Olfactory communication of the white rhinoceros (*Ceratotherium simum*)

by

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As candidate's supervisors, we have approved this thesis for submission;

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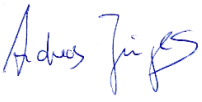
Signed:  Name: Prof. Andreas Jürgens Date: 7 February 2017

Abstract

Many mammal species communicate olfactorily via specialised scent glandular secretions, urine and/or dung. Despite a large body of work on olfactory communication, the extent to which mammals communicate via dung odours, and what information is transmitted, is unknown. White rhinos (*Ceratotherium simum*) have poor eyesight but an acute sense of smell and therefore rely heavily on olfactory signals. Moreover, white rhinos of all ages and sex defecate communally in middens, thus it is possible that these middens act as olfactory information centres for male-male, female-male, male-female and female-female communication. To explore these possibilities, I first analysed the odours emitted from the dung of free-ranging white rhinos. In doing so, I identified distinct odour profiles that indicated an individual's sex, age, male territorial status, and female oestrous state. Once I had identified the information transmitted, I then explored how long these signals lasted. In order for an olfactory signal to be effective it must persist in the environment for an extended period. To determine signal longevity I analysed the temporal changes of white rhino dung odours. I found that over a short period male dung odours had shorter longevity than female odours. Within males, territorial odours had shorter longevity than non-territorial, while non-oestrous female odours had a shorter longevity than oestrous odours. The high temperature and humidity of the wet season decreased the longevity of all adult dung odours. However, white rhinos did not adjust their visitation or defecation frequency during the wet season to counteract this decrease in longevity. Having identified the odours and how long they lasted, I then investigated the behaviour of white rhinos at middens to determine which individuals were primarily transmitting information and who were the intended targets. I found that middens were utilised predominately by adults. Moreover, the primary function of middens was for territorial males to transmit and obtain information (male-male and female-male communication), with secondary functions for non-territorial males to also assess female reproductive state, and females to assess the quality and number of potential mates (male-female communication). In addition to olfactory signals there was a spatial aspect to defecating in middens, where territorial males defecated in the centre of the midden and other individuals around the periphery. Further, territorial males regulated their dung output, with a higher defecation frequency and smaller dung volume than any other adult. Finally, I conducted an experiment to investigate the purpose of territorial male dung kicking. Using non-territorial adult male dung as a surrogate, I found that the dispersal of male white rhino dung caused olfactory signal amplification by increasing the emission of hydrocarbon acids. However, despite the benefits of odour amplification, dung dispersal also carried a cost of decreased odour longevity, ultimately decreasing signal longevity. Territorial males likely counteract this by defecating in middens during peak visitation times by other individuals. Ultimately, my results highlight the mechanism behind olfactory communication in white rhinos and the importance of middens in this communication system. Moreover, as many other mammal

species defecate communally, olfactory communication via dung odours is likely a widespread phenomenon.

Declaration: plagiarism

I, Courtney Jade Marneweck, declare that

a) The research reported in this dissertation, except where otherwise indicated, is my original work
b) This thesis has not been submitted for any degree or examination to any other university
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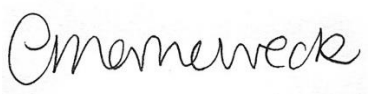
Courtney Jade Marneweck
February 2017

Preface

The research contained in this thesis was completed by the candidate while based in the Discipline of Ecology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa, under the supervision of Dr. Adrian M. Shrader and Prof. Andreas Jürgens. The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.



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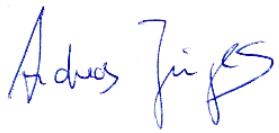

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Co-supervisor
February 2016

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After five years of working on this project many people have come and gone and left their mark in some way. Firstly, I would like to thank Ezemvelo field staff, Joseph Dlamini and Bom Ndwandwe for their protection, tracking skills, and many patient hours in the field. I will always remember fondly the day Joseph and myself waited over seven hours for a rhino calf to poo! Thanks to the section rangers Andile Mhlongo, Dennis Kelly and Dirk Swart for allowing me unlimited access to white rhinos in their sections, and the ecologist Dr. Dave Druce for his logistical support. Also to Ester van der Merwe for pointing me to rhino poo, Geoff Clinning for always helping me find my way there, and Stacey Klein Snakenborg for helping me carry it. I have been lucky enough to have spent countless hours with white rhinos in the field and nothing will ever compare to those special moments of tranquillity, sharing a shady spot with a sleeping rhino while the world ticks by.

