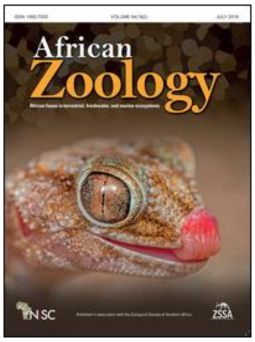

African Zoology

ISSN: 1562-7020 (Print) 2224-073X (Online) Journal homepage: https://www.tandfonline.com/loi/tafz20

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African Zoology 2019, 54(3): 175–179
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AFRICAN ZOOLOGY
ISSN 1562-7020 EISSN 2224-073X
https://doi.org/10.1080/15627020.2019.1635911

Short communication

The first report of urine overmarking of pro-oestrus female dung by a male white rhino

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Although observed in other Perrisodactyla, urine overmarking in white rhinos has not been described. Using a single opportunistic sighting, we were able to collect two dung samples from one oestrous female white rhino; one unmarked and one overmarked with male urine. Because of the behaviour of both the male and the female, we suggest that the female was in pro-oestrus. We hypothesised that the function of overmarking by the male was for oestrus concealment (i.e. odour masking), as observed in horses. Because dung from oestrous female white rhinos emits a higher proportion of alkanes than non-oestrous dung, we expected the proportion of alkanes emitted from oestrous dung to decrease after overmarking. In contrast, we found that after overmarking the proportion of alkanes emitted increased. We suggest that the function of urine overmarking in white rhinos could be to conceal all signals of reproductive condition, so that neither oestrous nor non-oestrous signals are recognisable, or that a signal is added to indicate that the female has been mated.

Keywords: *Ceratotherium simum*, odour masking, oestrus concealment

Online Supplementary Material: Supplementary table and figure available at: https://doi.org/10.1080/15627020.2019.1635911

Introduction

Overmarking occurs when one individual places its scent mark on top of a scent mark from another individual (Ferkin and Pierce 2007). There are several hypotheses regarding the function of overmarking by specific individuals. Same-sex overmarking gives an advantage to the individual overmarking in the form of physically masking the initial odour (Johnston et al. 1994); provoking competition (Ferkin et al. 2004); or, showing social rank (Rich and Hurst 1999). These hypotheses pertain mainly to same-sex overmarking, and suggest that the most recent odour added are investigated more frequently and afforded higher status by competitors. For example, meadow voles (*Microtus pennsylvanicus*) and prairie voles (*Microtus ochrogaster*) exposed to an overmark were able to distinguish the two different signals and preferred the overmark (Ferkin et al. 2001).

Opposite-sex overmarking is a form of mate attraction (Ferkin and Pierce 2007). As such, individuals overmark the scent marks of reproductive individuals of the opposite-sex in order to facilitate mating (i.e. overmarking occurs before mating). Overmarking pre mating has been reported in Cape mountain zebras *Equus zebra zebra*; Penzhorn (1984) and meadow voles (Ferkin et al. 2004). Furthermore, overmarking is thought to be a form of mate guarding, whereby overmarking masks or devalues the odour of the initial mark (Ferkin and Pierce 2007). This hypothesis predicts that individuals will overmark post reproduction in order to hide the reproductive status of an individual or indicate that the individual has been mated (Ferkin and Pierce 2007). For example, klipspringers (*Oreotragus oreotragus*) mark with secretions from their preorbital gland, males overmark female scent marks as a form of chemical mate guarding (Roberts and Dunbar 2000). Yet, it is also possible that if the signal is overmarked prior to mating, it might reduce competition from rival males that encounter the mark (Kimura 2001).

Overmarking as a function of oestrus concealment was suggested by Penzhorn (1984) with regard to the urine overmarking of male Cape mountain zebras on female urine and dung. Recent evidence from feral horses (*Equus caballus*) indicates a similar function, where overmarking the dung of oestrous females with male urine changes the odour profile such that it is more similar to the odour of non-oestrous female dung (Kimura 2001). Recent evidence also suggests that intersexual overmarking in female equids (African wild ass (*Equus africanus*), Grevy's zebra (*Equus grevyi*), plains

African Zoology is co-published by NISC (Pty) Ltd and Informa UK Limited (trading as Taylor & Francis Group)

Published online 05 Sep 2019

zebra (*Equus quagga*), mountain zebra (*Equus zebra*)) helps maintain social bonds and group cohesion (Tučková et al. 2018). Ultimately, overmarking appears to be an important behaviour in Perrisodactyla.

