Phytoestrogen binding capacity to in vitro produced southern white rhino estrogen receptors. A comparison of native and captive feeds.

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Project engaged to the Institute for Breeding Rare and Endangered African Mammals (IBREAM) and San Diego Zoo's Institute for Conservation Research (CRES) Reproductive Physiology Division

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ABSTRACT

The southern white rhinoceros population in captivity has a disappointing reproductive rate and the reason behind this is unknown. On the contrary, the wild population has grown steadily over the last two decades and is still growing. In captivity, white rhinos are given a diet of mainly alfalfa and soy-based products, which contains high concentrations of phytoestrogens. Phytoestrogens are known to bind estrogen receptors and exposure to phytoestrogens is known to impair reproduction in several different species.

In Lapalala Wilderness, South Africa, research was conducted to determine the exact grass species consumed by free-ranging white rhinos, and to define the relative distribution of grass species eaten throughout the observed study period. This aim was addressed by observing four female white rhinos during a period of 3 months. Samples of the grass species were collected, identified and extracted using 100% methanol. The relative binding of phytoestrogens present in the dried plant extracts on white rhino estrogen receptors was tested. This was done both with phytoestrogens extracted from alfalfa and with phytoestrogens extracted from the samples of the grass species from Lapalala Wilderness. As a result, we could compare the captive feeds and the native browse. The comparative results show that alfalfa binds approximately 100% of the in vitro produced estrogen receptors. The samples of the identified wild grass species bind at most only 20% of the receptors. This study does suggest that the high concentration of phytoestrogens in captive feeds and the low concentration in native feeds are possibly an explanation for the difference in reproductive success between wild ranging white rhinos and captive white rhinos.

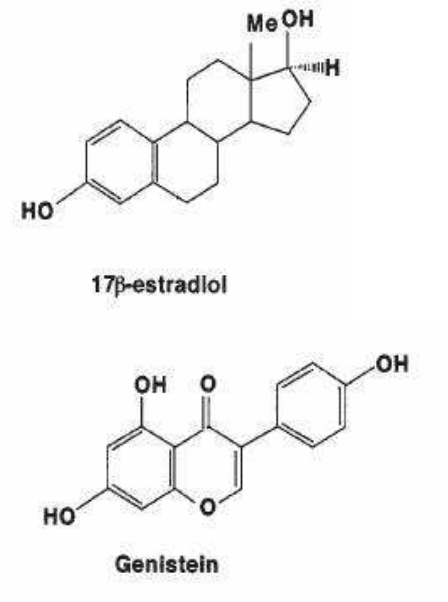
INTRODUCTION

Worldwide there are currently five rhinoceros species: the black rhinoceros, the white rhinoceros, the greater one-horned rhinoceros, the Javan rhinoceros and the Sumatran rhinoceros. Of these species, the black and white rhinos are native to Africa (*Skinner and Smithers, 1990*). The white rhino is divided into two subspecies, the southern white rhino (*Ceratotherium simum simum*) and the northern white rhino (*Ceratotherium simum cottoni*). The majority of the white rhino population is living in only four countries, South Africa, Namibia, Kenya and Zambia. After being hunted to near extinction by the end of the 19th century, the wild population has now grown to an estimated number of 17,500 (*Emslie, 2008*) Although the southern white rhino is now the least endangered of all the rhino-species, it is still sited as 'near threatened' on the IUCN Red list of threatened species (*IUCN, 2010*). The reason for this qualification is the continuing poaching threat due to a high and increased organization of criminal horn trading networks (*Milledge, 2007*). Although the free-ranging white rhino population has increased in the last couple of years (with an annual growth-rate of 6-10% (*Emslie and Brooks, 1999*), the IUCN still supports captive breeding as a safety measure, because of the political and social instability of the range countries (*Emslie and Brooks, 1999*). However, reproduction of captive southern white rhinos is very limited and this has resulted in a current decline of the captive population (*Emslie and Brooks, 1999*).

Conservation of the southern white rhino depends on two major approaches. The first is to effectively reduce poaching and protect and monitor wild populations (*Milledge, 2007; Emslie and Brooks, 1999; IUCN, 2010*). Secondly it is of major importance to enhance the reproduction of captive rhinos, and in particular of female rhinos born in captivity. As said before, captive populations can act as a safety net in case protection of the wild population is impaired (*Emslie and Brooks, 1999*) Furthermore, captive populations are important for education and raising public awareness (*Emslie and Brooks, 1999*).
In order to achieve a better reproduction of captive rhinos, it is critical to first determine the cause of the disappointing reproductive success of the rhino, which remains unresolved. Reproduction amongst captive-born females (F_1) is extremely slow, in some populations as few as 8% reproduces (*Schwarzenberger et al., 1999*). However the founding population (F_0) has shown successful breeding results in the past, and even now males continue to breed with wild-caught females (*Swaisgood et al., 2006*). The problem therefore is believed to lie within the F_1 females. Many possible explanations for the poor reproduction of captive rhinos have already been examined. For example, the reproductive hormones can show abnormalities across the reproductive cycle (*Schwarzenberger et al., 1999; Patton et al., 1999*), but this is not more common in F_1 females than in F_0 females, and therefore cannot explain the difference between these two groups. At this point the only clear consensus is that the cause of poor reproduction can be found somewhere in the post-copulation period, the F_1 females failing to conceive, or maintain the pregnancy (*Swaisgood et al., 2006*).

