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Estrogenicity of captive southern white rhinoceros diets and their association with fertility

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ABSTRACT

The captive southern white rhinoceros (SWR) population is not currently self-sustaining, primarily due to poor or absent reproduction of captive-born (F_{1+}) females. In this study, we investigate the role of dietary phytoestrogens in this reproductive phenomenon by characterizing activation of SWR estrogen receptors (ESRs) 1 and 2 by diet items from nine North American institutions and comparing female SWR fertility to total diet estrogenicity. Of the diet items tested, alfalfa hay and soy and alfalfa-based commercial pellets were found to be the most potent activators of SWR ESRs. In contrast, most grass hays tested were not estrogenic. The estrogenicity of total diets varied across the institutions surveyed and the degree of diet estrogenicity was positively associated with the percentage of the total diet comprised by pellets. Comparisons of fertility records of the institutions surveyed showed no significant relationship between diet estrogenicity and fertility for female SWR conceived or born in the wild (F_0). However, for F_{1+} females, there was a significant negative relationship between institutional diet estrogenicity and fertility. Taken together, these data suggest that developmental exposure to phytoestrogens may be the cause of poor fertility in captive-born female SWR. Whether the low fertility of the current population of captive-born female SWR is permanent or can be reversed by removing phytoestrogens from the diet remains unclear. However, our findings suggest that in order for the SWR population to become self-sustaining, the development and feeding of low phytoestrogen diets should be strongly considered.

1. Introduction

Over the past hundred years the southern white rhinoceros (SWR; *Ceratotherium simum simum*) has made a remarkable recovery from near extinction, growing from approximately 100 individuals to a current estimated population of approximately 20,400 (International Rhino Foundation, 2014; Renshaw, 1904). The creation of *in situ* reserves to protect wild animals and the establishment of *ex situ* captive breeding populations have played an important role in this species' conservation and current IUCN listing of 'near threatened' (Emslie, 2012). However, while the captive SWR population initially grew due to successful reproduction of founding females imported from the wild (F_0), it is currently not self-sustaining due to low fertility of captive-born females (F_{1+}) (Emslie and Brooks, 1999; Swaisgood et al., 2006). The recent escalation of wild SWR poaching to record levels underscores the critical importance of a self-sustaining captive population to ensure this species' survival. Thus, factors limiting reproduction must be identified.

Previous investigations have eliminated many of the factors anecdotally thought to contribute to low F_{1+} female fertility. One study has suggested captive male SWR fertility may be reduced (Hermes et al., 2005), though further investigations are needed to more clearly assess this possibility. With specific regard to female fertility, Swaisgood et al. (2006) found no evidence of social suppression of F_{1+} females by F_0's, no differences between sociosexual behaviors exhibited by F_0 and F_{1+} females and "proven" male SWR (males that have previously sired offspring) showed no preference for either F_0 or F_{1+} females. Analyses of fecal glucocorticoids showed higher levels in F_0 females compared F_{1+} females, although no differences in adrenal activity were observed between acyclic and normal cycling females or between nulliparous and parous females (Metrione and Harder, 2011).

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Reproductive health assessments, however, revealed numerous reproductive pathologies in a study of 48 captive female SWR that were consistent with prolonged exposure to estrogenic substances (Hermes et al., 2006). The suggested cause of the observed cysts, tumors and altered hormone cycles is prolonged non-reproductive periods resulting in continual ovarian activity and exposure to elevated levels of endogenous estrogens compared to their wild counterparts. A similar paradigm has been proposed for other captive species (Hermes et al., 2004), but the potential for exogenous, environmental sources of estrogenic substances to contribute to the observed SWR reproductive pathologies and fertility issues has not thus far been considered.

The capacity for estrogenic environmental chemicals to disrupt endocrine function and affect fertility of humans, wildlife and laboratory species is well documented (Colborn et al., 1993). These endocrine disrupting chemicals (EDCs) can interfere with a myriad of endocrine functions (Guillette, 2006) and can have profound impacts on reproduction through both organizational and activational effects. A striking example of an organizational effect is the impact of diethylstilbestrol (DES) usage in humans. Originally prescribed to women to prevent miscarriages, it was later determined that women who were exposed to the synthetic estrogen *in utero* experienced increased incidence of reproductive pathologies and impaired fertility (Colborn et al., 1993). Non-anthropogenic compounds can also act as EDCs and have profound effects on reproduction. In sheep, activational effects, including sub-fertility and infertility, have been observed following ewes grazing on clover (*Trifolium* spp.), a legume containing high levels of phytoestrogens (Adams, 1995a). These effects can typically be mitigated once ewes reduce clover consumption (Adams, 1995b). Developmental exposure to phytoestrogens can also result in organizational effects similar to those of DES exposure in humans in that they are typically more severe than activational effects and permanent (Adams, 1995b; Jefferson et al., 2012).

Phytoestrogens are likely exogenous sources of estrogenic EDCs to which SWR are exposed, as phytoestrogen-rich legumes like soy and alfalfa are common ingredients in captive diets. Moreover, many of the pathologies described in captive female SWR (Hermes et al., 2004, 2006) are similar to those observed in other species consuming high phytoestrogen diets, such as abnormal hormone cycles, development of uterine cysts and tumors and overall reduced fertility (see Burton and Wells, 2002 and Jefferson et al., 2012 for review). To investigate the potential role of phytoestrogens in the low fertility of F_{1+} SWR, we previously compared SWR estrogen receptor (ESR) activation to activation of ESRs of greater one-horned rhinoceros (GOHR); a species that reproduces well in captivity while receiving diets similar to those of SWR (Tubbs et al., 2012). In that study, maximal activation by purified phytoestrogens of SWR ESRs was greater than maximal activation of GOHR ESRs (Tubbs et al., 2012). Although these data provide an association between sensitivity to phytoestrogens and fertility, their significance remains unclear as SWR ESR activation was characterized by purified phytoestrogens, not actual diet items.