Thanks to Dr. Adam Shuttleworth for processing GC-MS samples and to my supervisors, Dr. Adrian Shrader and Prof. Andreas Jürgens. I am so grateful for your initial marriage of disciplines that brought life to this project and your support throughout the years.

I thank my family in England for their gracious acceptance of my absence. It was my goal for many years to get here and, with your continuous love, I have finally done it. Most importantly, I thank my husband, David. There are no words enough to express what you've done for me these five years. From pushing me to take on the project in the first place to being by my side as I hand it in. For the days and nights of discussion, advice, and support, through tears and frustration, I am eternally grateful.

Finally, I would like to dedicate this thesis to rhinos, in the hope that the contents benefit them in some way. I ache for those that have been taken and for those that are yet to be taken. I hope there is never a time that your gentle steps do not wander this earth, wild and free.

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Chapter 1 : Introduction

1.1. Literature review

1.1.1. Olfactory communication

Olfactory communication is the perception of molecules generated from one individual and transmitted through the air, which results in an alteration of the behaviour of another individual (i.e. the receiver) (Bossert and Wilson 1963). Many mammals utilise olfaction as a form of communication (Eisenberg and Kleiman 1972; Johnson 1973) and it offers several advantages in communicating relevant biological information. Firstly, it likely provides honest indicators of the characteristics of the depositor. For example, scent gland secretions of male lemurs (*Lemur catta*) indicate their genetic quality, allowing females to make optimal mate choices (Charpentier et al. 2008). A second advantage is that olfactory signals can provide spatial information such as territory ownership or information on the movements of a particular individual without that individual having to be present (Gosling and Roberts 2001; Moorcroft et al. 2006). Thirdly, it requires less energy than acoustic calls (Bradbury and Vehrencamp 2011) and, lastly, odours persist in the environment after the sender has gone (Johnston and Schmidt 1979; Linklater et al. 2013). A wealth of biological information is present in olfactory signals and available to individuals that are able to interpret such information.

However, there are also disadvantages to olfactory communication. For instance, signals cannot be focused in specific directions or towards specific individuals (Eisenberg and Kleiman 1972). Further, diffusion in the air causes a concentration gradient known as the active space and individuals must rely on wind speed and direction to expand this active space (Bossert and Wilson 1963). Additionally, environmental factors such as temperature and humidity also effect the odour of scent marks beyond the sender's control (Bossert and Wilson 1963; Martín and López 2013; Nimmermark and Gustafsson 2005). Chemical signals can also be costly to produce. Male mice (*Mus musculus*) suffer reduced body size and growth rate when they increase their scent mark rate (Gosling et al. 2000).

1.1.2. Odour sources

Odours (i.e. olfactory signals) originate from three main sources; specialised glandular secretions, urine, and dung. Specialised scent glands exist in many species. Anal pouches are present in all four hyaenid species (Eaton 1976; Stoeckelhuber et al. 2000; Theis et al. 2008; Wagner 2006). Specialised scent glands can also occur within the skin, (Buesching et al. 2002a), between digits (Reiter et al. 2003) and in the foot (Sunquist and Sunquist 2002). In contrast to the costs of producing specialised scent marks (Gorman 1990), marking with urine and/or dung requires little additional energy expenditure (Gorman 1990) as it is constantly produced. Spray urination is common in felids. Male tigers (*Panthera tigris*) spray urine along territory boundaries and females spray urinate more frequently just before oestrus,

suggesting an advertisement of territory ownership and reproductive state, respectively (Smith et al. 1989). In contrast, only male leopards (*Panthera pardus*) spray urinate and it was suggested that these marks were directed towards females rather than other males, as the rate of marking increased during the breeding season (Bothma and le Riche 1995).

Another source of odours is dung, which is commonly used by ungulates to advertise territory boundaries and ownership. Dung is large, readily produced, and persists in the environment, making it an ideal vector for olfactory signals (Gosling 1985). For example, male oribi (*Ourebia ourebi*) (Brashares and Arcese 1999), male red hartebeest (*Alcelaphus buselaphus caama*) (Gosling 1985) and male mountain gazelles (*Gazella gazelle*) (Habibi et al. 1993) all mark with dung at territory boundaries. Many carnivore species also utilise dung as a territory marker, such as wolves (*Canis lupus*) and coyotes (*Canis latrans*) (Barja et al. 2004; Ralls and Smith 2004).

