Spinescence and Total Phenolic Content Do Not Influence Diet Preference of a Critically Endangered Megaherbivore, but the Mix of Compounds Does

Peter F. Scogings[1] · Stuart Demmer[1,2] · Dawood Hattas[3]

Abstract

In contrast to understanding spinescence in savanna woody species, little is known about the functions of plant secondary metabolites (PSM). Negative effects of PSMs on individual animal performance potentially translate into negative effects on herbivore population growth. Hence, understanding PSM functions is important for the conservation of savanna megafauna. We tested the view that black rhinoceros (*Diceros bicornis*) diet preference is not affected by spinescence or total phenolic abundance. We hypothesized that the composition of phenolic mixtures, however, would affect preference. Furthermore, we tested our data from 71 woody species for a trade-off between structural and chemical defenses. Spinescence type, and spinescence generally, did not deter black rhino feeding. Using eco-metabolomic data, we found that total abundance of phenolics did not affect preference, but mixture composition did and that the probability of spinescence trading off against phenolics depended on the mixture. We note that our study was restricted to black rhino and that diet preferences of other mammal herbivores might be influenced by subtle differences in phenolic mixtures. However, our results did support a previous, more detailed study of phenolic profiles of six species showing the same patterns in relation to preference generalised across mammal herbivore species in savannas. Our results represent substantial advancement in the understanding of the roles of PSMs, especially flavonoid compounds, in the functioning of savanna ecosystems, and highlight the need to dig deeper into broad groups of traits such as spinescence or total phenolics to improve understanding of woody plant defenses in savannas.

Keywords *Diceros bicornis* · Eco-metabolomic · Flavonoid · Plant defense · Savanna

Introduction

Among woody species in African savannas, the ecological functions of structural traits, such as spinescence and dense branching, are more thoroughly understood than those of

✉ Peter F. Scogings
scogingsp@ukzn.ac.za

Stuart Demmer
dstuart99@gmail.com

Dawood Hattas
dawood.hattas@uct.ac.za

[1] School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa

[2] Centre for Functional Biodiversity, University of KwaZulu-Natal, Scottsville, South Africa

[3] Department of Biological Sciences, University of Cape Town, Rondebosch, South Africa

chemical traits, such as phenolic compounds. Structural traits are effective defenses against large mammal herbivores (LMH) that are browsers or mixed-feeders (Charles-Dominique et al. 2020; Wigley et al. 2019). Such traits affect diet preference and restrict short-term feeding rates (g min^{-1}) of several browsing mesoherbivore species (adult body mass < 1000 kg) (Basha et al. 2012; Cooper and Owen-Smith 1986; Fomum et al. 2015; Mkhize et al. 2014; Scogings et al. 2004; Skarpe et al. 2012; Wilson and Kerley 2003a). However, mixed-feeding megaherbivore species (adult body mass > 1000 kg), e.g. African elephant (*Loxodonta africana*), are not deterred by spines but may be deterred by other structural traits such as springy branches (Owen-Smith et al. 2020; Scogings and Sankaran 2020).

Spinescence encompasses several morphological structures of different origin and function (Bell 1991; Hanley et al. 2007), including spines (modified stipules in pairs at nodes), thorns (modified stems that often have leaves), and prickles (modified epidermis, typically recurved or hook-like). Spines are the most effective of these types against LMH in

savannas (Dziba et al. 2003; Skarpe et al. 2012; Wilson and Kerley 2003b), yet studies of spinescence in savanna woody species tend to ignore the different types (e.g. Charles-Dominique et al. 2016; Tomlinson et al. 2016; Wigley et al. 2018).

Contrary to structural traits, ecological functions of chemical traits, especially phenolic compounds, are poorly known for savanna woody species. Overall animal performance could be negatively affected by delayed feedback from ingested secondary metabolites (Scogings et al. 2004; Skarpe et al. 2007), such as phenolics that reduce browse digestibility and reduce long-term intake rate (kg day^{-1}) (Basha et al. 2013; Mkhize et al. 2015). Negative effects of digestibility-reducing compounds on individual performance potentially translate into negative effects on population growth (DeGabriel et al. 2014; McArt et al. 2009). Phenolics have little effect on diet preference or feeding rate of mesoherbivores (Basha et al. 2012; Fomum et al. 2015; Mkhize et al. 2014), but their effect depends on herbivore species and chemical concentration (Cooper and Owen-Smith 1985). While the effects of phenolics on diet preference are reasonably well known for domestic goats (*Capra hircus*) in savannas, little is known about their effect on diet preference of native savanna LMH. Elephants are selective in the species they eat, but the effects of phenolics on their diet preference are unclear (Owen-Smith et al. 2020), while volatile organic compounds are important (McArthur et al. 2019; Schmitt et al. 2018). Hence, developing a deeper understanding of plant secondary metabolite (PSM) functions in relation to savanna LMH is an important avenue of research, especially for the conservation of endangered megafauna such as black rhinoceros (*Diceros bicornis*) because the conservation of endangered species depends on the provision of adequate nutrition (Gyöngyi and Elmeros 2017).

Similar to studies of spinescence, studies of chemical traits in relation to savanna LMH have largely ignored variation within the traits, which could account for an unclear understanding of their ecological functions. Typically, such studies only considered very broad groups of compounds, such as condensed tannins or total phenols, quantified by very general assays (e.g. Gowda et al. 2019; Scogings et al. 2017; Wigley et al. 2019). Recently, however, it has been highlighted that such approaches have limited predictive abilities because of the diversity of compounds that constitute broad groups (DeGabriel et al. 2014; Marsh et al. 2020). Moreover, it is known that no plant species share the same mixture of compounds, although some species' mixtures are dominated by the same compounds, while other species' mixtures are dominated by entirely different compounds (Hattas et al. 2011; Salminen and Karonen 2011). Thus, exploring similarities and dissimilarities among species' metabolite mixtures (profiles) contributes greatly to understanding ecological interactions experienced by those species (Peters et al. 2018). However, few studies of savanna woody species have used the

eco-metabolomic approach and those that have demonstrated the benefits of quantifying individual compounds in mixtures over simply quantifying the total size of mixtures (e.g. Hattas et al. 2011, 2017). Hattas et al. (2011) suggested that condensed tannin and total phenol contents are unrelated to the palatability of plants to savanna LMH, but certain subgroups of flavonoid compounds (e.g. flavones) were characteristic of preferred species, while other compounds (e.g. flavonols) were characteristic of less preferred species.