A possible cause of the poor reproductive success of southern white rhinoceros is exposure to phytoestrogens, which are compounds produced by plants with a close structural resemblance to the hormone estrogen. The steroid hormone estrogen and its receptor have an important influence on the growth, differentiation and functioning of many target cells, including tissues of the female reproductive tract (*Korach et al., 1994*). Dietary phytoestrogens like coumestrol,

genistein and zearalenone can diffuse into cells and bind estrogen receptors like endogenous estrogens (*Kuiper et al., 1997, 1998*) and this way compete with their normal function. Exposure to dietary phytoestrogens is known to impair reproduction in several different species by binding estrogen receptors and disrupting their normal function in different ways (*Whitten et al., 2001; Rosselli et al., 2000*). Ewes for example may suffer from temporary or permanent infertility without any clinical signs after prolonged exposure to estrogenic pasture due to a changed responsiveness to hormones (*Adams, 1995*), soy-derived phytoestrogens can disrupt reproductive efficiency and uterus function in cattle during the estrous cycle and early pregnancy (*Woclawek-Potocka et al., 2005*) and perinatal phytoestrogen exposure causes alterations in the development of reproductive organs in minks (*Ryökkynen et al., 2005*). The binding capacity to the estrogen receptors (*Kuiper et al., 1997*) and the cellular and biomechanical mechanisms by which they influence the reproductive function differs amongst the phytoestrogens and amongst animal species (*Rosselli et al., 2000*) and as a result, it is difficult to predict the exact effects of exposure.



Picture 1. Structure formula of 17β-estradiol and genistein, a phytoestrogen

Captive rhinos are likely exposed to phytoestrogens through their diet, which contains alfalfa and soy-based products (*Personal communication with C. Tubbs; www.sandiegozoo.org*), known to contain lots of phytoestrogens (*Kurzer and Xu, 1997; Adams, 1995*). The goal of this research is to evaluate the potential role these high levels play on the poor reproduction of captive rhinos. In particular the goal was to compare the relative binding capacity to estrogen receptors of phytoestrogens derived from native browse of free-ranging white rhinos with phytoestrogens extracted from captive rhino diets. To compare this, we first determined the relative amount of different grass species that free-ranging white rhinos forage on. In addition, the phytoestrogen content of these grasses was estimated by evaluating their binding capacity on estrogen receptors produced using an intro assay. In collaboration with the San Diego Zoo we will compare these results with the binding capacity of phytoestrogens present in captive rhino diets.

PURPOSE OF THE RESEARCH PROJECT

- To identify the different grass species eaten by southern white rhinos in Lapalala Wilderness
- To determine relative consumption of different grasses by Lapalala southern white rhinos
- To evaluate differences throughout the observed study period in grass foraging
- To collect samples of consumed grasses and extract phytoestrogens from the samples.
- To test the binding capacity of these extracts on in vitro produced white rhinoceros estrogen receptors.

- To compare phytoestrogen content in native browse of the free-ranging rhino to captive feeds
- To quantify differences throughout the observed study period in native browse phytoestrogen content

METHODS AND MATERIALS

Study site and animals

All foraging studies and grass collections occurred at Lapalala Wilderness in South Africa. Lapalala Wilderness is situated near the town of Vaalwater in Limpopo Province (*maps.google.com*) and is a part of the Waterberg mountainous area. Lapalala Wilderness covers approximately 36000 ha and is the largest private game reserve in South Africa. The reserve has an estimated annual rainfall of 500 mm with a mid-summer (January-February) seasonality (*Lapalala Wilderness masterplan, 2004*) This research took place in the months September-November 2009. The rainy period started in October 2009.

The reserve currently has a population of approximately 48 white rhinoceroses and 18 black rhinoceroses, and the animals are closely monitored and managed (*Lapalala Wilderness masterplan, 2004*). Lapalala Wilderness is currently closed for tourism because of landclaims over a large portion of the area (*www.lapalala.com*). As a consequence the animals can live freely with minimal human interaction. Conservation of rare and endangered animals has been included in the original mission statement of Lapalala Wilderness established in 1981, and as stated in the annual reports of 2007 and 2008 (*Lapalala annual report 2007; 2008*), Lapalala is willing to establish research programmes with external organizations like the Institute for Breeding Rare and Endangered African Mammals (IBREAM). This research is one of those programmes and was approved by dr. Anthony Roberts. In this research four female white rhinos (Grikie, Radimpe, Munyani and Mokibelo) were observed on a regular basis (each individual approximately twice a week) in the period of August 2009-December 2009, with the help of an experienced tracker, employed by IBREAM specifically for this purpose. The results of two of the rhinos (Munyani and Mokibelo) are put together, as they were almost always found together, foraging on the same spot. The tracking was done from a jeep and by foot by using 'spoor-tracking' from the roads or in the field and visual ID.