The present study aims to further address the potential role of dietary phytoestrogens in the low fertility of captive-born SWR. We were particularly interested in investigating the relationship between diet and fertility between F_0 and F_{1+} SWR given that both groups typically receive the same diets at a particular institution yet can exhibit marked difference in fertility. To do this, we first characterized estrogenicity of individual diet items fed to captive SWR using SWR-ESR receptor activation assays. In addition, we collected feeds from nine North American institutions experiencing variable success in breeding SWR to assess total diet estrogenicity. Finally, since controlled, long term experimental treatment studies examining the effects of EDC exposure on reproduction are not feasible in a species like rhinoceros, we utilized historical breeding records at those nine institutions to determine if a relationship exists between dietary phytoestrogen exposure and fertility.

2. Materials and methods

2.1. Extraction of captive feeds

Samples of individual diet items (pasture grasses, hays and commercial pellets) were dried in a convection oven at 50 °C for 24 h and then pulverized using a hand-held mortar and pestle. Pelleted foods were assigned into one of three classes based on the most abundant ingredients reported on the manufacturer's nutrition labels. The classes were soy-based, alfalfa-based, or soy/alfalfa combination pellets. Dried plant material was then extracted at a ratio of 1 g per 4 mL of methanol while rocking at room temperature in glass test tubes overnight. Extracts were separated from solids by filtration though Whatman filter paper that was presoaked in methanol. Aliquots of extract (0.5 mL) were added to a clean test tube and dried under forced air for 24 h. Dried extracts of the individual diet items were resuspended in DMSO to a concentration of 1 g starting material/mL DMSO. A set of clean test tubes receiving only 4 mL of methanol were included in each extraction and used for the preparation of vehicle treatment controls in receptor activation studies described below.

2.2. Preparation of whole diet extracts

Nine institutions within North America were surveyed to determine composition of whole diets fed to SWR. The institutions were: the San Diego Zoo Safari Park (Escondido, CA), Lion Country Safari (West Palm Beach, FL), White Oak Conservation Holdings (Yulee, FL), Busch Gardens (Tampa, FL), Center for Conservation of Tropical Ungulates (Punta Gorda, FL), the Wilds (Cumberland, OH), Fossil Rim Wildlife Center (Glen Rose, TX), Knoxville Zoo (Knoxville, TN) and Disney's Animal Kingdom (Lake Buena Vista, FL). Samples of individual diet items from each institution were obtained and extracted as described above. Extracts dissolved in DMSO were then combined proportionately by mass based on each institution's reported SWR diet composition. For example, if an institution fed 10 kg hay and 10 kg pellets, a 50:50 (vol:vol) mixture of hay and pellet extracts was made. SWR at some survey institutions obtain a significant proportion of their diet from grazing on open pasture during certain times of the year. Since quantifying the amount of fresh pasture plants consumed was not possible, we estimated that the amount grazed, in addition to other food items fed, would bring the total mass of food consumed to 22.7 kg/day. This amount corresponds to a mass of food equaling 1–2% of their body mass, which SWR normally consume daily (Owen-Smith, 1973). For institutions that offer varied diets according to reproductive status, we limited our analysis to the diet prescribed to females during the gestation period. Finally, the consistency of the percentage of different items fed at each institution was confirmed for the time period for which fertility was calculated (described below).

2.3. Cell culture and receptor activation studies

Activation assays with SWR ESRs 1 and 2 were performed as described previously (Tubbs et al., 2012). HEK 293 cells were maintained in minimum essential medium (MEM) with 10% fetal bovine serum (FBS). Cells (100 µl of 6×10^5 cells per/mL) were added to each well of a 96 well plate. After 24 h cells were co-transfected with 5 µg pCMX-β-galactosidase, 5 µg pGL2-3xERE luciferase reporter plasmid (Addgene plasmid 11354; (Hall and McDonnell, 1999)) and 0.5 µg of SWR ESR-pcDNA3.1(+) expression plasmid

(Invitrogen) per plate using TransIT 2020 transfection reagent (Mirus Bio LLC, Madison, WI) and incubated for an additional 24 h. Transfected cells were then treated with 1 nM 17β-E_2, 0.2–2.0 mg/mL extracts of individual food items or whole diets or a vehicle control treatment of 0.1% DMSO in MEM supplemented with 10% of charcoal-resin stripped FBS. For each diet item analyzed, a group of cells were co-treated with 2.0 mg/mL of extract and the ESR antagonist, ICI182780. After 24 h cells were lysed and assayed for luciferase and β-galactosidase activity as described previously (Grun et al., 2002). Luciferase activity of treatments relative to vehicle-only treatment and normalized to β-galactosidase activity was used to calculate fold receptor activation. All data were normalized to fold activation of a 1 nM E_2 treatment and expressed as a percentage of that response as reported previously (Tubbs et al., 2012).

2.4. Studbook analyses

The International Studbook for White Rhinoceros (Association of Zoos and Aquariums, 2014) was used to calculate historical and current fertility (from 13 October 1965 through 16 May 2015) of female SWR residing at the nine institutions whose diets were analyzed. All institutions that participated in the study are participants in the Association of Zoos and Aquariums (AZA) SWR Species Survival Plan® (SSP). Each of these institutions are committed to breeding SWR in accordance with recommendations from the SSP for individual SWR based on genetic analyses. In addition, all institutions surveyed have had past or recent success in breeding SWR and manage their SWR in environments known to support reproduction, such as large enclosures and herds consisting of multiple females housed with single males.