1.1.3. Volatile organic compounds

Olfactory signals are transmitted through volatile organic compounds (VOCs). The volatility of a compound depends on several factors, such as its molecular weight and vapour pressure, where VOCs of high molecular weight and low vapour pressure are less volatile (Apps et al. 2015; Stoddart 1976). In order for an olfactory signal to be effective it must persist in the environment in the absence of the sender (Eisenberg and Kleiman 1972). VOCs with high molecular weight and low vapour pressure are less volatile and therefore persist in the environment longer than more volatile VOCs. The suggested ideal range for olfactory signals is a molecular weight between 50 and 300 g/mol (Wheeler 1977), where 300 g/mol is approaching the upper limit for volatility. These factors have important repercussions for the information in odours being stable over time (i.e. the heavier a compound, the longer it persists in the environment), which ultimately means a stable signal. However, an odour signal that lasts too long loses its potential to indicate proximity and an odour signal that is lost too quickly loses its ability to be effective in transferring information before a target individual can encounter it. Therefore, the VOCs utilised become function specific. For example, alarm signals are highly volatile (low molecular weight) so that they can dissipate once the threat has gone (Verheggen et al. 2010). Contrastingly, territorial signals generally have low volatility (high molecular weight) so that they can persist in the environment and remain as active signals of territory ownership in the absence of the owner (Hurst et al. 1998).

The longevity of odours also seems to be source specific, where specialised glandular secretions last for several weeks (Buesching et al. 2002b; Epple et al. 1980; Ferkin et al. 1995; Johnston and Lee 1976; Roberts 1998) and urine only several days (Beauchamp and Berüter 1973; Drickamer 1986; Goodwin et al. 2012; Kwak et al. 2013; Lydell and Doty 1972). Empirical examples of dung odour longevity, however, are lacking, with only one example of black rhinos (*Diceros bicornis*) responding to dung up to 32 days old (Linklater et al. 2013). The associated effects of an olfactory signal's longevity in the environment is likely to have behavioural consequences. However, due to our lack of

understanding with regard to the longevity of dung odours, it is unclear exactly what information is transferred via dung odours and for how long.

1.1.4. Information transmitted via odours

A wide range of information is accessible from olfactory signals. This information can be fixed (i.e. sex) or variable (i.e. age, reproductive or territorial status), where fixed information could have a genetic basis and variable information effected by hormonal changes (Brown et al. 1996). Males and females possess different chromosomes (i.e. male XY and female XX) with the X and Y chromosomes providing genetically determined differences in expressed genes and consequently causing differences in odours (Harris et al. 2014; Jordan et al. 2011; Vaglio et al. 2016; Yamazaki et al. 1986). Variable information is also identifiable from odours, age for example (Kean et al. 2011; Macdonald et al. 2008; Osada et al. 2003), as well as territorial status (Humphries et al. 1999) and female reproductive state (Achiraman et al. 2010; Archunan and Rajagopal 2013; Karthikeyan et al. 2013).

Different characteristics are identified by either a change in the concentration of a VOC(s) or the sudden appearance of a VOC(s) at the onset of the characteristic. For example, two urinary VOCs (viz. di-n-propyl phthalate and 1-iodoundecane) appear at the onset of oestrus in cows (*Bos Taurus*) (Kumar et al. 2000). Contrastingly, m- and p-cresols emitted from horse (*Equus cabalus*) urine increase in concentration during oestrus (Mozūraitis et al. 2012). Additionally, odours unique to specific individuals have been reported in several species (giant pandas *Ailuropoda melanoleuca* (Hagey and Macdonald 2003), Eurasian otters *Lutra lutra* (Kean et al. 2015), ring-tailed lemurs (Scordato et al. 2007) and raccoons *Procyon lotor* (Kent and Tang-Martínez 2014)).