White rhinos (*Ceratotherium simum*) defecate in communal middens (i.e. latrines) where they deposit and obtain information, including the territorial or oestrus state of the depositor (Marneweck et al. 2017a, 2018). In general, the dung of white rhino females in oestrus emits a higher proportion of hydrocarbon alkanes than non-oestrous female dung (Marneweck et al. 2017a; unpublished data). Furthermore, the emission of the alkane 2,6-dimethylundecane from female white rhino dung is an important indicator of oestrus (Marneweck et al. 2017a), where oestrous dung odours contain a larger proportion than non-oestrous (median proportion [interquartile range]: non-oestrous 0.0005 [0.0014], n = 23; oestrous 0.0030 [0.0124], n = 7; Marneweck et al. 2017a; unpublished data).

Overmarking of dung in middens has been observed when a territorial male is being challenged (Owen-Smith 1975; Marneweck pers. obs.). In this situation, the territorial male places his dung on top of the challenger's dung. However, male overmarking of female dung has not been reported in white rhinos, although observed in other Perrisodactyla (Penzhorn 1984; Kimura 2001). In addition, prior to copulation, female white rhinos emit repeated little squirts of urine (Owen-Smith 1973), which might be a form of oestrus advertisement. However, as with the dung of these oestrous females, overmarking of this urine by males has also not been reported.

An opportunistic sighting allowed us to collect two dung samples from one oestrous female white rhino; one unmarked and one overmarked with male urine. Because of the polygynous mating system of white rhinos (White et al. 2007), it is unlikely that intersexual overmarking is for mate attraction. Males establish territories within which they actively pursue oestrous females that move through. Despite territorial males having primary access to these females, some sneaky copulations by subordinate males resident within the territories can take place (Guerier 2012). As a result, we hypothesised that the function of male overmarking of female dung was oestrus concealment as a form of chemical mate guarding, similar to what has been observed in horses (i.e. to mask the oestrus signal; Kimura (2001)). Hence, we predicted that (1) the overall proportion of alkanes emitted by the overmarked dung would decrease, specifically (2) the proportion of 2,6-dimethylundecane would decrease to levels found in the odour of non-oestrous female dung.

Methods

We conducted this study in the 896 km^2 Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. During our two-year study, we observed, on foot, over 200 separate defecation events, with at least ten of these from oestrous females. Despite the sample size, we only recorded urine overmarking by males once. Here, we opportunistically collected two dung odour samples from a single wild, free-ranging female white rhino in oestrus during June 2012. The first sample was collected at 15:25, the second collected at 15:50 from a separate defecation ~60 m away. At the second defecation, a territorial male sniffed the dung pile, performed flehmen, then overmarked the female's dung with his urine. We are unsure as to the reason why the male overmarked the second dung pile and not the first. From several former observations of this male over the extended study period in 2012, we established that he was a territory holder, by reason of the territorial behaviours he performed (i.e. dung kicking and spray urination) (Owen-Smith 1971; Kretzschmar et al. 2001; Marneweck pers. obs.). However, while overmarking, his urine was excreted in a stream, as in non-territorial males and females, not sprayed in a mist, as is the case for territorial males. We did not observe this male interact with any other individuals during the extended study period, nor did we determine the location of his territory boundary. As such, it is possible that he was outside of his territory, consequently was acting as a subordinate (i.e. not spray urinating), or this event occurred during a territory challenge or takeover. Although stream urination is not ordinarily performed by territorial males, they can periodically urinate in a stream (Owen-Smith 1973).