For the purposes of this study, fertility rate was defined as the number of offspring produced per female reproductive year. Summarized fertility rates were calculated for F_0 and F_{1+} females at each institution [total number of offspring produced/total number of female reproductive years]. Although most calves are born to cows between the ages of 10 and 30 (Tubbs, *personal obs.*), females between 4 and 43 years of age were considered reproductive based on the ages of the youngest (4.01 years) and the oldest (42.8 years) cows giving birth, according to studbook records. Females from 6 of the 9 institutions surveyed spanned the entire 4–43 year age range while females at 3 other institutions had females aged 4–28, 4–30 and 4–35 years (Institutions 2, 3 and 6 in Fig. 4). To ensure fertility was only calculated for individuals with the potential to breed, only years during which females had access to a male identified by the SSP as a potentially suitable mate were included in the analyses. Females were excluded from analyses during any years in which access to a potential mate could not be confirmed or were housed at an institution without a history of breeding success.

For F_0 females, fertility rates were applied to each institution in which they resided during their reproductive years. In other words, if a female spent half her reproductive years at 'Institution 1' and half at 'Institution 2', her individual fertility rate at each of those institutions would be integrated into each institution's summarized fertility rate separately. However, in order to address the possible effects of developmental (as opposed to activational) exposure to phytoestrogens, the fertility rates of F_{1+} females were applied to the summarized fertility rate of the institution in which they were conceived, regardless of any transfers that occurred among participating institutions during their reproductive years. Four F_{1+} females that were included in the analyses that were transferred during gestation, and each was gestated at the institution where they were conceived for at least 4.8 months. Finally, females conceived in the wild and born in captivity (n = 2) were designated as F_0 based on the location where conception occurred. In total, 136 female SWR were included in the fertility analyses. F_0 and F_{1+} females accounted for 73 and 63 of the individuals included, respectively.

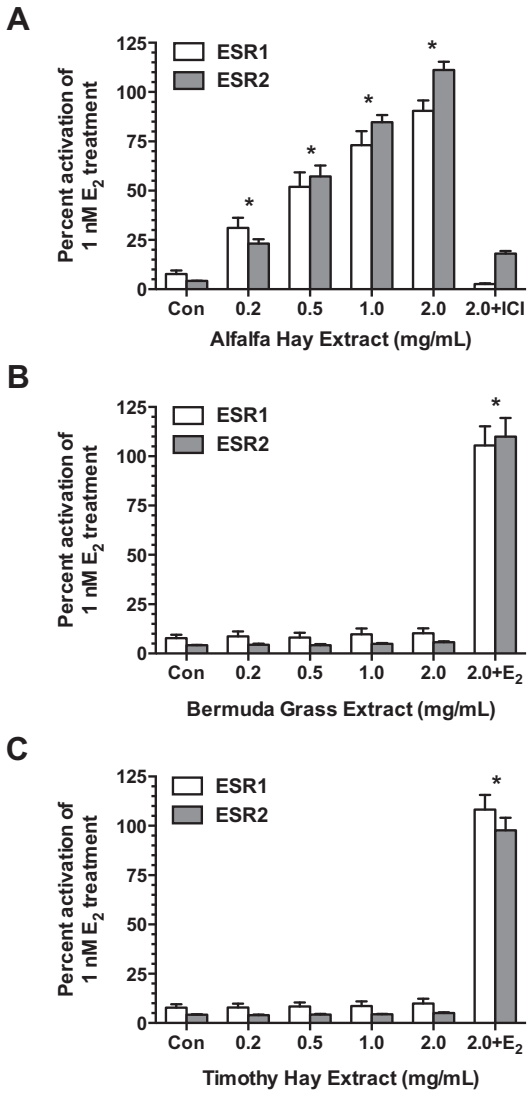

Fig. 1. Activation of southern white rhinoceros ESR1 and ESR2 by extracts of (A) alfalfa, (B) Bermuda hay and (C) Timothy hay. Cells expressing SWR ESR1 or ESR2 were treated with increasing concentrations (0.2–2.0 mg/mL) of dried extract resuspended in DMSO, vehicle (Con; 0.1% DMSO) or 2.0 mg/mL extract and 10 nM of the ESR antagonist ICI182780 or 1 nM E_2. Data are presented as mean ± SEM of the fold activation of each treatment relative to a 1 nM E_2 treatment. Significantly different means compared to control treatments for each receptor were determined using a one-way ANOVA and Dunnett's multiple comparisons (*$p < 0.05$; n = 3).

2.5. Statistical analyses

All regressions and statistical analyses were performed using Graph Pad Prism Software (San Diego, CA). Data represent mean ± SEM of at least three independent experiments and are considered significant if $P < 0.05$. Comparisons of SWR ESR activation were analyzed by one-way ANOVA and Dunnett's multiple comparisons post-test.

3. Results

3.1. Estrogenicity of captive feeds

For all grasses and hays tested, activation of SWR ESRs 1 and 2 was greatest by extracts of alfalfa (*Medicago sativa*) (Fig. 1A), with significant activation occurring at all concentrations tested. Co-treatment of HEK293 cells expressing SWR ESRs with 2.0 mg/mL