Picture 2. Rhino spoor on the road

Picture 3. Munyani found sleeping

Grass collection

Each individual rhino was located at least once a week in the early morning or late afternoon, when they were most active (*Owen-smith 1973; 1988*). When a rhino was found and we were able to stay close by, the animal was observed for one hour to define its grazing behavior. On t=0, t=30 min and t=60 min we determined whether or not the rhino was foraging and if so, the area where it had been foraging was

marked visually using landmarks like trees, shrubs and rocks (*Shrader, 2003*). When the rhino was moved away from the spot on a safe distance, we looked closer and tried to determine what grass species it was grazing on. In our original protocol a "grazed species" was defined as one that a rhino eats for 10 consecutive bites, a method also used in previous studies (*Shrader, 2003*). In the field this proved to be hard, if not impossible, because often the rhino was at a big distance during observations, which resulted in a poor vision of the rhino's mouth. Due to the low level of experience of the researchers in discerning feeding patches and specifically distinguishing 10 consecutive bites, we determined only one species on every spot of which we were sure it had been grazed on. Samples of the entire plant were taken from the grass species for identification at base-camp. Identification of the grass species was done by distinguishing characteristics of the plants such as stems, leaves and reproductive structures like seeds and flowers using the *"Guide to Grasses of Southern Africa" (Van Oudtshoorn, 1999).* During all the observations we documented the date and time of collection, the rhino which was grazing on it, the grass-species, and the area of collection. For further analysis of the grazed species, we collected other samples, a plastic zipper bag was filled up with plant material from above the soil (at least 5-10 g). Each grass species that had been eaten was sampled at least once a month. The samples of the grasses were documented with a code including date, time of collection, the area of collection and the grass-species and stored at -20°C in plastic zipper bags back at base-camp. After completing the collection period, the samples were transported by car to the laboratory of Onderstepoort in a cooler box for further preparation.

Phytoestrogen extraction

At Onderstepoort, the grass samples were dried in an oven at 50°C for at least 24 hours. Some samples needed to be dried longer to make sure they were completely dry. Once the samples were completely dry, they were cut into a fine powder using a Typhoon wooden block and a cutting knife. The pulverized samples were then sieved using a small kitchen-sieve, and a total amount of 1 gram of the remaining powder was added to 4 mL of 100% methanol in a glass test tube (*Kimble Chase Borosilicate glass, Product no. 73500-1275*). It was covered with Parafilm and mixed on a vortex for 10 seconds. The samples were then allowed to sit overnight at room temperature. After the methanol extraction, the tubes were centrifuged for 10 minutes at 3000 x g. In addition to the centrifuging we used a metal column to push down the remaining plant material for 2-3 seconds that was still preventing us from pipetting the supernatant. Thereafter, we removed 500 µL of supernatant from each sample with a pipette and transferred this into microcentrifuge tubes (*1,5 mL, Eppendorf Microcentrifuge Safe-Lock tubes*). Tubes were then left open in a fume hood for a couple of days to dry. After they were dried completely, they were sealed and sent in a package by mail to the San Diego Zoo's Institute for Conservation Research (CRES) for further analysis.

Picture 4. Cutting the grass on a Typhoon wooden block with a cutting knife

Sample testing on estrogen receptors in vitro

Upon arrival at CRES, the dried plant extracts were resuspended in dimethyl sulfoxide (DMSO) resulting in a concentration of 1 g/mL of extracted dried material to DMSO. After this, the resuspended extracts were diluted in high salt TEDG buffer (400 mM KCl, 10 mM Tris, 10% glycerol, 1 mM sodium molybdate, 1,5 mM EDTA, 1 mM PMSF, and 1 mM DTT) (*Wilson et al., 2002; Rider et al., 2009*) to a final concentration of 1000 ug/mL. This was incubated in the presence of southern white rhino estrogen receptors and 0.5 nM of tritium(3H)-labled estradiol. A set of wells was incubated without any extract to determine total binding (TB) or with 100 nM non-radiolabeled estradiol to determine non-specific binding (NSB). The reactions were allowed to proceed in a 96-well plate for 20 hours at 4°C. After 20 hours, 5%:0.5% charcoal:dextran in binding buffer was added to each well and incubated for 5 min at 4°C. Plates were then centrifuged at 1000 x g for 5 minutes at 4°C to separate bound 3H-estradiol from free. 50uL of supernatant (containing receptor bound to 3H-estradiol or extract) was removed from each well and placed in a scintillation counter. The amount of radioactivity in the supernatant is inversely proportional to how well each extract binds to the receptor.

Specific binding (SB) of 3H-estradiol was calculated by subtracting the radioactivity in the NSB wells from the total radioactivity bound in the supernatant for each extract. The percentage displacement for each extract was determined by dividing the SB of the TB wells (TB-NSB) by the SB of the extract wells (extract-NSB).

Statistical Analysis

All data are presented as means±standard error of measurement. Significant differences (*=$p<0.05$) in estrogen receptor binding compared to 100nM estradiol treatment were determined using GraphPad Prism software (San Diego, CA) using a one-way ANOVA and Dunnett's multiple comparison post-test.

RESULTS

Grass species eaten

With a total of 200 observations, southern white rhinoceros were observed eating 28 different grass species (Table 1). Only once during the entire research period we observed a rhino eating a shrub species. We found during the period from the 28th of August 2009 to the 13th of November 2009, that 30,00 % of the diet consisted of Digitaria eriantha, 14,50 % of Cynodon dactylon and 8,50% of Panicum maximum. Fig. 1 shows relative consumption of the species during different months. In all three months Digitaria eriantha was the most consumed grass species. The relative amount increased over the months. The relative amount of Cynodon dactylon decreased.

We also found that individual rhinos prefer different species of grass (Fig. 2). For example, Grikie's diet consisted of 55,10% Digitaria eriantha, whilst the diet of Munyani and Mokibelo showed a lot more variation and only consisted of that specific species for 7,14%. Radimpe's diet contained 42,55% of Digitaria eriantha, but also 23,40% of Cynodon dactylon. Munyani and Mokibelo showed 17,86% of Cynodon dactylon, but we never observed Grikie eating Cynodon dactylon.