We identified the female as an adult (>7 years), based on body size and horn development (Hillman-Smith et al. 1986), she was accompanied by a female calf and a female subadult (approximately three- and six-years-old, respectively). We identified the oestrus state via the behaviour of the male. For white rhinos, there is a consort period of several days where a territorial male will move with an oestrous female. During this time, he follows her closely, restricts her movement beyond his territory boundary, makes several mounting attempts (Owen-Smith 1973). There is usually only one successful copulation during this courtship and, subsequently, the male can continue to follow the female loosely, but does not attempt to mount further (Owen-Smith 1973). We observed the male follow the female closely (i.e. within 10–20 m), for approximately 80 min. During this time, the male attempted to restrict the movement of the female, performed flehmen in response to her dung, attempted to mount her once. The mounting attempt took place as the female was walking slowly with the male following her closely. His penis was extended, but he did not attempt intromission. Shortly after this attempt, the female defecated for the first time. The male investigated the dung, to which he performed flehmen, while the female continued walking slowly away. She then defecated a second time and, when the male reached the second dung pile, he overmarked it with his urine. He did not overmark the first dung pile. We did not observe any squirt urination from the female, we did not record any vocalisations by either adult.

Although the mate guarding hypothesis predicts that overmarking as a function of mate guarding would occur post mating, we cannot confirm whether the female in question had already mated with the male moving with her, but this is unlikely, because males usually cease mounting attempts after successful copulation (Owen-Smith 1973). Hence, it is possible that he overmarked her dung to reduce competition from other resident males. Because the consort period in white rhinos lasts for 1 to 2 weeks, it does not begin at the onset of oestrus. The onset of oestrus is described as regular advances and hiccing vocalisations from the male (Owen-Smith 1973). The frequency of these behaviours increases until mating occurs, then the male does not attempt to mount again after successful copulation (Owen-Smith 1973). Because we observed only one mounting attempt (no copulation), no vocalisations, we assume the female was in pro-oestrus.

We collected odour samples using a dynamic headspace extraction method (Amirav and Dagan 1997) to collect air for 25 min from approximately 800 g (one bolus) of fresh (<5 min old) dung enclosed in a polyacetate bag using a micro-air sampler (Supelco PAS-500) with a realised flow rate of 150 ml min^{-1}. The volatile organic compounds (VOCs) emitted from the dung were captured in a small thermodesorption trap filled with 1 mg of Tenax® and 1 mg of Carbotrap®. We confirmed that both dung samples were from the same adult female by following her and observing her defecate.

We analysed the thermodesorption traps using gas chromatography-mass spectrometry (GC-MS). We carried out analysis on a Bruker 450 GC with a 30 × 0.25 mm internal diameter (film thickness 0.25 µm) Varian VF-5 ms column, connected to a Varian VF-1 ms column (11 × 0.25 mm internal diameter, film thickness 0.25 µm) coupled to a Bruker 300 quadrupole mass spectrometer in electron-impact ionisation mode at 70 eV. Thermodesorption traps were placed in a Varian 1079 injector equipped with a ChromatoProbe thermal desorption device. The flow of helium carrier gas was 1 ml min^{-1}. We held the injector at an initial temperature of 250 °C for 20 min. The split vent was programmed to start with a 10: 1 split for 2 min and then to switch to splitless mode for 2 min to allow for thermal desorption, followed by a 100:1 split after 4.2 min to clean the injector. After an initial temperature at 45 °C the temperature of the GC oven was increased to 260 °C by 7 °C min^{-1} and, after reaching 260 °C, held at this temperature for a total run time of 35 min. We identified VOCs using Varian Workstation software with the NIST 2011 mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011; MS search software version 2.0 d). We verified the identification of VOCs with retention times of authentic standards and published Kovats retention indices wherever possible (Supplementary Table S1).

To compare the odour of the overmarked dung, we created an MDS plot, using the R package *vegan*; Oksanen et al. (2015), including dung odour samples from both oestrous (n = 3) and non-oestrous (n = 15) females collected during the same season for comparison (Marneweck et al. 2017a, 2017b). Some females may have been miss-identified as non-oestrous, by reason of the fact that they were observed alone, which might explain the inclusion of some non-oestrous markers close to the oestrous core in Figure 1. Alternatively, it could be that oestrous and non-oestrous odours are very similar and, as a result, will overlap. However, because of the small sample size of oestrous females in the dry season (n = 3), it makes it difficult to differentiate. Because we believe the female in this study to be in pro-oestrus, this could further explain the close relationship with non-oestrous odours, (i.e. it could be a continual drift from non-oestrus, to pro-oestrus, to oestrus, to an-oestrus).