Many odours can convey multiple messages, such as secretions from the sub-caudal scent glands of European badgers (*Meles meles*) that convey information on sex, age, body condition and reproductive status (Buesching et al. 2002a). Further, urinary marks of mice convey information on male territory ownership, female reproductive state, age, health condition and even individual identity (Achiraman et al. 2010; Brennan 2004; Humphries et al. 1999; Kavaliers and Colwell 1995; Osada et al. 2003).

1.1.5. Communal centres of olfactory information

The transfer of information via olfactory signals is improved if depositors can ensure a high probability that their messages will be received (Alberts 1992). One way that many species do this is to leave their signals at a communal site ensuring that conspecifics will find them. A common form of such a site is an area of communal defecation termed latrine or midden. The use of these middens is common in many mammalian species including ungulates (e.g. oribi (Brachares and Arcese 1999), brown brocket deer *Mazama gouazoubira* (Black-Decima and Santana 2011), Arabian gazelles *Gazella arabica* (Wronski

et al. 2013)) and carnivores (e.g. coyotes (Ralls and Smith 2004), European badgers (Roper et al. 1993), swift foxes *Vulpes velox* (Darden et al. 2008), brown hyenas *Hyaena brunnea* (Hulsman et al. 2010)).

Dung can be deposited alone at middens (e.g. oribi (Brachares and Arcese 1999)) or together with urine (e.g. brown brocket deer (Black-Decima and Santana 2011)) and/or specialised glandular secretions (e.g. European badger (Roper et al. 1986)). Despite the common use of middens by different species, the function of middens is species specific. For example, oribi use middens for territorial defence (Brashares and Arcese 1999), whereas bushbucks (*Tragelaphus scriptus*) use middens for inter-sexual communication (i.e. male-female communication) (Wronski et al. 2006). Middens can be found in the approximate centre of a territory or home range (e.g. swift foxes (Darden et al. 2008)), along the boundary (e.g. oribi (Brachares and Arcese 1999)), or scattered throughout (e.g. white rhinos *Ceratotherium simum* (Kretzschmar et al. 2001; Owen-Smith 1973)), and their location has implications for their function. For instance, middens at the edge of a territory may be used for territorial defence whereas middens in the centre may be used for social group communication (Darden et al. 2008; Dröscher and Kappeler 2014; Roper et al. 1993).

1.1.6. Ritualised behaviours

Many ungulate species that utilise middens also perform ritualised behaviours whilst depositing their scent marks. Linked urination-defecation, or 'dunging ceremonies', are reportedly performed by oribi, Thompsons gazelle (*Eudorcas thomsonii*), Grants gazelle (*Nanger granti*) and springbok (*Antidorcas marsupialis*) (Estes 1991; Skinner and Chimimba 2005). The sequence typically starts with a female urinating and defecating, which then triggers other members of the group to do the same, with a male ending the sequence. Some even leave physical marks while scent marking at middens. Oribi, Thompsons gazelles and sable antelopes (*Hippotragus niger*) scrape the ground before defecating (Estes 1991; Monfort and Monfort 1974), while springboks and black wildebeests (*Connochaetes gnou*) scrape the ground after defecating (Estes 1991; Skinner and Chimimba 2005). Dik-diks (*Madoqua kirkii)* scrape over female marks (Tinley 1969) and suni (*Neotragus moschatus*) scrape after a territorial intrusion (Somers et al. 1990). Both sexes of black rhino scrape with their back feet after defecating (Freeman et al. 2014) while for white rhinos, only territorial males scape when defecating (Owen-Smith 1971). Although scraping behaviour is common in ungulates its function remains understudied.

Over-marking (when one individual places a scent mark on top of another individuals') is a common strategy used by scent marking mammals (Ferkin and Pierce 2007; Jordan et al. 2011; Vogt et al. 2014). By the very nature of cumulative dung piles, over-marking is inevitable, but in some cases has important functions. European badgers over-mark the dung of neighbouring groups at boundary middens, potentially to eliminate rival odours (Roper et al. 1993). Further, male oribi over-mark the dung of females with their own dung, possibly to conceal their reproductive state from rival males (Brachares and Arcese 1999).

