of very large body size on
535 ecology. In RSK Bar, HJB Birks, EF Connor, RT Paine, (Eds.) Cambridge Studies in
536 Ecology, Cambridge Press, Cambridge, UK.
537
538 6. Hass TC, Ferreira SM (2016) Combating rhino horn trafficking: the need to disrupt
539 criminal networks. *PLoS ONE* **11(11)**: e0167040.
540
541 7. Tubbs C, Hartig P, Cardon M, Varga N, Milnes M (2012) Activation of southern white
542 rhinoceros (*Ceratotherium simum simum*) estrogen receptors by phytoestrogens: potential
543 role in the reproductive failure of captive-born females? *Endocrinology,* **153**: 1444-1452.
544
545 8. Shrader AM, Owen-Smith N, Ogutu JO (2006) How a mega-grazer copes with the dry
546 season: Food and nutrient intake rates by white rhinoceros in the wild. *Funct Ecol*, **20(2)**:
547 376-384.
548
549 9. Owen-Smith N (1973) The behavioural ecology of the white rhinoceros. PhD. Thesis,
550 University of Wisconsin-Madison.
551
552 10. Tham DM, Gardner CD, Haskell WL (1998) Potential health benefits of dietary
553 phytoestrogens: a review of clinical, epidemiological, and mechanistic evidence. *Int J*
554 *Clin Endocrinol Metab*, A Review of the Clinical, Epidemiological, and Mechanistic
555 Evidence, **83(7)**:2223-2235.
556
557 11. Adams NR (1995a) Organizational and activational effects of phytoestrogens on the
558 reproductive tract of the ewe. *Proc Soc Exp Biol Med,* **208**: 87-91.
559
560 12. Adams NR (1995b) Detection of the effects of phytoestrogens on sheep and cattle. *J*
561 *Anim Sci*, **73**: 1509-1515.
562
563 13. Patisaul HB, Jefferson W (2010) The pros and cons of phytoestrogens. *Front*
564 *Neuroendocrinol*, **31**: 400-419.
565

14. Jefferson WN, Patisaul HB, Williams CJ (2012) Reproductive consequences of developmental phytoestrogen exposure. *Reproduction* **143(3)**: 247-260.

15. Patton ML, Swaisgood RR, Czekala NM, White AM, Fetter GA, Montagne JP, Rieches RG, Lance VA (1999) Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and obervations of mating behavior. *Zoo Biol*, **18**: 111-127.

16. Schwarzenberger F, Walzer C, Tomasova K, Vahala J, Meister J, Goodrowe KL, Zima J, Strauß G, Lynch M (1998) Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum)*. *Anim Reprod Sci*, **53**: 173-190.

17. Hermes R, Hildebrandt TB, Walzer C, Göritz F, Patton ML, Silinski S, Anderson MJ, Reid CE, Wibbelt G, Tomasova K, Schwarzenberger F (2006) The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simu, C.s. cottoni*). *Theriogenology*, **65**: 1492-1515.

18. Atkinson C, Frankenfeld CL, Lampe JW (2005) Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol and Med,* **230(3)**: 155-170.

19. Day AJ, Cañada FJ, Díaz JC, Kroon PA, Mclauchlan R, Faulds CB, Plub GW, Morgan MRA, Williamson G (2000) Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Letters*, **468**: 166-170.

20. Clavel T, Henderson G, Engst W, Doré J, Blaut M (2006) Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. *FEMS Microbiol Ecol* **55**: 471-478.

21. Cohen LA, Crespin JS, Wolper C, Zang EA, Pittman B, Zhao Z, Holt PR (2007) Soy isoflavone intake and estrogen excretion patterns in young women: Effect on probiotic administration. *in vivo*, **21**: 507-512.

22. McFall-Ngai M, Hadfield MG, TCG Bosch, Carey HV et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. *PNAS* **11(9):** 3229-3236.

23. Landete JM, Medina M, Gaya P, de Las Rivas B, Muñoz R (2016) Bioactivation of phytoestrogens: Intestinal bacteria and health. *Crit Rev Food Sci Nutr* **56(11)** 1826-43.

24. Williams CL, Dill-McFarland KA, Vandewege MW, Sparks DL, Willard ST, Kouba AJ, Suen G and Brown AE (2016) Dietary Shifts May Trigger Dysbiosis and Mucous Stools in Giant Pandas (*Ailuropoda melanoleuca*). *Front. Microbiol*. **7**:661.

25. Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH (2012) The influence of diet on gut microbiota. *Pharmacol Res* **69**: 52-60.

26. Suzuki TA, Worobey M. (2014) Geographical variation of human gut microbial composition. *Biol. Lett*. **10**: 20131037.

27. Hazarika BC, Saikia PK (2012) Food habit and feeding patterns of great Indian one-horned rhinoceros (*Rhinoceros unicornis*) in Rajiv Gandhi Orang National Park, Assam, India. *ISRN Zoology*, **2012**: 259695.

28. Metcalf JL, Song SJ, Morton JT, Weiss S, Sequin-Orlando A, Joly F, Feh C, Taberlet P, Coissac E, Amir A, Willerslev E, Knight R, McKenzie V, Orlando L (2017) Evaluating the impact of domestication and captivity on the horse gut microbiome. *Sci Rep*, **7**: 15497.