Results and discussion

The odour of the overmarked female dung sample was more similar to that of non-oestrous female dung (Figure 1). After overmarking, two alkanes were eliminated from the female's

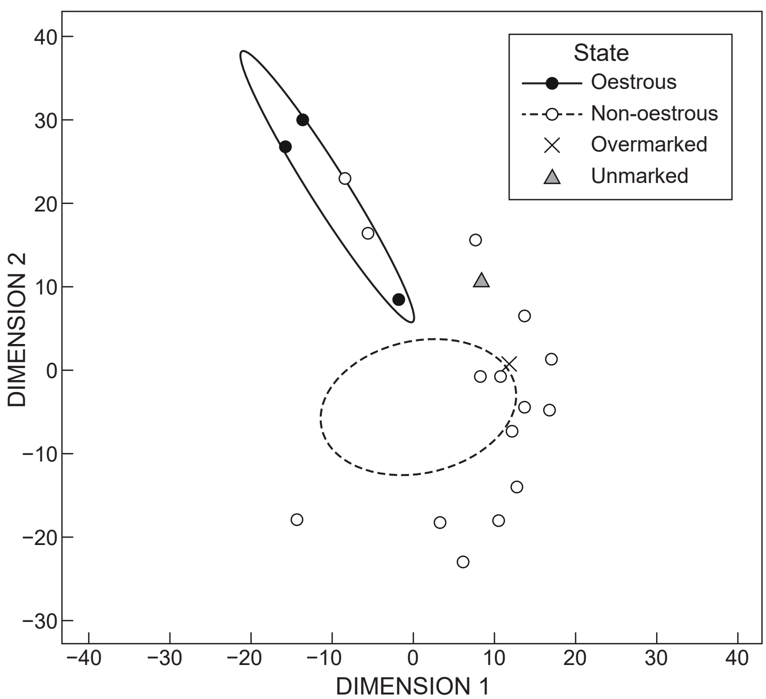

Figure 1: Multidimensional scaling (MDS) plot based on Bray–Curtis similarities of volatile organic compounds (VOCs) emitted from female white rhino dung during the dry season. Encompassing circles represent 95% confidence intervals; stress = 0.15

Table 1: The tentatively identified volatile organic compounds (VOCs) belonging to the alkane function group, and their relative proportion, present in dung odour of unmarked and overmarked female dung

Name	CAS number	Weight ($g\ mol^{-1}$)	Proportion contribution to dung odour: Sample one (unmarked)	Proportion contribution to dung odour: Sample two (overmarked)
Nonane	111-84-2	128	0.0083	0.0092
Tridecane	629-50-5	184	0.0079	0.0383
Hexane	110-54-3	86	0.0073	0.0040
2,6,10-Trimethyldodecane	3891-98-3	212	0.0039	0.0148
2,6-Dimethylundecane	17301-23-4	184	0.0027	0.0066
Dodecane -	112-40-3	170	0.0012	0.0000
2,3-Dimethylundecane	17312-77-5	184	0.0008	0.0022
Tetradecane	629-59-4	198	0.0007	0.0025
3-Methylpentane -	96-14-0	86	0.0006	0.0000
3-Methyldecane	13151-34-3	156	0.0005	0.0016
Hexadecane	544-76-33	226	0.0005	0.0071
Heptadecane	629-78-7	240	0.0002	0.0027
Octadecane	593-45-3	254	0.0001	0.0010
(3-Methylbutylidene)cyclopentane +	53366-51-1	138	0.0000	0.0051
2-Methylundecane +	7045-71-8	170	0.0000	0.0006
6-Methyloctadecane +	10544-96-4	268	0.0000	0.0005
		Total	0.0347	0.0962

- denotes VOC eliminated after overmarking
+ denotes VOC appeared after overmarking

dung odour (3-methylpentane and dodecane; Table 1). Additionally, three alkanes appeared after overmarking, that were not present in the unmarked sample ((3-methylbutylidene) cyclopentane, 2-methylundecane, 6-methyloctadecane; Table 1). The total proportion of alkanes emitted from the unmarked dung was 0.0347, this increased to 0.0962 after overmarking (177% increase; Table 1). Furthermore, the proportion of 2,6-dimethylundecane increased by 143% after overmarking; from 0.0027 to 0.0066 (Table 1). For a list of all tentatively identified VOCs and their relative proportions, see Supplementary Table S1.