1.1.7. White rhinos

The white rhinoceros is the world's largest purely graminivorous animal and is therefore limited to grassland savannahs (Skinner and Chimimba 2005). White rhinos are the most social of all the rhinoceros species, and mixed age and sex social groups are common (Owen-Smith 1973). Territorial males are solitary and hold small defined territories ranging from 0.7-2.6 km^2 (Owen-Smith 1973). They mark their territories by spray urinating at boundaries and defecating in middens throughout their territory (Kretzschmar et al. 2001; Owen-Smith 1973). In contrast, adult females live in large home ranges of 6.1-20.5 km^2 that overlap extensively with those of other adult females and can include the territories of multiple males (Owen-Smith 1973).

White rhinos have poor eyesight but an acute sense of smell and therefore rely heavily on olfactory signals (Owen-Smith 1973). White rhinos of all ages and sex defecate in middens (Owen-Smith 1973). As a result, it is possible that these middens act as information centres for male-male, female-male, male-female, and female-female communication. When a territorial male defecates in the centre of the midden, the dung is scattered by a kicking action with the back feet (Owen-Smith 1971; Owen-Smith 1973). This could be a ritualised marking behaviour, or have an important over-marking function. Either way, this behaviour has been understudied. Middens are found throughout a white rhino's territory and not only localised at boundaries (Kretzschmar et al. 2001), suggesting that middens are used for more than just adult male territory demarcation. Therefore, middens may hold records of territory ownership, the reproductive state of females, and potentially the individual identities of rhinos in the area (Owen-Smith 1973). If this is true, then middens would be important centres of information exchange for white rhinos, yet they remain understudied.

White rhino middens are large, conspicuous, and numerous. Owen-Smith (1975) recorded 30 middens within the territory of one adult male, and they can be up to 20 m in diameter (Marneweck et al. unpublished data). The combination of these factors make white rhinos a perfect model species to study olfactory communication. Despite this, studies of white rhino olfactory communication have been mostly descriptive with little investigation regarding dung VOCs emitted as signals. For example, Cinková and Policht (2014) suggested that white rhinos were able to distinguish sex from dung odours, however, they did not quantify this. Both behavioural studies by Kretzschmar et al. (2001) and Grün (2006) found that vigilance of territorial bulls increased after they found foreign male dung in their territory (including enhanced spray urination and foot dragging), suggesting both the identification of a male and a potential threat. However, neither study quantified which VOCs were eliciting such a behavioural response.

Owen-Smith (1973) described a consort period where a territorial male attaches to a female coming into oestrus for 1-2 weeks before mating occurs. During this time, he follows and restricts her from leaving his territory. Based on this behavioural data, it is likely that males are able to detect the

onset of oestrus from female odours as there are no female behavioural cues to indicate oestrous. Grün (2006) findings support this, where males spent a significant amount of time investigating the dung from breeding females, suggesting the identification of oestrous odours. As an individual's age is potentially identifiable through dung odours in black rhinos (Linklater et al. 2013), it is possible that age is also attainable through dung odours in white rhinos. Territorial male white rhinos have been reported to defecate in middens on average every second day (Owen-Smith 1973), which suggests that dung odours have a short longevity. However, to date none of this has been quantified.

Despite all of the behavioural evidence from studies conducted on white rhinos, there is a gap in our knowledge regarding which VOCs are behind the olfactory signals and how white rhinos use these signals. White rhinos employ a polygynous mating strategy where males defend a territory and mate with multiple females within his territory (White et al. 2007). With this in mind, communication would likely be male-male, in order to advertise territory ownership, and female-male, for males to gather information on the reproductive state of females. Additionally, male-female, for females to assess mate quality, and female-female communication, for social group cohesion, are also possible. Still, our knowledge is limited as to how white rhinos utilise middens. We know very little about what information they can obtain from these middens, or at whom the information is targeted. For example, do all individuals leave information for the territorial male only, or do all individuals pick up information from the middens? In this study I aimed to create a rounded understanding of white rhino olfactory communication, and the function that middens play within white rhino behavioural ecology.