Structural and chemical defense traits are widely purported to trade off against one another among savanna woody species, but tests for the existence of the trade-off are few and the evidence is ambiguous (Tomlinson et al. 2016; Wigley et al. 2018). The basis for expecting the trade-off is finite resource availability, especially carbon for allocation among different defenses (Moles et al. 2013; Moreira et al. 2014). On the assumptions that spinescence is dependent on growth and is a cheap defense because their anti-herbivory benefit outweighs their production and maintenance costs (Charles-Dominique et al. 2020; Midgley and Ward 1996), and preferred species are faster growing and invest in cheap defenses (Bryant et al. 1983; Coley et al. 1985; Herms and Mattson 1992), then preferred species are more likely to be spinescent, and rejected species defended with PSM. Furthermore, phenolic-rich, non-spinescent species rejected by LMH are more likely to occur in mesic (> 650 mm mean annual rainfall, MAR) savannas dominated by broad-leaved species (as opposed to fine-leaved species bearing bipinnate microphyllous leaves), because soils are leached and insect herbivory is more abundant than mammal herbivory (Tomlinson et al. 2016; Wigley et al. 2018). Conversely, spinescence, which has been shown to have evolved in African savannas around the time that the dominant LMH taxa appeared in Africa (Charles-Dominique et al. 2016), is characteristic of semi-arid savannas (< 650 mm MAR) dominated by 'palatable' fine-leaved species, where mammal herbivory is abundant (Tomlinson et al. 2016; Wigley et al. 2018). Among savanna woody species, Tomlinson et al. (2016) found spinescent species have lower leaf mass fractions, while other species have lower specific leaf areas, which they attributed to lower C:N ratios in the former and higher C:N ratios in the latter species, hence supporting the distinction between preferred-and-spiny vs rejected-and-unspiny strategies. Like Tomlinson et al. (2016), Wigley et al. (2018) found spinescent species had higher nitrogen than other species, making them potentially more preferred, but otherwise, they found no direct evidence for either a trade-off between phenolics and spinescence or any association with soil fertility.

The black rhino is a critically endangered megaherbivore species in African savannas (Ferreira et al. 2017). Because plant defenses potentially have negative impacts on herbivore population dynamics (DeGabriel et al. 2014; Illius et al. 2002), developing a better understanding of its feeding

behavior and the effects of different defenses is critical for its conservation (Gyöngyi and Elmeros 2017). Black rhino feed by biting twigs, including spinescent ones, and include plant species known to be tannin-rich, yet they select the species they eat (Buk and Knight 2010; Duthé et al. 2020; Ganqa et al. 2005; Kingdon et al. 2013; Landman et al. 2013; Muya and Oguge 2000; van Lieverloo et al. 2009). Black rhino are expected to tolerate plant species of high spinescence or low digestibility because of their large body size and mouthparts (Codron 2020; Kingdon et al. 2013; Owen-Smith and Chafota 2012; Steuer et al. 2014; Wilson and Kerley 2003b), but factors affecting their selectivity are unknown.

Therefore, we aimed to develop a better understanding of structural and chemical factors affecting diet preference of black rhino and to determine if we could detect a trade-off between spinescence and phenolics. First, we tested the general view that black rhino diet preference is not affected by spinescence of any type or total abundance of phenolics. We analyzed the effects of spinescence (in general and by type) and total abundance of phenolics (determined by high-performance liquid chromatography; HPLC) on black rhino preference among 71 woody species. Second, we hypothesized that the composition of the phenolic mixtures would affect preference, and tested this by analyzing the effects of phenolic compound diversity, richness, and evenness on diet preference and by analyzing dissimilarities of phenolic mixtures among preference classes. Lastly, we tested our eco-metabolomic data for a trade-off between structural and chemical defenses, and for relationships or associations between the type of defense and leaf type (broad vs fine) or geographical distribution (semi-arid vs mesic).

Methods and Materials

Each woody species (Table 1) was classified as having spines, thorns, prickles, or nothing, according to Coates Palgrave (2002). Pooled leaf samples were collected from three plants of each species that was considered to be an important component of the vegetation in terms of rhino diet choice in five protected areas holding black rhino populations in southern Africa (i.e. no species was sampled in more than one protected area). The protected areas were Etosha National Park, Waterberg Plateau National Park, Great Fish River Reserve, Tembe Elephant Park, and Hluhluwe-iMfolozi Park, which span a gradient of MAR of 400–900 mm. All species were sampled in one growing season (December 2008 to March 2009), except that *Acacia natalitia*, *Balanites maughamii* and *Catunaregam obovata* were sampled in November 2010. Upon collection, samples were dried over silica gel for three days and then transferred to storage bottles. Dry samples were ground to a fine powder in a ball mill (Retsch GmbH, Germany). Each species' profile of phenolic

compounds was obtained by HPLC according to Hattas et al. (2011) but without mass spectrometry. The method is optimized for detection of low-molecular-weight (LMW) phenolics, including hydrolyzable tannins (gallotannins and ellagitannins), flavonols (e.g. myricetin and quercetin), flavones (e.g. apigenin and luteolin), flavanols (flavan-3-ols, e.g. catechin and gallocatechin), flavanones (e.g. eriodictyol and naringenin), flavanonols (dihydroflavonols, e.g. dihydromyricetin and dihydroquercetin) and derivatives. Flavanols are the polymeric units of condensed tannins (CT), which have higher molecular weights and are known for their digestibility-reducing properties (Routaboul et al. 2012). Although our HPLC method does not permit quantification of CT, flavonol content is a predictor of CT content in some African savanna woody species included in our sample (Scogings et al. 2014), implying that flavonol and flavanol abundances may be useful indicators of CT content.