Picture 5. Panicum maximum

Species identified eating	Percentage
Aristida Congesta subsp. Barbicollis	2,50%
Aristida Congesta subsp. Congesta	1,00%
Aristida Stipitata	0,50%
Chloris Virgata	0,50%
Cynodon Dactylon	14,50%
Digitaria Eriantha	30,00%
Digitaria Longiflora	1,50%
Elionurus Muticus	0,50%
Enneapogon Cenchroides	1,50%
Enneapogon Scoparius	3,50%
Eragrostis Chloromelas	0,50%
Eragrostis Curvula	1,00%
Eragrostis Gummiflua	1,00%
Eragrostis Lehmanniana	2,50%
Eragrostis Trichophora	0,50%
Heteropogon Contortus	2,00%
Hyparrhenia Hirta	0,50%
Hyperthelia Dissoluta	1,00%
Loudetia Simplex	3,00%
Melinis Nerviglumis	0,50%
Melinis Repens	0,50%
NO ID/ different species	3,50%
not foraging	11,50%
Panicum Coloratum	0,50%
Panicum Maximum	8,50%
Schizachyrium Jeffreysii	0,50%
Setaria Lindenbergiana	0,50%
Setaria Sphacelata var. Sphacelata	4,00%
SHRUB	0,50%
Trachypogon Spicatus	0,50%
Urochloa Mosambicensis	1,00%
Total	100,00%

Table 1. Relative consumption of different grass species in the period from the 28th of August 2009 to the 13th of November 2009

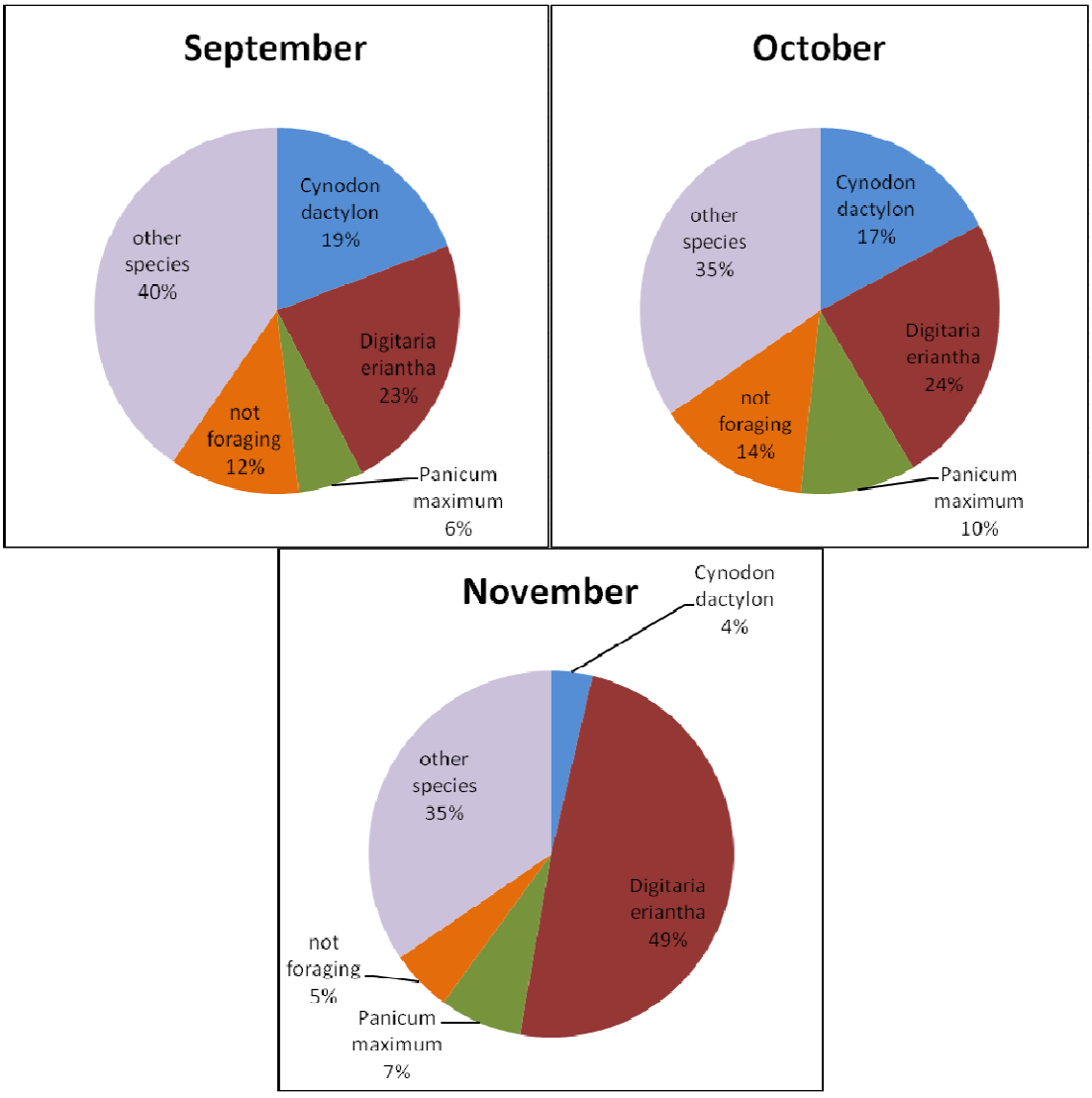


Figure 1. Relative consumption of grass species in September 2009, October 2009 and November 2009