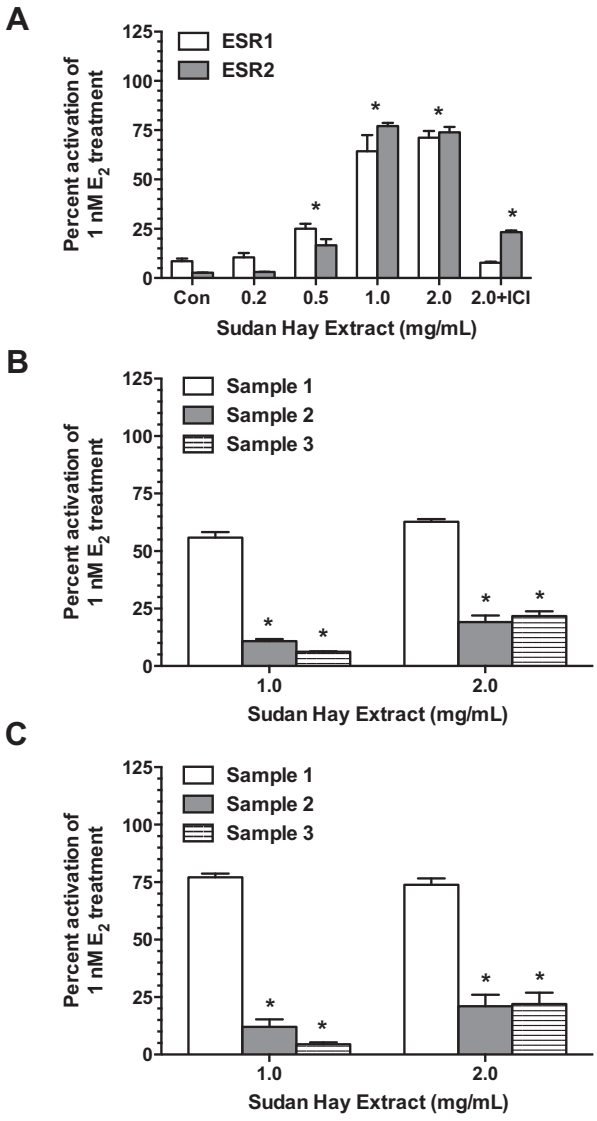

Fig. 2. Activation of southern white rhinoceros ESR1 and ESR2 by extracts of Sudan hay. Cells expressing SWR ESR1 or ESR2 were treated with increasing concentrations (0.2–2.0 mg/mL) of dried extract resuspended in DMSO, vehicle (Con; 0.1% DMSO) or 2.0 mg/mL extract and 10 nM of the ESR antagonist ICI182780 (A). Variable activation of ESR1 (B) and ESR2 (C) of three different extracts of Sudan hay. Data are presented as mean ± SEM of the fold activation of each treatment relative to a 1 nM E_2 treatment. Significantly different means compared to control (A) or Sample 1 (B-C) treatments for each receptor were determined using a one-way ANOVA and Dunnett's multiple comparisons ($*p < 0.05$; n = 3).

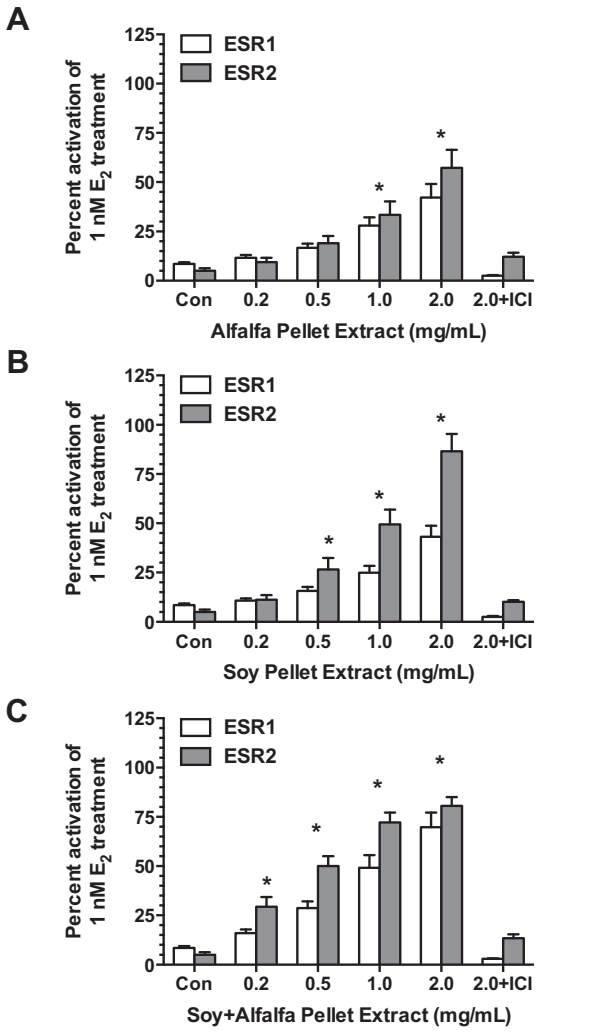

Fig. 3. Activation of recombinant southern white rhinoceros ESR1 and ESR2 by extracts of three different types of pellets. Cells expressing SWR ESR1 or ESR2 were treated with increasing concentrations (0.2–2.0 mg/mL) of dried extract resuspended in DMSO, vehicle (Con; 0.1% DMSO) or 2.0 mg/mL extract and 10 nM of the ESR antagonist ICI182780. The pellets tested were (A) primarily alfalfa-based, (B) primarily soy-based and (C) pellets with both alfalfa and soy as major ingredients. Data are presented as mean ± SEM of the fold activation of each treatment relative to a 1 nM E_2 treatment. Significantly different means compared to control treatments for each receptor were determined using a one-way ANOVA and Dunnett's multiple comparisons ($*p < 0.05$; n = 3).

alfalfa extract and the ESR antagonist ICI182780 significantly reduced activation compared to the 2.0 mg/mL alfalfa only treatments. Neither extracts of Bermuda grass (*Cynodon dactylon*) or Timothy hay (*Phleum pretense*) stimulated ESR1 or 2 activation at any concentration tested, nor did they inhibit receptor activation by E_2 following co-treatment with 2.0 mg/mL of extract and 1 nM E_2 (Fig. 1B-C). Extracts of Sudan hay (*Sorghum* × *drummondi*) activated both ESR1 and 2 to levels similar to those of alfalfa extracts (Fig. 2A). However, testing of all subsequent lots of Sudan hay received at different times by the same institution resulted in significantly lower ESR activation than initial tests (Fig. 2B-C).

All extracts of commercial pellets tested in this study significantly activated both SWR ESRs 1 and 2 (Fig. 3A-C). Pellets for which soy or a combination of soy and alfalfa products were listed as the primary ingredients were more potent activators of SWR ESRs than alfalfa-based pellets. Receptor activation by all pellet extracts was inhibited by co-treatment with the ESR antagonist, ICI182780.