Quantification of phenolic compounds was by peak integration at 270 nm wavelength using Agilent ChemStation for LC 3D systems, Rev. B.04.03. Only peaks at retention times (Rt) > 5.0 min were included because peaks at shorter Rt were not readily detected at 270 nm, which meant that hydrolyzable tannins (gallotannins and ellagitannins) were largely excluded from this analysis. However, as far as we know these are not abundant in savanna woody species (Hattas et al. 2011). Individual compounds were not identified because our objective was broadly to determine if total phenolic content, indexed by the total area of peaks, or composition of phenolic mixtures, indexed by Rt, differed among preference classes. Moreover, preliminary attempts to identify compounds using an in-house library revealed many unknown compounds.

Published data on black rhino preference are scarce because the elusiveness of the species makes observation difficult. Therefore, we classified plant species as preferred, neutral, or rejected, based primarily on expert opinion (African Rhino Specialist Group) and supported by limited published information (Buk and Knight 2010; Duthé et al. 2020; Ganqa et al. 2005; Landman et al. 2013; Muya and Oguge 2000; van Lieverloo et al. 2009). The published information was used mainly for moderating the expert opinion to derive generalized ranks. There was high concordance in ranks among 34 species that were included in our study and at least one other study, and no species was changed from rejected to preferred or *vice versa*. Of the 71 species, 30 were classed as preferred, 19 as neutral, and 22 as rejected.

Statistical analyses were conducted in R version 3.5.2 (R Core Team 2018). We modeled the presence and absence of spinescence types using the "glm" function from the "stats" package with residuals modeled using the binomial distribution and logit link functions to ensure proportional fits. Because plant spinescence is varied, we modeled the probability of possessing spinescence across preference classes, where spinescence type was prickles, spines, or thorns.

Table 1 Woody plant species sampled for phenolic profiling by HPLC, their spinescence and preference by black rhino, distribution in semi-arid or mesic savannas, and their leaf morphology and phenology

Species	Family	Spinescence	Preference	Distribution	Leaf morphology	Leaf phenology
Acalypha glabrata	Euphorbiaceae	None	Preferred	Mesic	Broad	Not determined
Albizia versicolor	Mimosoideae	None	Rejected	Mesic (mostly)	Broad	Deciduous
Azima tetracantha	Salvadoraceae	Thorns	Neutral	Widespread	Broad	Evergreen
Balanites maughamii	Zygophyllaceae	Thorns	Rejected	Mesic	Broad	Evergreen
Bauhinia petersiana	Caesalpinioideae	None	Rejected	Semi-arid (mostly)	Broad	Evergreen
Boscia albitrunca	Capparaceae	None	Neutral	Semi-arid	Broad	Evergreen
Brachylaena ilicifolia	Asteraceae	None	Rejected	Semi-arid (mostly)	Broad	Evergreen
Burkea africana	Mimosoideae	None	Rejected	Widespread	Broad	Deciduous
Capparis sepiaria	Capparaceae	Prickles	Neutral	Widespread	Broad	Evergreen
Catunaregam obovata	Rubiaceae	Thorns	Preferred	Mesic	Broad	Not determined
Coddia rudis	Rubiaceae	None	Neutral	Mesic	Broad	Deciduous
Colophospermum mopane	Caesalpinioideae	None	Rejected	Semi-arid	Broad	Deciduous
Combretum apiculatum	Combretaceae	None	Neutral	Semi-arid (mostly)	Broad	Deciduous
Combretum collinum	Combretaceae	None	Rejected	Widespread	Broad	Deciduous
Combretum psidioides	Combretaceae	None	Rejected	Widespread	Broad	Deciduous
Commiphora neglecta	Buseraceae	Thorns	Preferred	Widespread	Broad	Deciduous
Croton menyharthii	Euphorbiaceae	None	Rejected	Semi-arid (mostly)	Broad	Not determined
Dichrostachys cinerea	Mimosoideae	Thorns	Preferred	Widespread	Fine	Deciduous
Diospyros lycioides	Ebenaceae	None	Rejected	Widespread	Broad	Deciduous
Dombreya rotundifolia	Sterculiaceae	None	Preferred	Widespread	Broad	Deciduous
Ehretia rigida	Boraginaceae	None	Neutral	Widespread	Broad	Deciduous
Euclea crispa	Ebenaceae	None	Rejected	Widespread	Broad	Evergreen
Euclea divinorum	Ebenaceae	None	Rejected	Widespread	Broad	Evergreen
Euclea natalensis	Ebenaceae	None	Rejected	Mesic (mostly)	Broad	Evergreen
Euclea undulata	Ebenaceae	None	Neutral	Semi-arid (mostly)	Broad	Evergreen
Flueggea verrucosa	Euphorbiaceae	None	Neutral	Semi-arid	Broad	Deciduous
Gnidia anthylloides	Thymelaeaceae	None	Rejected	Not determined	Broad	Not determined
Grewia avellana	Tiliaceae	None	Preferred	Semi-arid (mostly)	Broad	Deciduous
Grewia bicolor	Tiliaceae	None	Preferred	Widespread	Broad	Deciduous
Grewia flava	Tiliaceae	None	Preferred	Semi-arid	Broad	Deciduous
Grewia flavescens	Tiliaceae	None	Preferred	Semi-arid (mostly)	Broad	Deciduous
Grewia retinervis	Tiliaceae	None	Preferred	Semi-arid	Broad	Deciduous
Grewia robusta	Tiliaceae	None	Preferred	Semi-arid	Broad	Deciduous
Grewia villosa	Tiliaceae	None	Neutral	Semi-arid	Broad	Deciduous
Gymnosporia capitata	Celastraceae	None	Neutral	Semi-arid	Broad	Evergreen
Gymnosporia glaucophylla	Celastraceae	None	Neutral	Semi-arid (mostly)	Broad	Evergreen
Gymnosporia maranguensis	Celastraceae	Thorns	Neutral	Semi-arid (mostly)	Broad	Evergreen
Gymnosporia senegalensis	Celastraceae	Thorns	Rejected	Semi-arid (mostly)	Broad	Evergreen
Jatropha capensis	Euphorbiaceae	None	Preferred	Semi-arid	Broad	Not determined
Lippia javanica	Verbenaceae	None	Rejected	Mesic (mostly)	Broad	Not determined
Monechma genistifolium	Acanthaceae	None	Preferred	Not determined	Broad	Not determined
Ochna pulchra	Ochnaceae	None	Rejected	Semi-arid	Broad	Deciduous
Olea europea	Oleaceae	None	Neutral	Widespread	Broad	Evergreen
Ormocarpum trichocarpum	Papilionoideae	Thorns	Preferred	Semi-arid	Broad	Deciduous
Philenoptera nelsii	Papilionoideae	None	Rejected	Semi-arid	Broad	Deciduous
Plumbago auriculata	Plumbaginaeae	None	Preferred	Semi-arid (mostly)	Broad	Deciduous
Portulacaria afra	Portulaceae	None	Neutral	Semi-arid	Broad	Evergreen
Rhigozum obovatum	Bignoniaceae	Thorns	Preferred	Semi-arid	Broad	Deciduous