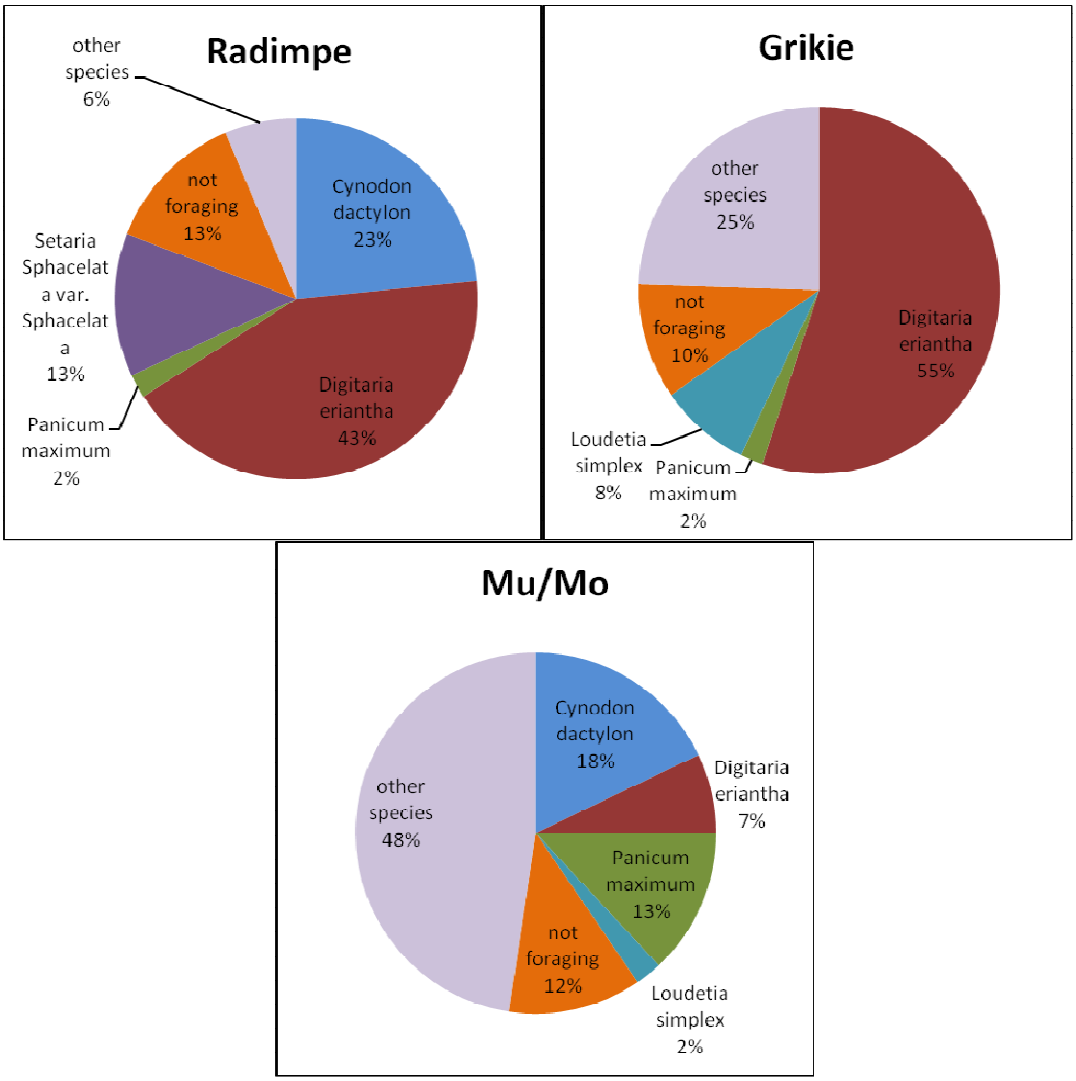


Figure 2. Relative consumption of grass species in the period from the 28th of August 2009 to the 13th of November 2009 by Radimpe, Grikie and Munyani/Mokibelo

Phytoestrogen binding capacity

During the entire research period we collected a total of 54 samples (Appendix I), which we processed in the laboratory, following the protocol for phytoestrogen extraction as described before. Because of financial and organizational issues, for my research project we were only able to test a few of these samples using the estrogen receptor binding assay. Specifically, we tested three samples of three grass species, Digitaria eriantha, Cynodon dactylon and Panicum maximum. These species were selected based on the fact that they compromised the highest proportion of the rhino diets in this study. One sample per month (September, October and November), thus a total of nine samples were tested. In addition we also tested samples of Panicum maximum, Urochloa mosambicensis, Digitaria longiflora and Aristida congesta. We chose three of these species (P. maximum, U. mosambicensis, and D. longiflora) based on them comprising the highest proportion (~25-30%) of white rhino diets in previously published reports (*Owen-Smith, 1973*).

The percentage of displacement was tested and as we can see in Figure 4, the binding varies between 0 and around 21 %. Two of the three samples of D. eriantha and P. maximum did not have sufficient quantities of phytoestrogens to displace any of the radio labeled estradiol from the receptors.