29. Flint H, Scott K, Louis P, Duncan S (2012) The role of the gut microbiota in nutrition and health. *Nat Rev Gastroenterol and Hepatol,* **9**, 577–589.

30. Braune A, Blaut M (2016) Bacterial species involved in the conversion of dietary flavonoids in the human gut, *Gut Microbes*, **7**: 216-234.

31. Mumford SL, Sundaram R, Schisterman EF, Sweeney AM, Barr DB, Rybak ME, Maisog JM, Parker DL, Pfeiffer CM, Louis GMB (2014) Higher urinary lignan concentrations in women but not men are positively associated with shorter time to pregnancy. *J Nutr* **144**: 352-358.

32. Setchell KDR, Brown NM, Zimmer-Nechemias L, Brashear WT, Wolfe BE, Kirschner AB, Heubi JE (2002) Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am J Clin Nutr*, **76**: 447-53.

33. Shutt DA, Braden WH (1968) The significance of equol in relation to the oestrogenic responses in sheep ingesting clover with a high formononetin content. *Aust J Agric Res,* **19**: 545-53

34. Shutt DA, Weston RH, Hogan JP (1970) Quantitative aspects of phyto-oestrogen metabolism in sheep fed on subterranean clover (*Trifolium subterraneum* cultivar clare) or red clover (*Trifolium pretense*). *Aust J Agric Res,* **21**: 713-22.

35. Woclawek-Potocka I, Bah MM, Korzekwa A, Piskula MK, Wiczkowski W, Depta A, Skarzynski DJ (2005) Soybean-derived phytoestrogens regulate prostaglandin secretion in endometrium during cattle estrus cycle and early pregnancy. *Ex Biol Med*, **230**: 189-199.

36. Hausmann B, Pelikan C, Rattei T, Loy A, Pester M (2018) Growth arrest in the active rare biosphere. bioRxiv 284430; doi: https://doi.org/10.1101/284430.

37. Jin SJ, Nobuko K, Hattori M (2007) Enantioselective oxidation of enterodiol to enterolactone by human intestinal bacteria. *Biol Pharm Bull*, **30**(11) 2204-2206.

38. Harley EH, de Waal M, Murray S, O'Ryan C (2016) Comparison of whole mitochondrial genome sequences of northern and southern white rhinoceroses (*Ceratotherium simum*): the conservation consequences of species definitions. *Conserv Genet* **17**: 1285-1291.

39. Hildebrandt TB, Hermes R, Colleoni S, Diecke S, Holtze S, Renfree MB, Stejskal J, Hayashi K, Drukker M, Loi P, Göritz F, Lazzari G, Galli C (2018) Embryos and embryonic stem cells from the white rhinoceros. *Nat Commun* **9**:2589.

40. Saragusty J, Diecke S, Drukker M, Durrant B, Ben-Nun IF *et al*. (2016) Rewinding the process of mammalian extinction. *Zoo Biol* **35**: 280-292.

41. Stevenson DM, Weimer PJ (2007) Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *App Microbiol Biotechnol,* **75**, 165-174.

42. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *J Appl Environ Microbiol,* **79**, 5112-5120.

43. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *J Appl Environ Microbiol*, **75**, 7537-7541.

44. Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res,* **35**, 7188-7196.

45. Good IJ (1953) The population frequencies of species and the estimation of population parameters. *Biometrika*, **40**, 237-264.

46. Oksanen J, Blanchet FG, Kindt R, Legendere P, Minchin PR, O'Hara RB *et al.* (2015). Vegan: community ecology package, 2.2-1 edn.

47. McMurdie PJ, Holmes S (2013) phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, **8**(4): e61217.

48. Bray JR, & Curtis JT (1957). An ordination of upland forest communities of southern Wisconsin. *Ecol Monogr,* **27**, 325-349.

49. Jaccard P (1912). The distribution of the flora in the alpine zone. *New Phytologist*, **11**, 37-50.

50. Lozupone CA, Hamady M, Kelley ST, Knight R (2007). Quantitative and qualitative B diversity measures lead to different insights into factors that structure microbial communities. *J Appl Environ Microbiol*, **73**(5) 1576-1585.

51. Shepard RN (1966) Metric structures in ordinal data. *J Math Psychol*, **3**, 287-315.

52. Kuhn M (2008) Caret package. *J Stat Softw*, **28**(5).

53. Palme R, Touma C, Arias N, Dominchin MF, Lepschy M (2013) Steroid extraction: Get the best out of faecal samples. *Wiener Tierärztliche Monatsschrift-Veterinary Medicine Austria*, **100**: 238-246.

54. Merl S, Scherzer S, Palme R, Mostl E (2000) Pain causes increased concentrations of glucocorticoid metabolites in equine feces. *J Equine Vet Sci*, **20**, 586-590.

55. Lahti L, Shetty S et al. (2017) Tools for microbiome analysis in R. Version 1.1.10012. URL: http://microbiome.github.com/microbiome

56. R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org