Table 1 (continued)

Species	Family	Spinescence	Preference	Distribution	Leaf morphology	Leaf phenology
Rhoicissus tridentata	Vitaceae	None	Neutral	Widespread	Broad	Deciduous
Schotia capitata	Caesalpinioideae	None	Neutral	Semi-arid	Broad	Evergreen
Sclerocarya birrea	Anacardiaceae	None	Preferred	Widespread	Broad	Deciduous
Scutia myrtina	Rhamnaceae	Prickles	Neutral	Mesic (mostly)	Broad	Evergreen
Searsia longispina	Anacardiaceae	Thorns	Rejected	Semi-arid	Broad	Evergreen
Searsia pentheri	Anacardiaceae	None	Rejected	Semi-arid (mostly)	Broad	Evergreen
Searsia tenuinervis	Anacardiaceae	None	Rejected	Semi-arid	Broad	Deciduous
Senegalia ataxacantha	Mimosoideae	Prickles	Preferred	Widespread	Fine	Deciduous
Senegalia burkei	Mimosoideae	Prickles	Preferred	Mesic	Broad	Deciduous
Senegalia caffra	Mimosoideae	Prickles	Preferred	Mesic	Fine	Deciduous
Senegalia nigrescens	Mimosoideae	Prickles	Preferred	Semi-arid (mostly)	Broad	Deciduous
Spirostachas africana	Euphorbiaceae	None	Preferred	Semi-arid	Broad	Deciduous
Terminalia sericea	Combretaceae	None	Rejected	Widespread	Broad	Deciduous
Vachellia borleae	Mimosoideae	Spines	Preferred	Semi-arid	Fine	Deciduous
Vachellia gerrardii	Mimosoideae	Spines	Preferred	Widespread	Fine	Deciduous
Vachellia grandicornuta	Mimosoideae	Spines	Neutral	Semi-arid (mostly)	Fine	Deciduous
Vachellia karroo	Mimosoideae	Spines	Preferred	Widespread	Fine	Deciduous
Vachellia natalitia	Mimosoideae	Spines	Preferred	Mesic	Fine	Deciduous
Vachellia nilotica	Mimosoideae	Spines	Preferred	Widespread	Fine	Deciduous
Vachellia reficiens	Mimosoideae	Spines	Preferred	Semi-arid	Fine	Deciduous
Vachellia robusta	Mimosoideae	Spines	Preferred	Mesic (mostly)	Fine	Deciduous
Vachellia tortilis	Mimosoideae	Spines	Preferred	Semi-arid	Fine	Deciduous
Ziziphus murcronata	Rhamnaceae	Prickles	Neutral	Widespread	Broad	Deciduous

Pairwise comparisons and probability differences to $p(x) = 0.5$ incorporating Tukey adjustments were conducted using the "emmeans" function from the "emmeans" package (Lenth, 2019).

For phenolic effects, we modeled $\log_e$-transformed total abundance of phenolics (sum of HPLC peak areas) and $\log_e$-transformed phenolic diversity where the initial diversity was greater than 0 (calculated as Shannon H′ using the "diversity" function from the "vegan" package; Oksanen et al. 2019) using the "aov" function from the "stats" package. Phenolic richness (number of peaks) of plants which contained more than one chemical species and phenolic evenness (Pilou's evenness, Shannon H′/log(richness)) were modeled using the "glm" function. Richness and evenness residuals were modeled using Poisson and binomial error distributions, respectively. To ensure positive and proportional responses we used $\log_e$ and logit link functions for richness and evenness models, respectively.

To investigate differences in mixtures of phenolic compounds in preference classes, we calculated Raup-Crick dissimilarities on phenolic compound presence/absence data between plant species using the "vegdist" function in "vegan" and using Rt as a surrogate for the compound name. The dissimilarity represents $1 - p(j)$, the probability of observing at least j shared phenolic compounds in both plant species. Only peaks with areas >5% of the total area were considered present; peaks with areas <5% were excluded from calculations. We excluded duplicate dissimilarity pairs to prevent data duplication and used the resulting dissimilarities to investigate whether species within preference classes produced similar compounds to other species within the same preference class and as well as those species in other preference classes. We did this by modeling phenolic dissimilarity differences between species by preference class comparison (i.e. rejected-rejected, rejected-neutral, rejected-preferred, neutral-neutral, neutral-preferred, preferred-preferred) using the "glmer" function in "lme4" (Bates et al. 2015). Plant species was included as a random intercept to control for repeated comparisons to other species using the same species. Residuals were modeled using a binomial error distribution. Logit link functions were used to ensure proportional responses.

To complement these dissimilarity analyses we modeled the area detected across the Rt ranges of the preference classes using the "gamm4" function in the "gamm4" package (Wood and Scheipl 2017), fitting Rt using thin-plate regression splines. Errors were modeled using a gaussian distribution. Areas were $\log_e$-transformed to improve model fit and ensure

positive responses. Species was included as a random intercept.

To detect trade-offs between structural and chemical defenses across and within preference classes, we modeled the probability that species will possess spinescence (regardless of type) as a function of preference by each phenolic variable (richness, total abundance, H′ and evenness) using the "glm" function with residuals modeled using binomial error distributions and a logit link function to ensure proportional responses. Pairwise comparisons of slopes were conducted and presented on the link scale following Tukey adjustments using the "emtrends" function. We then modeled the probability that a species within a browsing preference category would be broad-leaved vs being fine-leaved using the methodology described above for modeling spinescence probability across preference classes.