C. dactylon showed a percentage of displacement in all three the samples.
Extracts of alfalfa (which is a component of captive rhino diets and known to contain high concentrations of phytoestrogens) showed a relative binding to the rhino estrogen receptors of approximately 100%. On the contrary, the relative binding of the grass species collected in Lapalala wilderness varied between 0 and 21% (Fig. 3, Fig. 4).

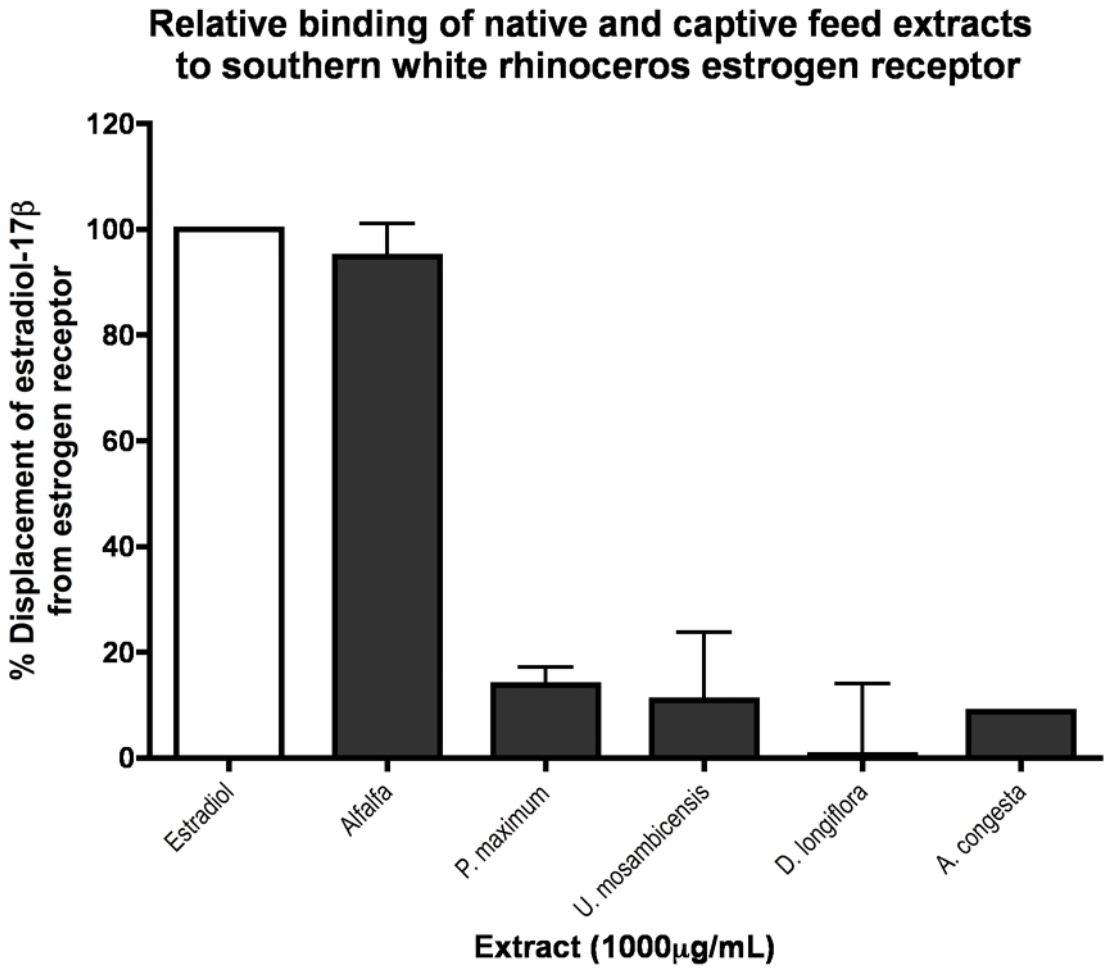


Figure 3. Relative binding of estradiol, alfalfa (captive feed extract) and 4 different grass species (native feed extracts) to southern white rhinoceros receptor