3.2. Estrogenicity of whole diets

The proportions of commercial pellets, grasses, hays and alfalfa that comprise captive diets varied across institutions. The proportion of pellets fed ranged from 0 to 53.8% for the institutions surveyed, while the proportion of grasses/hay and alfalfa ranged from 46 to 88.2% and 0 to 11.8%, respectively (Fig 4A-B). A two-factor ANOVA showed a significant effect on activation of both ESRs 1and 2 by concentration of extract tested ($F(3, 588)$, $p < 0.001$; $F(8, 588)$, $p < 0.001$) and institutional diet ($F(3, 500)$, $p < 0.001$; $F(8, 500)$, $p < 0.001$). Estrogenicity of institutional diets differed with activation of ESR1 ranging from 13.4 to 74.6% and activation of ESR2 ranging from 6.4 to 100.9% of a 1 nM E_2 treatment by 2.0 mg/mL of extracts (Fig. 4A-B). Whole diet estrogenicity (expressed as the sum of the mean activation of ESR1 *or* ESR2 by

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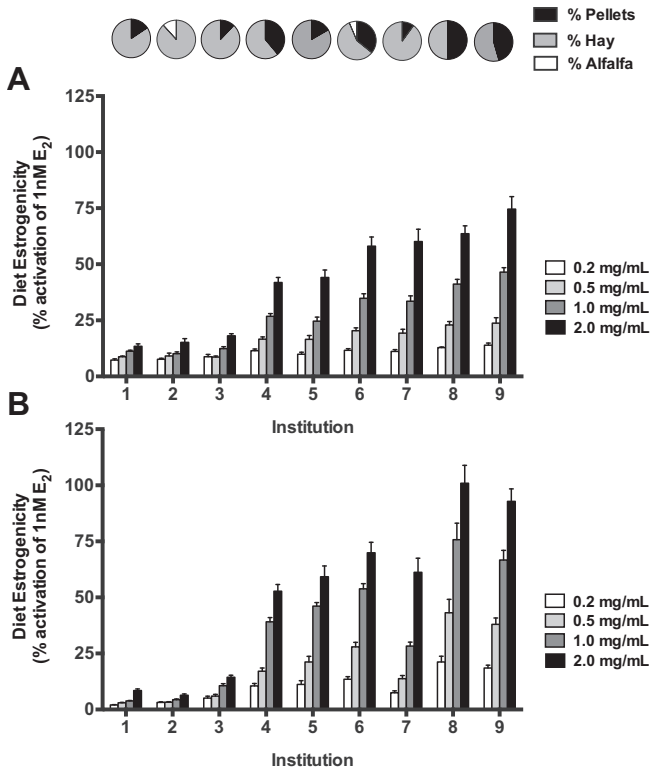

Fig. 4. Activation of recombinant southern white rhinoceros ESR1 (A) and ESR2 (B) by extracts of diets from nine North American institutions. Cells expressing SWR ESR1 or ESR2 were treated with increasing concentrations (0.2–2.0 mg/mL) of dried extracts of each diet item combined in the proportion they are fed to reflect estrogenicity of whole diets. Relative proportions of each type of food (pellets, hay or alfalfa) are shown as pie charts above. Data are presented as mean ± SEM of the fold activation of each treatment relative to a 1 nM E_2 treatment. A two-way ANOVA determined there was a significant effect of both institution and concentration on activation of both ESR 1 ($p < 0.001$) and ESR2 ($p < 0.001$).

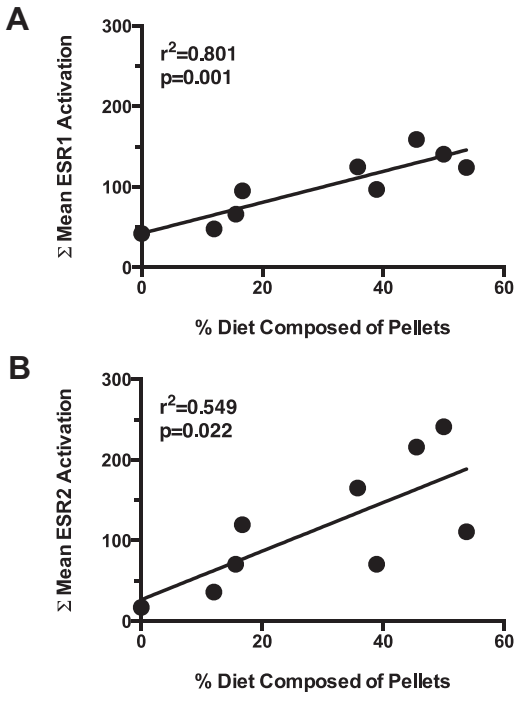

Fig. 5. Relationship between estrogenicity of diets and the percentage of pellets in diets of nine North American institutions. Estrogenicity is expressed as the sum of mean activation of (A) ESR1 ($p = 0.001$) and (B) ESR2 ($p = 0.022$) for all concentrations tested (0.2–2.0 mg/mL) for each diet extract. A Pearson's correlation was performed using GraphPad Prism.

each concentration of extract tested) was positively correlated to the percentage of commercial pellets found in diets for both SWR ESR1 ($r^2 = 0.801$, $p = 0.001$) and ESR2 ($r^2 = 0.549$, $p = 0.022$) (Fig. 5A-B).

3.3. Association between diet estrogenicity and fertility

There was no significant relationship between diet estrogenicity and fertility rate for females born in the wild or conceived in the wild and born in captivity (F_0) ($r^2 = 0.350$, $p = 0.092$) (Fig. 6A). In contrast, there was a significant negative relationship between diet estrogenicity and fertility rate for female SWR born in captivity (F_{1+}) ($r^2 = 0.534$, $p = 0.025$) (Fig. 6B).