Finally, the probability of possessing physical defences in relation to five geographical distributions was assessed using the "glm" function with residuals modeled using a binomial error distribution and a logit link function to ensure proportional responses. Distribution classes were semi-arid, mostly semi-arid, widespread (occurring across semi-arid and mesic areas), mostly mesic, and mesic, converted to a numeric predictor ranging from 1 to 5. Significance was determined at $\alpha = 0.05$ for all analyses.

Results

Compared to rejected species, neutral species had equal probabilities of possessing spinescence, but preferred species had higher probabilities of possessing spinescence (LR $\chi^2_2 = 9.108$, $p = 0.011$; Fig. S1 in Online Resource 1). Neither spinescence type (LR $\chi^2_2 = 0.655$, $p = 0.721$) nor the interaction of the two predictor variables (LR $\chi^2_4 = 9.264$, $p = 0.055$, Fig. S1) revealed differences among preference classes in probabilities of possessing spinescence.

Total phenolic abundance did not differ significantly across preferences ($F_{2,66} = 2.473$, $p = 0.092$, Fig. 1a). However, phenolic richness differed significantly (LR $\chi^2_2 = 12.309$, $p = 0.002$, Fig. 1b), with preferred species possessing higher phenolic richness than neutral species whilst the number of chemical species possessed by rejected species did not differ from either neutral or preferred species. Shannon diversity ($F_{2,64} = 0.476$, $p = 0.624$, Fig. 1c) and Pilou's evenness (LR $\chi^2_2 = 0.110$, $p = 0.947$, Fig. 1d) did not differ significantly among preference classes. Phenolic compositional dissimilarities differed significantly across comparisons of preference classes ($\chi^2_5 = 33.767$, $p < 0.0001$; Fig. 2). Rejected-rejected and rejected-neutral dissimilarities were less than rejected-preferred dissimilarities. Neutral-preferred dissimilarities were greater than rejected-rejected dissimilarities. Neutral-neutral

and preferred-preferred dissimilarities were similar to all other comparisons.

Modelled phenolic profiles of all preference classes deviated significantly from a straight line (Fig. 3). Profiles of rejected and neutral classes (rejected: estimated df = 5.291, F = 5.741, $p < 0.001$; neutral: estimated df = 3.422, F = 4.599, $p = 0.0035$) peaked at Rt of 22 and 20 min, respectively, while the preferred class profile (estimated df = 5.325, F = 7.293, $p < 0.001$) peaked at 18 and 41 min.

The probability of possessing spinescence was not significantly affected by phenolic richness across all species (LR $\chi^2_1 = 0.144$, $p = 0.705$), but differed significantly across preference classes (LR $\chi^2_2 = 12.820$, $p = 0.002$). The probability of possessing spinescence across the phenolic richness gradient varied across browsing preference classes (LR $\chi^2_2 = 12.019$, $p = 0.002$; Fig. 4) with the most detectable difference in slopes occurring between neutral and preferred species (slope difference = −0.533 (−1.158, 0.092), Z-ratio = −2.000, $p = 0.112$). There was no effect of total abundance of phenolics on the probability of possessing spinescence (LR $\chi^2_1 = 0.007$, $p = 0.932$), however, spinescence possession probability differed significantly across preference classes (LR $\chi^2_2 = 12.400$, $p = 0.002$) and the interaction between preference class and total abundance was significant ($\chi^2_2 = 20.930$, $p < 0.001$; Fig. 4). The most detectable difference in slope was between neutral and preferred species (slope difference = −2.260 (−4.740, 0.211), Z-ratio = −2.144, $p = 0.081$). Shannon diversity did not predict spinescence possession probability ($\chi^2_{1,67} = 0.018$, $p = 0.894$) whilst browsing preference class (LR $\chi^2_2 = 12.679$, $p = 0.002$) and the interaction of phenolic diversity and preference class (LR $\chi^2_2 = 8.023$, $p = 0.018$; Fig. 4) predicted spinescence possession probability. Again, the most detectable difference in slopes occurred between neutral and preferred species (slope difference = −3.192 (−6.530, 0.147), Z-ratio = −2.240, $p = 0.065$). Phenolic evenness significantly predicted spinescence possession probability (LR $\chi^2_1 = 4.673$, $p = 0.031$), as did preference class (LR $\chi^2_2 = 16.070$, $p < 0.001$) and the interaction between preference class and phenolic evenness (LR $\chi^2_2 = 6.625$, $p = 0.036$; Fig. 4). The most detectable difference was between rejected and preferred species (slope difference = 35.657 (−16.897, 88.301), Z-ratio = 1.587, $p = 0.251$).

Black rhino preferences for broad- versus fine-leaved species differed significantly across browsing preference classes ($\chi^2_{2,66} = 8.469$, $p = 0.014$, Fig. S2). Rejected plant species were equally as likely as neutrally selected plants to have broad leaves, but compared to preferred plant species, rejected species were more likely to possess broad leaves. Furthermore, the probability that a rejected or neutrally selected species was broad-leaved was greater than 50% whereas the preferred plant species' probabilities did not differ from 0.5 indicating that preferred species could be either broad- or fine-leaved. Finally, across geographical distributions ranging