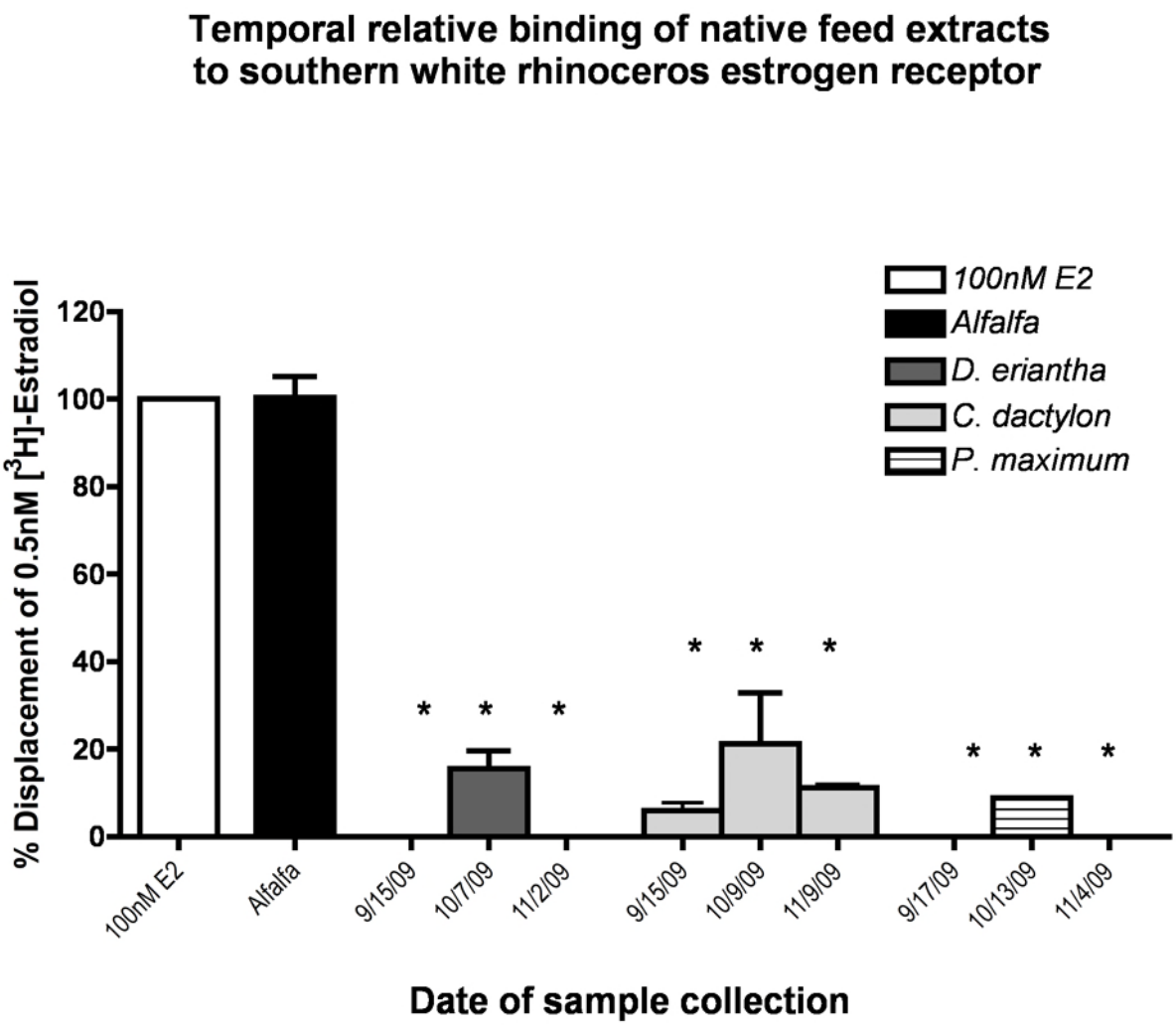


Figure 4. Relative binding of 3 different grass species in 3 different months to southern white rhinoceros estrogen receptor

DISCUSSION

This study suggests that the diet of wild foraging white rhinos differs per individual. The diet of a wild foraging rhino likely depends on the availability of different grass species in the home range where the rhino is foraging. Other circumstances influence the choice of the rhino as well. The availability of grass species for example can differ between seasons (*Pedersen, 2009*). As the dry season progresses, the availability and quality of grasslands declines and the rhino tends to forage in high-quality short and woodland grasslands (*Shrader et al., 2006*), whilst as the start of the wet season rhinos feed more on regrowth of formerly burnt grasslands (*Shrader et al., 2006*). Shrader also states that as the dry season progresses, white rhinos become less selective and they utilize a wider variety of feeding areas and grass species (*Shrader, 2003*). The density of a rhino population is also likely to have an influence (*Shrader, 2003*). Vegetation varies in different areas. According to Owen-Smith (*1988*) during dry season white rhinos occupy sweetveld areas where the soil nutrient-rainfall

combination causes grasses to build up only moderate levels of indigestible fiber in their leaves. In sourveld areas where grasses are more fibrous and less nutritious during the dry season, white rhinos feed mostly in those areas of the landscape where soil nutrients accumulate, for example around termitaria and along the margins of drainage sump grasslands.

In this research we found a variety in the grass species the rhinos seemed to prefer in the different months we observed them, but it is difficult to say how exactly this difference in results is caused. First of all we only followed four rhinos, which means that they all have a big influence on the total results. For example the decrease of amount of Cynodon dactylon eaten in November, can maybe be explained by the fact that Radimpe was not found on the big field anymore. The field contained a water hole and the dominating grass species was Cynodon dactylon. In November water was available in the whole area, which made the rhinos stay more in the bush, where other grasses might be more present. As described in *Shrader (2003)* the choice of different grasslands is influenced by the seasonal availability. Also he states that white rhinos select high quality food when it's available.

The results clearly show a big difference between the relative binding of native and captive feeds to estrogen receptors of white rhinos. The phytoestrogens present in alfalfa bind almost 100% of the receptors, whilst the phytoestrogens in the grass species bind only 0 to 20%. The amount of phytoestrogens in the grass species we tested, doesn't show a big difference between the different species. That suggests that for the further comparison of native and captive feeds, it is of less importance which particular species is used, as long as the rhino is actually foraging on it and it is part of the native pasture.

From this research it is difficult to see a seasonal influence on the amount of phytoestrogens in the grasses. Because there is no clear pattern to discover over the months and all the phytoestrogen concentrations stay below 20% binding capacity, it is likely to assume that there is no significant seasonal difference in phytoestrogen content. This also supports the observation that the wild feeds do not contain high concentrations of phytoestrogens at all. There is a number of things we should keep in mind though. First of all the samples were not taken on the same spot every month. There might be other influences on the amount of phytoestrogens then only the species, for example the soil type. During the processing of the samples for extraction, the grasses were cut by hand using a wooden block and a cutting knife. There was no equipment available to do this in a more homogenous way. Furthermore we didn't collect grasses in the remaining part of the year (December-July), so there is still the possibility that the amount of phytoestrogens is higher in other periods of the year.