4. Discussion

The recovery of the southern white rhinoceros (SWR) from near extinction within the last century is a conservation success story. However, SWR are once again threatened by increasing levels of poaching, leaving the viability of wild populations in question (Ferreira et al., 2012). Historically, *ex situ* SWR breeding programs have served as valuable assurance populations, but the captive population is not self-sustaining, largely due to low fertility of females born in captivity. In this study, we examined the potential of dietary phytoestrogens to contribute to the poor reproductive success of SWR. Our data indicate that the diets at many institutions are estrogenic and that estrogenicity of the diet is negatively associated with fertility of captive-born (F_{1+}) females.

Previously we have identified dietary phytoestrogens as potential contributing factors to the low fertility exhibited by F_{1+} female SWR (Tubbs et al., 2012, 2014). We found that purified phytoestrogens were more potent activators of SWR ESRs compared to greater one-horned rhinoceros (GOHR) ESRs; a species that receives similar diets in captivity, but has higher fertility rates. Here, we examined activation of SWR ESRs by extracts of food items routinely eaten at nine North American institutions to more accurately assess SWR exposure to dietary phytoestrogens. Of the different diet components tested, only Bermuda and Timothy grasses failed to activate SWR ESR1 or ESR2 at the concentrations tested. Sudan grass, on the other hand, exhibited moderate estrogenic activity, though subsequent testing of different lots of dried hay resulted in weak estrogenic activity similar to the other grasses tested. Sudan grass and other members of the genus Sorghum are known to produce phytoestrogens (Yang et al., 2012). Whether phytoestrogen production can vary in this species, as observed in other plants due to differences in water content and season (Leopold et al., 1976), which was not accounted for in this study, is unknown, but could explain differences in estrogenicity observed here.

Another possibility is that the more estrogenic sample contained exogenous substance(s), like the estrogenic mycotoxin zearalenone. Grain crops contaminated with zearalenone-producing fungi, such as *Fusarium* spp., are known to have estrogenic effects in a variety of agricultural animals (Kuiper-Goodman et al., 1987). Regardless of the cause of variability in estrogenicity of Sudan grass extracts, these data highlight an important consideration to be made when identifying specific diet items low in phytoestrogens, which is that estrogenicity of a particular ingredient can vary and should be analyzed across multiple received lots.

Extracts of alfalfa hay exhibited the highest estrogenic activity, and at the higher concentrations tested, activated SWR ESR2 to a level exceeding that of 1 nM E_2. The dominant phytoestrogen

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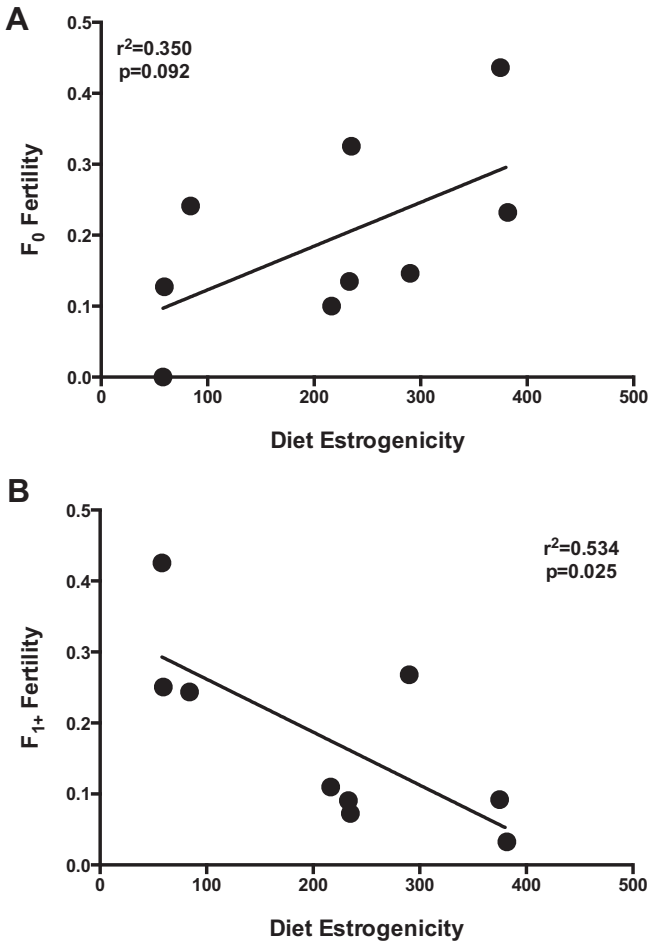

Fig. 6. Relationship between estrogenicity of diets and fertility of (A) wild-born (F_0) and (B) captive-born (F_{1+}) southern white rhinoceros from nine North American institutions. Estrogenicity is expressed as the sum of mean activation of ESR1 and ESR2 of all diet extract concentrations tested (0.2, 0.5, 1.0 and 2.0 mg/mL) for each diet extract. Fertility was calculated as the number of calves born to females of reproductive age per year. A Pearson's correlation was performed using GraphPad Prism.

found in alfalfa is coumestrol, which is the most potent phytoestrogen activator of SWR ESRs (Tubbs et al., 2012). Similar to alfalfa extracts, coumestrol can stimulate a level of ESR2 activation greater than that of a 1 nM E_2 treatment, albeit at micromolar concentrations (Tubbs et al., 2012), suggesting SWR may be particularly sensitive to alfalfa-based items. Interestingly, SWR ESR activation by extracts of alfalfa-based pellets was not greater than soy-based pellets. Instead, SWR ESR2 was most sensitive to soy-based pellets, while both ESR1 and 2 were more sensitive to extracts from pellets listing both soy and alfalfa as major ingredients than extracts from predominately alfalfa-based pellets. Soy contains high amounts of the isoflavones daidzein and genistein, which also activate SWR ESRs, but at lower levels than coumestrol (Tubbs et al., 2012). In this study, the concentrations of specific phytoestrogens in each variety of pellet and the efficiency of our method of extracting phytoestrogens from diets items were not analyzed. Therefore, it is unclear whether differences in estrogenicity are due to overall differences in phytoestrogen content, or are related to the fact that extracts contain mixtures of phytoestrogens and other chemicals that may have combinatorial effects on receptor function and physiological outcomes (Crews and Willingham, 2000; Charles et al., 2002, 2007). Further research is needed to establish relationships between concentrations and combinations of specific phytoestrogens in diets, diet extracts, in the circulation following consumption and estrogenicity via ESR activation. This knowledge would be useful in predicting the physiological consequences associated with a proposed diet and for developing diets with minimal estrogenic activity.