Fig. 1 Phenolic (a) total abundance, (b) richness, (c) Shannon diversity and (d) Pilou's evenness of plants occurring in black rhino (*Diceros bicornis*) browsing preference classes. Solid black points indicate mean responses. Error bars indicate 95% confidence intervals of means. Empty points indicate raw data points and are jittered horizontally to show overlap. Note log scale applied to (a), (b) and (c)
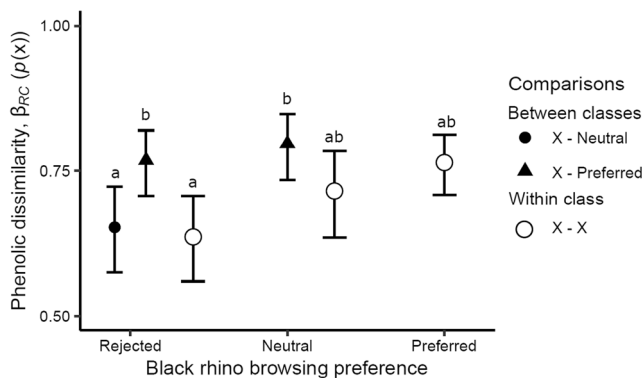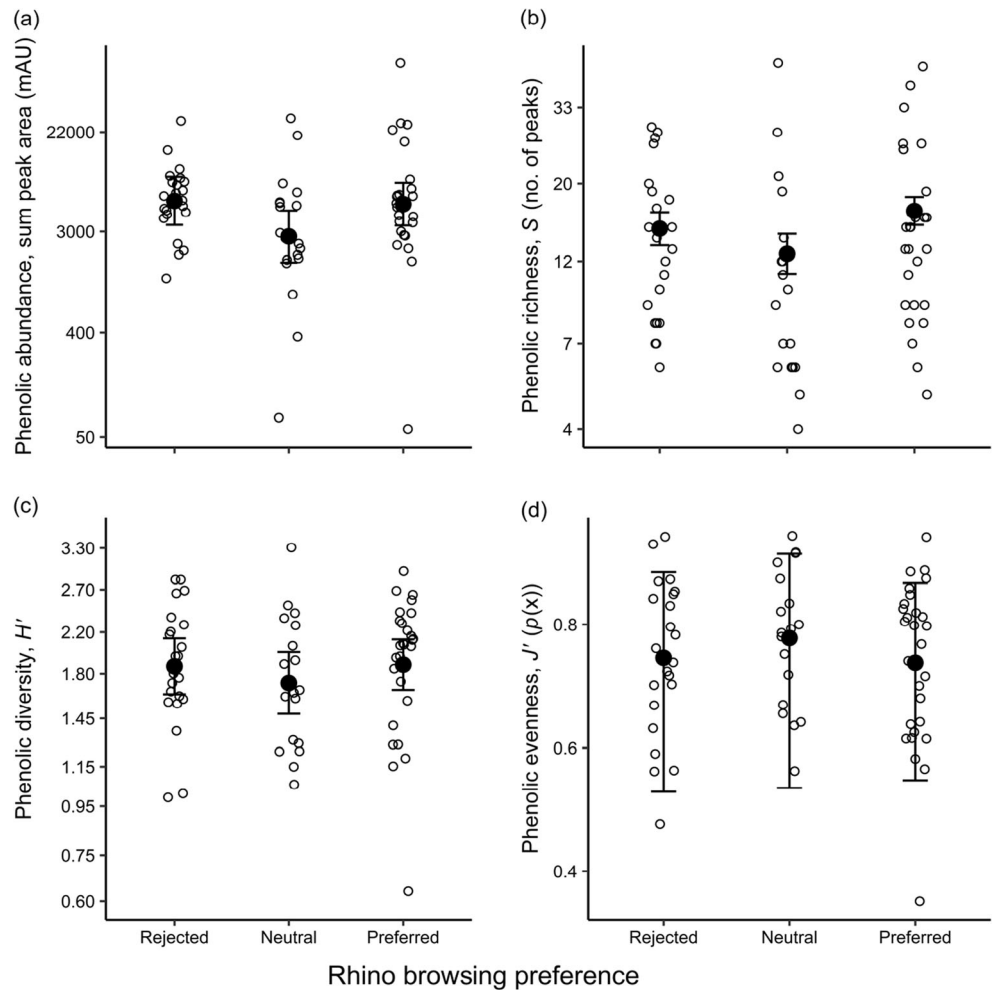

Fig. 2 Raup-Crick proportional dissimilarities based on phenolic presence/absence differences between species within each black rhino browsing preference class (empty circles – e.g. each rejected species compared all other rejected species) and between species in different preference classes (filled circles and triangles – e.g. filled circle represents comparisons between rejected and neutrally selected species). *p*(x) near 0 indicates greater phenolic similarity, *p*(x) near 1 indicates greater phenolic dissimilarity. Error bars represent 95% confidence intervals of the proportion. Different letters above error bars indicate significant differences between means. Raw data points excluded for clarity

from semi-arid to mesic the probability of possessing physical defenses was greater in more mesic regions (LR $\chi^2_2 = 4.458$, $p = 0.035$, Fig. S3).

Discussion

We aimed to develop a better understanding of factors affecting the diet preference of black rhino and to determine if a trade-off between spinescence and phenolics could be detected. We first tested the general view that black rhino diet preference is not affected by either spinescence or abundance of total phenolics. Our data supported this view in that abundance of phenolics and type of spinescence did not affect preference, and spinescence, in general, did not deter black rhino feeding. The high probability of spinescence in preferred species is congruent with the view that spinescent species are palatable (Tomlinson et al. 2016; Wigley et al. 2018), but our results indicated they also have a high abundance of LMW phenolics. That observation can be interpreted in several ways. It could be argued that our results suggest that