CONCLUSION AND FUTURE DIRECTIONS

From our findings we can conclude that the phytoestrogens are present in captive diets presenting higher concentrations than in the grasses wild rhinos forage on. This conclusion is supported by the findings that there is no significant seasonal difference in phytoestrogen concentrations and no significant difference between the different grass species. In all cases there is a significant difference between the native and captive feeds.

This combined with the fact that the reproduction rate of the wild white rhino population is much higher than that of captive population (*Emslie and Brooks, 1999*), and moreover the fact that exposure

to phytoestrogens is known to impair reproduction in several different species (*Adams, 1995; Woclawek-Potocka et al., 2005; Whitten et al., 2001*), makes it impossible to rule out the high levels of phytoestrogens in captive feeds as a possible cause for their disappointing reproductive results. This possibility needs to be further supported and from this perspective, the next step will be to find out the direct effects of phytoestrogens binding the rhino estrogen receptors.
Suggestions for further research are to do more nutritionally focused research on the diet of wild southern white rhinos. Since there was a noticeable difference in food preference between the individuals, it would be useful to find out why. Is it because of a different vegetation in the home-range of each rhino? And if there is, is it possible to define the preferred grass species for every type of vegetation?
Another, maybe more reliable way of determining the diet of wild foraging white rhinos is by dung analysis. By micro-histological analysis of undigested plant material in fecal samples (*Holechek et al., 1982; Maia et al., 2003; Chetri, 2006; Pedersen, 2009*) we can determine which grass species are part of the diet. Another often used method (*Walker et al., 1998, 2002; Landau et al., 2004;*) to make a detailed profile of the diet is the use of faecal near infrared spectroscopy (F.NIRS).

Furthermore, if we want more reliable information about the seasonal influences on the amount of phytoestrogens, we could collect a sample of all preferred grass species every month, for at least one year. Now we only collected for 3 months, so we cannot say anything about the rest of the year. We should try and rule out as many variables as possible. For example, the samples could be taken on the same spot every time, we should also try to find a way to cut the grass even more to a powder.

ACKNOWLEDGEMENTS

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Picture 6. Thomas Litshani observing Munyani

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Appendix I

Sample:	Sample code:	Scientific names:
tube		
1	**0914E00OH**	NO ID
2	**0914E30OH**	Digitaria Longiflora
3	**0914E60OH**	Eragrostis Gummiflua
4	**0915E60OH**	Digitaria Eriantha
5	**0915E00OF**	Cynodon Dactylon
6	**0916E00ML**	Aristida Congesta subsp. Barbicollis
7	**0916E30ML**	Aristida Congesta subsp. Congesta
8	**0916E60ML**	Aristida Congesta subsp. Congesta
9	**0917E00DL**	Panicum Maximum
10	**0917E30DL**	Chloris Virgata
11	**0917E60DL**	Panicum Maximum
12	**0922E00OF**	Aristida Stipitata
13	**0924E00RR**	Eragrostis Curvula
14	**0924E30RR**	Digitaria Longiflora
15	**0928E00OH**	Digitaria Eriantha
16	**0928E00RR**	Cynodon Dactylon
17	**0930E00RC**	SHRUB
18	**0930E30RC**	Eragrostis Lehmanniana
19	**1001E30OF**	Cynodon Dactylon
20	**1001E60OF**	Eragrostis Chloromelas
21	**1006E00OH**	NO ID: Different species
22	**1006E60OH**	Loudetia Simplex
23	**1006E00RC**	Aristida Congesta subsp. Barbicollis
24	**1006E60RC**	Heteropogon Contortus
25	**1007E30OF**	Digitaria Eriantha
26	**1008E30OH**	Loudetia Simplex
27	**1008E60OF**	Cynodon Dactylon
28	**1009E00RR**	Cynodon Dactylon
29	**1012E30RR**	Hyperthelia Dissoluta
30	**1012E60RR**	Eragrostis Lehmanniana
31	**1013E00RC**	Panicum Maximum
32	**1013E30RC**	Setaria Lindenbergiana
33	**1014E00HW**	Loudetia Simplex
34	**1015E00RC**	Eragrostis Curvula
35	**1015E30RC**	Eragrostis Lehmanniana
36	**1015E60RC**	Heteropogon Contortus
37	**1019E00DC**	Urochloa Mosambicensis
38	**1020E00HW**	Digitaria Eriantha
39	**1020E60HW**	Enneapogon Cenchroides
40	**1026E00RC**	Enneapogon Scoparius
41	**1027E00RC**	Loudetia Simplex
42	**1027E30RC**	Panicum Maximum
43	**1028E00RC**	Enneapogon Scoparius
44	**1102E00HE**	Digitaria Eriantha
45	**1102E30OF**	Digitaria Longiflora
46	**1104E00OFa**	Setaria Sphacelata var. Sphacelata
47	**1104E00OFb**	Panicum Maximum
48	**1105E00HE**	Schizachyrium Jeffreysii
49	**1109E00RR**	Heteropogon Contortus
50	**1109E60RR**	Cynodon Dactylon
51	**1111E00EQ**	Hyperthelia Dissoluta
52	**1111E30EQ**	Urochloa Mosambicensis
53	**1111E60aEQ**	Melinis Repens
54	**1111E60bEQ**	Eragrostis Trichophora