The estrogenicity of total diets from the nine institutions varied, although each diet tested activated SWR ESRs *in vitro*. The degree to which a given diet was estrogenic was positively associated with the percentage of the total diet comprised by pellets. This finding was anticipated since soy and/or alfalfa products are typically major ingredients in pellets as discussed above. Although alfalfa was one of the most estrogenic diet items tested, it is usually not a major component of SWR diets. Dietary recommendations suggest alfalfa not exceed 20% of the total mass of SWR diets (Clauss and Hatt, 2006) as rhinoceros consuming diets high in alfalfa often exhibit mineral imbalances (Dierenfeld et al., 2005). Of the institutions surveyed here, only two fed any alfalfa hay and the institution that fed the most alfalfa (#2), which comprised 11.8% of the total mass of the diet, had one of the least estrogenic diets. How the estrogenicity of the diets examined here compare to diets in the wild, where SWR are reproducing well, is unknown. SWR are a grazing species and consume primarily grasses. Although it's been shown that South African pasture grasses can exhibit estrogenic activity (Millar, 1967), preliminary studies conducted in our lab examining ESR binding by extracts of the predominant grasses in wild SWR diets (Owen-Smith, 1973) failed to show any appreciable receptor binding (Tubbs et al., *unpublished obs.*). Taken together, these data suggest that in order to reduce the quantity of dietary phytoestrogens captive SWR receive, the proportion of the diets composed of pellets should be reduced and the amount of grasses or hays fed should be increased.

Although reduced male fertility may also be a contributing factor (Hermes et al., 2005), the poor self-sustainability of the captive SWR population has been primarily attributed to poor reproduction of females born in captivity. The extent of the problem is unclear with estimates of the percentage of reproductive F_{1+} females ranging from as little as 8% to as many as 38% of the total population (Schwarzenberger et al., 1999; Swaisgood et al., 2006; Hermes et al., 2006; Metrione and Harder, 2011). Despite the existence of a studbook that documents all North American SWR births since the 1960's, accurately assessing fertility of captive SWR females presents many challenges. First, not all institutions with SWR are breeding institutions, either by choice, or because they do not maintain their animals under conditions known to be required for successful reproduction, which include large enclosures and multiple females housed with a single male at a time. In addition, day-to-day management decisions that can affect fertility cannot be easily accounted for using a retrospective studbook analysis. For example, the studbook indicates whether a certain female and male were both present at a given institution during a particular time, but it does not qualify or quantify the degree of access the potential breeding pair had to one another. We chose to restrict our analyses to institutions with successful SWR breeding programs that follow the breeding recommendations of the SWR SSP. In all, 63 of the 193 North American captive-born SWR were included in our analyses and represent the individuals with the best opportunity to breed successfully. Among the nine institutions we included, there was no difference in overall mean reproductive rates of F_0 and F_{1+} females (data not shown). Nonetheless, of the F_{1+} females examined, only 28 individuals (44%) have produced at least one offspring in their lifetime. This estimate is higher than those noted in previous studies suggesting the possibility that reproductive success has gradually improved as management is made more appropriate. However, the females that do not reproduce and yet are housed at these institutions where reproduction is expected represent a critical population whose reproductive failure has not been explained. Swaisgood et al.

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(2006) concluded that the root cause of this issue is the result of development in captivity and occurs post-copulation. In this study, we specifically explored the possibility that development in a phytoestrogen-rich environment is the cause of poor reproduction.

For wild-born female SWR, we found no significant relationship between estrogenicity of diet and fertility. In contrast, there was a significant negative relationship for captive-born females, suggesting that the more estrogenic a female's diet, the less likely her female offspring are to reproduce in their lifetime. Whether a similar relationship exists for captive-born males is difficult to assess, since standard management practices require single males to be housed with multiple females and therefore not all males become part of a breeding program. The ability of phytoestrogens, and other estrogenic substances, to influence female development and reproduction are well documented in many species. The effects of developmental exposure to phytoestrogens are wide ranging and include alterations in the estrous cycle, reproductive behavior, gonadal function, reproductive tract development and function and embryo survival (reviewed in Jefferson et al., 2012). Many of the observed effects are consistent with those documented in captive female SWR (Hermes et al., 2004, 2006), supporting the overall hypothesis that phytoestrogens are contributors, if not the primary cause of low fertility in captive-born SWR. If phytoestrogens are a cause of low SWR fertility, the severity of this phenomenon, in terms of whether it is reversible, is not known. Developmental exposures resulting in organizational effects are often permanent and normal function is not restored by reducing or eliminating exposure (Colborn et al., 1993; Adams, 1995b; Guillette et al., 1995). However, the magnitude of those effects is likely to vary from individual to individual and it is possible that the chronic exposure to dietary phytoestrogens F_{1+} SWR receive post-weaning results in activational effects that also impair reproduction, but are reversible. Therefore, changing to low phytoestrogen diets now could perhaps rescue fertility in at least some of the captive-born females in the current population that have never reproduced, which would be tremendously useful in establishing a self-sustaining captive SWR population.