Fig. 3 Modelled phenolic profiles of plant species across black rhino diet preference classes ((a) rejected, (b) neutral, (c) preferred). Retention time indicates the time at which phenolics were detected. Peak area indicates the amount of the phenolic that was detected. Dotted vertical lines indicate retention times where the greatest amount of phenolics were detected. Shaded regions represent 95% confidence intervals of the prediction. Note log scale applied to the y-axis
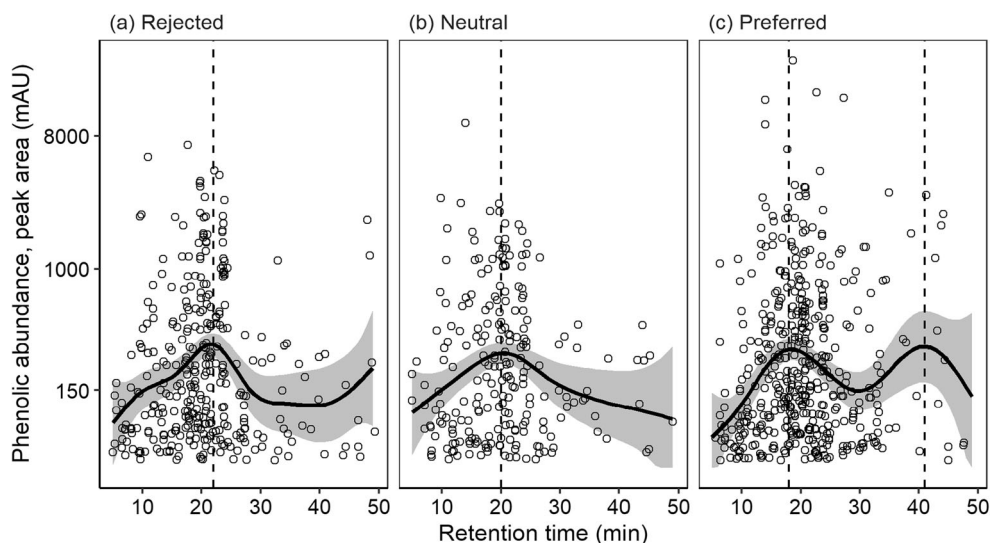
LMW phenolics do not function as defenses against black rhino (and neither does spinescence), but rather that other compounds such as CT, terpenoids, or alkaloids act as defenses, for example in the case of African elephant diet selection (Schmitt et al. 2020). It could also be argued that total abundance of measured phenolics is not an appropriate measure of chemical defense, but rather that specific compounds, or combinations thereof, in the mixture that makes up total abundance act as defenses (DeGabriel et al. 2014; Marsh et al. 2020). The latter view does not preclude the possibility that CT, terpenoids or alkaloids may also be involved, but given that we measured only LMW phenolics, we could not explore that possibility. The likely scenario for the preferred species is that, rather than trading off structural defenses against
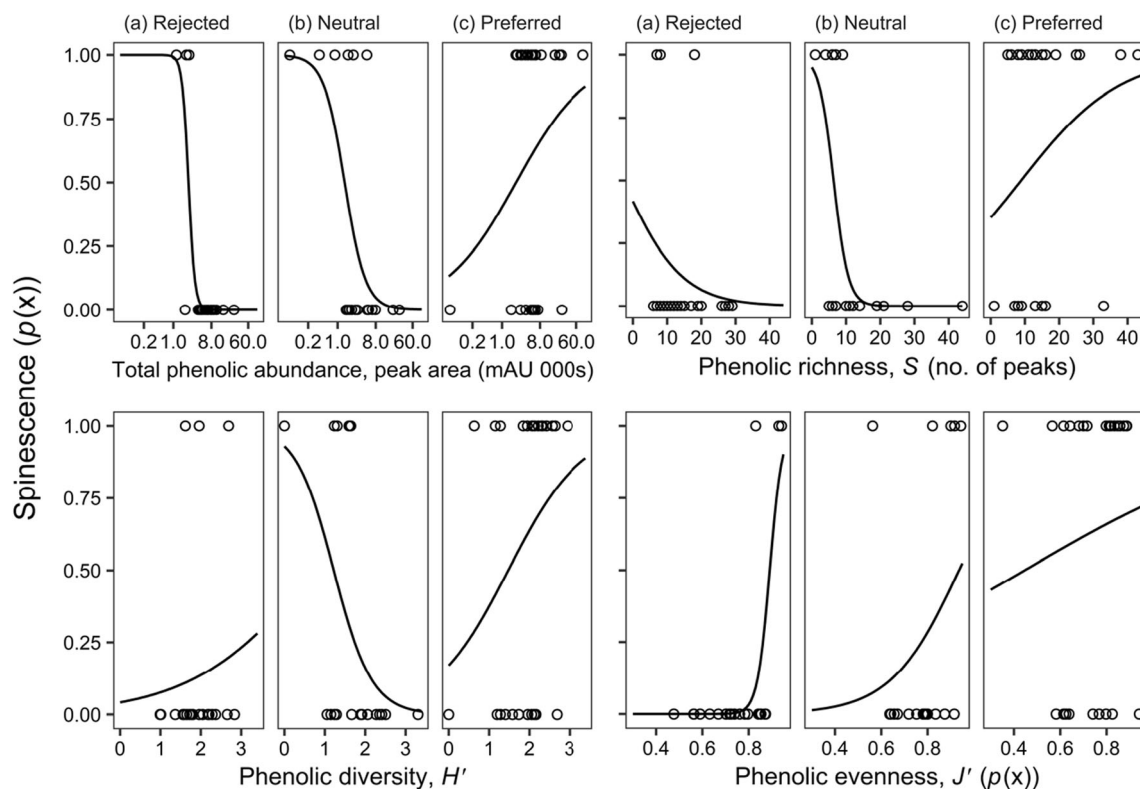

Fig. 4 Probability of plants possessing spinescence ($p(x) = 1$) versus not possessing spinescence ($p(x) = 0$) in relation to total abundance (top left panels, note log scale along x-axis and units in thousands), richness (top right panels), Shannon diversity (bottom left panels) and Pilou's evenness (bottom right panels) of phenolic compounds for black rhino diet preference classes ((a) rejected, (b) neutral, (c) preferred). Lines indicate change in spinescence. Empty points indicate raw data points

chemical defenses, they have multiple defense traits that combine to form defense syndromes, or co-adapted complexes of traits (Moles et al. 2013; Tomlinson et al. 2016; Wigley et al. 2018). Such syndromes could also include associational resistance (Atsatt and O'Dowd 1976), as observed among some species of *Grewia* in African savannas (Sankaran et al. 2013; Tews et al. 2004; Watson and Brown 2000). Moreover, the higher phenolic richness observed among preferred species suggests that richer mixtures are easier to neutralize than poorer mixtures of a few prominent compounds (Dearing et al. 2005; Nobler et al. 2019).