Although overmarking increased the proportion of 2,6-dimethylundecane emitted, the proportion was above the average found in oestrous dung odour. Accordingly, overmarking did not conceal oestrous by mimicking non-oestrous dung odour, as expected. Rather, it could be that urine overmarking in white rhinos makes the oestrous condition unrecognisable, or perhaps adds a signal to show that the female has already been mated.

Interestingly, the overmarked dung odour was still similar to female dung odour in general, the overmarking behaviour did not create a new, unique odour (see Supplementary Figure S1). The most important indicator of territorial status in male dung odour is the alkane nonane, where territorial male dung emits a larger proportion than non-territorial (mean proportion + SE non-territorial 0.0120 ± 0.0027, n = 29; territorial 0.0158 ± 0. 0031, n = 30; Marneweck et al. 2017a, unpublished data). The proportion of nonane in the dung odour of the oestrous female increased by only 10% after overmarking, did not reach the level of nonane emitted from either non-territorial or territorial male dung. This suggests that the indicator for territorial status might be different in urine than in dung.

Because of the polygynous mating system of white rhinos, it is most likely that intersexual overmarking is for mate guarding, only this did not occur in the way we expected. White rhino males hold exclusive territories, whereas females hold larger, unexclusive home ranges that encompass several male territories (Owen-Smith 1975). Territorial males have primary access to females within their territory, but sneaky copulations by subordinate males resident within the territories do occur (Guerier 2012). As a result, by rendering a female's reproductive condition unrecognisable, a male would likely reduce the possibility of these subordinate males trying to mate with her. Yet, it could also be that the female defecated in a midden along the male's territory boundary. Neighbouring territorial males often explore the middens along their territorial boundaries (Owen-Smith 1973). Therefore, by overmarking her dung, the territorial male could have been trying to prevent the neighbouring male from detecting that the female was reproductively receptive. If so, this would then prevent aggressive interactions with the neighbouring male.

We fully acknowledge that the sample size is a key limitation of our study, but present these findings as a way to urge additional investigation on the subject. Future studies could investigate the chemical implications of overmarking, as well as the function of urine marking in white rhinos. Yet, we suggest that this research be conducted on wild, free-roaming populations, not captive white rhinos, because the chemical components/ concentrations in the urine of captive individuals would likely differ to free-ranging individuals. The reason is that testosterone levels are significantly affected by social housing (i.e. number of females or other males present; Kretzschmar et al. (2004), Hermes et al. (2005), Christensen et al. (2009)). In addition, captive females do not show normal oestrous cycles, with cycles being erratic, shorter or longer than average, or females being acyclic (Brown et al. 2001). These hormonal fluctuations will also make it difficult to collect odours representative of free-roaming oestrous females. Finally, the volatile compounds emitted from dung and urine would likely be further impacted by the fact that captive animals do not eat a natural diet. Because odours are influenced by diet, such a non-natural diet would likely affect the volatile organic compounds emitted from dung and urine, compared with a natural diet (MacDonald et al. 2008; Kean et al. 2011).

Despite the limited sample size, our results suggest that urine overmarking in white rhinos could be a function of mate guarding, they give emphasis to the potential for urine to portray a different message than dung as a scent marking source. Ultimately, this is the first study to describe, hypothesise on the function of, urine overmarking behaviour in white rhinos.

Acknowledgements — This research was approved by the University of KwaZulu-Natal Ethical Approval of Research on Animals (reference: 059/14/Animal). The authors declare no conflicts of interest.

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Manuscript received: 14 March 2019, revised: 21 June 2019, accepted: 21 June 2019
Associate Editor: A Ganswindt