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References

Adams, N.R., 1995a. Detection of the effects of phytoestrogens on sheep and cattle. J. Anim. Sci. 73, 1509–1515.
Adams, N.R., 1995b. Organizational and activational effects of phytoestrogens on the reproductive tract of the ewe. Exp. Biol. Med. 208, 87–91.
Association for Zoos and Aquariums. 2015, 2014. International Studbook for White Rhinoceros, .
Burton, J.L., Wells, M., 2002. The effect of phytoestrogens on the female genital tract. J. Clin. Pathol. 55, 401–407.
Charles, G.D., Gennings, C., Zacharewski, T.R., Gollapudi, B.B., Carney, E.W., 2002. An approach for assessing estrogen receptor-mediated interactions in mixtures of three chemicals: a pilot study. Toxicol. Sci. 68, 349–360.
Charles, G.D., Gennings, C., Tornesi, B., Kan, H.L., Zacharewski, T.R., Bhaskar Gollapudi, B., et al., 2007. Analysis of the interaction of phytoestrogens and synthetic chemicals: an in vitro/in vivo comparison. Toxicol. Appl. Pharmacol. 218, 280–288.
Clauss, M., Hatt, J.M., 2006. The feeding of rhinoceros in captivity. Int. Zoo Yearb. 40, 197–209.
Colborn, T., vom Saal, F.S., Soto, A.M.., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384.
Crews, D., Willingham, E., 2000. The red-eared slider turtle: an animal model for the study of low doses and mixtures. Am. Zool. 40, 421–428.
Dierenfeld, E.S., Atkinson, S., Craig, A.M., Walker, K.C., Streich, W.J., Clauss, M., 2005. Mineral concentrations in serum/plasma and liver tissue of captive and free-ranging rhinoceros species. Zoo Biol. 24, 51–72.
Emslie, R., 2012. *Ceratotherium simum*. IUCN 2012. IUCN Red List of Threatened Species, <www.iucnredlist.org>.
Emslie, R., Brooks, M., 1999. African Rhino Status Survey and Conservation Action Plan. IUCN.
Ferreira, S.M., Botha, J.M., Emmett, M.C., 2012. Anthropogenic influences on conservation values of white rhinoceros. PLoS One 7, e45989.
Grun, F., Venkatesan, R.N., Tabb, M.M., Zhou, C., Cao, J., Hemmati, D., Blumberg, B., 2002. Benzoate X receptors alpha and beta are pharmacologically distinct and do not function as xenobiotic receptors. J. Biol. Chem. 277, 43691–43697.
Guillette, L.J., 2006. Endocrine disrupting contaminants—beyond the dogma. Environ. Health Perspect. 114, 9–12.
Guillette, L.J., Crain, D.A., Rooney, A.A., Pickford, D.B., 1995. Organization versus activation: the role of endocrine-disrupting contaminants (edcs) during embryonic development in wildlife. Environ. Health Perspect. 103, 157–164.
Hall, J.M., McDonnell, D.P., 1999. The estrogen receptor beta-isoform (ERβ) of the human estrogen receptor modulates eralpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140, 5566–5578.
Hermes, R., Hildebrandt, T.B., Goritz, F., 2004. Reproductive problems directly attributable to long-term captivity–asymmetric reproductive aging. Anim. Reprod. Sci. 82–83, 49–60.
Hermes, R., Hildebrandt, T.B., Blottner, S., Walzer, C., Silinski, S., Patton, M.L., Wibbelt, G., Schwarzenberger, F., Göritz, F., 2005. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation. Theriogenology 63, 219–238.
Hermes, R., Hildebrandt, T.B., Walzer, C., Goritz, F., Patton, M.L., Silinski, S., et al., 2006. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). Theriogenology 65, 1492–1515.
International Rhino Foundation, 2014. Annu. Rep. 1–11.
Jefferson, W.N., Patisaul, H.B., Williams, C.J., 2012. Reproductive consequences of developmental phytoestrogen exposure. Reproduction 143, 247–260.
Kuiper-Goodman, T., Scott, P.M., Watanabe, H., 1987. Risk assessment of the mycotoxin zearalenone. Regul. Toxicol. Pharmacol. 7, 253–306.
Leopold, A.S., Erwin, M., Oh, J., Browning, B., 1976. Phytoestrogens: adverse effects on reproduction in California quail. Science 191, 98–100.
Metrione, L.C., Harder, J.D., 2011. Fecal corticosterone concentrations and reproductive success in captive female southern white rhinoceros. Gen. Comp. Endocrinol. 171, 283–292.
Millar, R.P., 1967. Oestrogenic activity in central African highveld grasses. Rhodesia Zambia Malawi J. Agric. Res. 5, 179–183.
Owen-Smith, R., 1973. The Behavioural Ecology of the White Rhinoceros. University of Wisconsin, Madison.
Renshaw, G., 1904. Natural History Essays. Sherratt and Hughes, London, pp. 1–30.
Schwarzenberger, F., Walzer, C., Tomasova, K., Zima, J., Goritz, F., Hermes, R., et al., 1999. Can the problems associated with the low reproductive rate in captive white rhinoceroses (*Ceratotherium simum*) be solved within the next five years. Verh. Ber. Erkrg. Zootiere. 39, 283–289.
Swaisgood, R.R., Dickman, D.M., White, A.M., 2006. A captive population in crisis: testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. Biol. Conserv. 129, 468–476.
Tubbs, C., Hartig, P., Cardon, M., Varga, N., Milnes, M., 2012. Activation of southern white rhinoceros (*Ceratotherium simum simum*) estrogen receptors by phytoestrogens: potential role in the reproductive failure of captive-born females? Endocrinology 153, 1444–1452.
Tubbs, C., McDonough, C.E., Felton, R., Milnes, M.R., 2014. Advances in conservation endocrinology: the application of molecular approaches to the conservation of endangered species. Gen. Comp. Endocrinol. 203, 29–34.
Yang, L., Allred, K.F., Geera, B., Allred, C.D., Awika, J.M., 2012. Sorghum phenolics demonstrate estrogenic action and induce apoptosis in nonmalignant colonocytes. Nutr. Cancer. 64, 419–427.