Our second prediction was that, if spinescence and abundance of phenolics did not affect diet preference, then the mixture of phenolics would. This prediction was supported because the compositional dissimilarities between rejected or neutral species vs preferred species were highest. In addition, when we modeled the LMW phenolic profiles of each preference class, we found a difference of four minutes between the modelled maxima in the profiles of rejected vs preferred species. The Rt at which the modeled profiles peaked (around 18, 20, and 22 min for preferred, neutral and rejected species, respectively) suggested the dominance of flavonol and flavone compounds in all three preference classes. However, the Rt values also indicated that the relative abundances of flavonols and flavones varied, with flavones being noticeably present among the preferred species and flavonols being more noticeable in the other two preference classes (Table 1 in Hattas et al. 2011). Hence, our results support a previous study of a much more restricted set of only six species (Hattas et al. 2011), as well as studies of willow species in Sweden (Stolter 2008; Stolter et al. 2005). Although the mechanisms by which flavonols influence LMH diet preference are unclear, they are possibly related to delayed post-ingestive feedback from digestibility reduction (Mengistu et al. 2017; Provenza 1995; Tan and Chang 2017; Villalba et al. 2015). Given that flavonols may be correlated with CT in some species (Scogings et al. 2014) further suggests that avoidance may be linked to reduced digestibility. Nevertheless, pending further investigation, we infer that flavones and flavonols distinguish preferred from neutral and rejected species, at least in terms of black rhino diet selection, but probably also in relation to other LMH species (Hattas et al. 2011).

Furthermore, our results suggest that other classes of compounds are active in determining the diet preference of black rhino. The upward trend between 40 and 50 min in the predicted profile of rejected species, which was most likely influenced by *Ochna pulchra* because it had large peaks at 48 to 49 min, indicating the presence of unknown compounds influencing rejection. Also influential in the predicted profile of rejected species were three *Searsia* species that had several small peaks between 40 and 46 min, suggesting the presence of flavanones (Table 1 in Hattas et al. 2011). In addition, the modeled peak around 41 min in the predicted profile of preferred species also suggested the presence of flavanones, such as pinocembrin, in some species

(Hattas et al. 2011). This was likely strongly influenced by *Vachellia borleae*, which had several large peaks between 38 and 45 min. In addition, *Vachellia recifiens*, *Rhigozum obovatum,* and three *Grewia* species were influential in the modeled profile in that they had several small peaks between 35 and 41 min. Identification of the compounds that eluted later than 35 min requires further research to determine which classes of compounds other than flavones and flavonols affect diet preference of black rhino, and other LMH in savannas.

Our test for a trade-off between structural and chemical defenses indicated that the interactions in each of the four models were significant, although none of the pairwise comparisons were significant ($p > 0.05$). For phenolic abundance and phenolic diversity, however, the p-values (0.081 and 0.065) were marginal and suggested a trend in the data toward opposite responses in neutral and preferred classes. It is our view that suggestions of such trends should not be completely ignored, especially if $0.10 > p > 0.05$ is simply an artifact of small sample size or, as in this study, the specific set of species selected (Mudge et al. 2012). The results of all our analyses depended on the species sampled for the study. With a different set of 71 species, or with more species or fewer species, the p-values could have been significant ($p > 0.05$), or not, but this requires further investigation to validate the trends.

Nevertheless, the significant interactions ($p < 0.05$) between preference class and total abundance of phenolics indicated that whether the probability of spinescence trades off against phenolics depends on the preference class. Given that phenolic abundance was not different among preference classes, but the composition of the mixtures was, we infer that the probability of spinescence trading off against phenolics depends on the type of LMW phenolics that dominate the mixture. When the phenolics are mainly flavonols, then spinescence is costly for savanna woody species, but when flavonols are not the dominant phenolics, then both defenses could be effective. We are not aware of similar findings in the literature, and plausible explanations for a trade-off when the LMW phenolics are mainly flavonols remain unclear.

Assuming spinescence is a relatively cheap defense (Charles-Dominique et al. 2020; Midgley and Ward 1996), then why invest in flavonols and not spines, and why invest particularly in flavonols and not flavones or flavanols (the polymeric units making up condensed tannins)? These three flavonoid classes are products of different branches of flavonoid biosynthesis (Yonekura-Sakakibara et al. 2019) and all have multiple functions in plants and various effects on animals (Hostetler et al. 2017; Mouradov and Spangenberg 2014). Both flavones and flavonols can have digestibility-reducing effects in herbivores (Mengistu et al. 2017; Tan and Chang 2017), which is also a well-known function of flavanol polymers (Routaboul et al. 2012). Relative costs of producing different types of flavonoids are unknown, as far as we can ascertain, but it has been suggested that flavonoids as a group are expensive in terms of costs to plant

growth (Tohge et al. 2017). We could assume that flavonol (and flavanol) synthesis costs more than flavone synthesis because more enzymes are required (Yonekura-Sakakibara et al. 2019), yet flavonols were found to be influential in savanna LMH selection of woody species, instead of either flavones or spinescence. A plausible explanation for the selection of flavonols over flavones remains elusive.

Our analysis of preference in relation to leaf type and spinescence revealed that most of the rejected/neutral species in our study were broad-leaved and non-spinescent, while most (two-thirds) of the preferred species were predominantly broad-leaved and non-spinescent, with the remainder being fine-leaved and spinescent (cf. Table 1). We note that some broad-leaved species generally regarded as rejected may be browsed by black rhino or other herbivores, especially during times of food scarcity (Joubert et al., 2018; Katjiua and Ward, 2006; Owen-Smith 2008). Furthermore, the contemporary geographical distributions of rejected/neutral species are either mainly in semi-arid areas or widespread from semi-arid to mesic, while distributions of preferred species are mainly semi-arid or widespread (Coates Palgrave 2002). Thus, the long-standing dichotomous view that phenolics dominate in mesic savannas where LMH are not abundant, and spinescence dominates in semi-arid savannas where LMH are abundant, is challenged. Why so many of our studied species were effectively defended by flavonoids against black rhino, and probably other LMH, and yet are not restricted to mesic areas, remains to be explained.

In conclusion, this work represents a substantial advancement in our understanding of the roles of PSMs, especially flavonoid compounds, in the functioning of savanna ecosystems. Our results highlight why it is necessary to dig deeper into broad groups of traits such as spinescence or total phenolics in order to develop a better understanding of plant defenses (Moles et al. 2013), more so when trying to understand the functions of PSMs in savannas. Future research needs to investigate the preferences of other large mammal herbivores in relation to low molecular weight phenolics.

Code Availability Not applicable.

Declarations

Conflicts of Interest/Competing Interests The authors declare no conflict of interest.

Availability of Data and Material Data supporting the results are archived in Figshare (https://doi.org/10.6084/m9.figshare.14131799.v1).

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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