1.2. Aims and Objectives

The broad aim of my PhD study was to better understand white rhino olfactory communication. Specifically, which volatile organic compounds (VOCs) are emitted from dung signals and which are potentially used to communicate specific information. Further, I focussed on how white rhinos utilise communal defecation sites (middens) to access this information. Despite advances in the technology analysing VOCs, no study has investigated the VOCs used by white rhinos, a species that exclusively utilises communal defecation sites (middens) and is renowned for its heavy reliance on olfactory communication. Several behavioural studies exist, investigating the responses to translocated dung, but we still do not know the potential use for middens in white rhino behavioural ecology. To address this, I conducted four studies to investigate different aspects of white rhino olfactory communication. Each of my four focal data chapters addresses one of the following objectives.

i. Identify specific (sex, age, male territorial status and female reproductive status) dung odour profiles from white rhinos and validate the findings with synthetically reproduced odour profiles (male territorial and female oestrus) for an *in-situ* bioassay (Chapter 2).
ii. Assess changes in temporal dung odour profiles from white rhinos of differing states (i.e. sex, male territorial and female reproductive status) (Chapter 3).

iii. Investigate the specific role of middens in white rhino olfactory communication (Chapter 4)
iv. Investigate the function of territorial male dung kicking (Chapter 5).

1.3. Thesis Structure

I have written the focal data chapters (Chapters 2-5) of this thesis in the form of individual scientific papers with each containing an abstract, introduction, methods, results, discussion and references section. The rationale behind this approach is to ease the process of submission for publication in peer-reviewed journals. As a result, there will be some repetition between chapters, especially in the method sections and references. Chapter 1 introduces this study, providing background to how mammals use olfactory communication. Chapter 2 identifies the dung odour profiles of specific characteristics of sex, age, male territorial status, and female reproductive state (published in Proceedings of the Royal Society B). Chapter 3 then examines how these state-specific dung odours change over time, and the impacts this could have on behaviour. Chapter 4 describes the behavioural use of middens (i.e. who is picking up and who is depositing information), and Chapter 5 investigates the purpose of territorial male dung kicking. Finally, Chapter 6 concludes the findings and describes the implications of the study. References have been formatted to the style of the Journal of Chemical Ecology, except for Chapter 2, which is formatted to Proceedings B.

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Chapter 2 : Dung odours signal sex, age, territorial and oestrous state in white rhinos

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Dung odours signal sex, age, territorial and oestrous state in white rhinos

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Mammals commonly communicate olfactorily via urine. However, the extent to which they communicate via dung, another waste product, is unknown. Behavioural studies suggest that mammals can obtain information from dung odours but are unclear about the information transmitted. Moreover, an understanding of the volatile organic compounds (VOCs) released from dung is limited. To address this, we analysed the odours emitted from the dung of free-ranging white rhinos, and found that 2,3-dimethylundecane sig- nalled an individual's sex, heptanal discriminated age class, nonane defined male territorial status and 2,6-dimethylundecane indicated female oestrous state. To validate these findings, we artificially reproduced key elements of the territorial and oestrous odour profiles (i.e. profiles likely to elicit behaviour- al responses from receivers). We then exposed free-ranging territorial males to these odours. In response, males elicited behaviours associated with the specific odours (e.g. territorial male (potential threat): reduced latency in assuming vigilance; oestrous female (potential mate): increased investigation). These results indicate that the VOCs identified from the dung of free-ranging individuals do transmit key information. Moreover, as white rhinos of all ages and sexes defecate communally, middens probably act as information centres. Furthermore, as many other mammals defecate communally, olfactory communication via dung odours is likely a widespread phenomenon.

1. Introduction

Olfactory signals are widely used by mammalian species [1 – 4] and the importance of urine in mammalian olfactory communication is well established [5 – 8]. Yet it is not clear to what extent dung, another waste product, relays specific information about individuals. There are examples of dung odours indicating oestrus in cows (*Bos taurus*) [9] and horses (*Equus caballus*) [10]. However, these are domesticated animals, and wild animals remain understudied. A large amount of information can be obtained from dung samples; for example, hormone metabolites indicating stress [11] or signalling dominance [12]. Yet it is unclear if these conditions are represented in the volatile organic compounds (VOCs) emitted from dung (i.e. odour). It has been suggested that the reason mammals defecate communally is to communicate olfactorily [13,14]. However, data on the VOCs emitted from dung are limited, especially in species using communal defecation sites (i.e. middens).

A wide range of mammals (e.g. oribi antelope (*Ourebia ourebi*) [1]; coyote (*Canis latrans*) [2]) use communal defecation sites and it is thought that these middens may play a key role in olfactory communication. For example, male and female Arabian gazelles (*Gazella arabica*) use middens for different purposes, males for territorial defence and females for social group communication [15]. Bushbuck (*Tragelaphus scriptus*) females leave information (i.e. defecate/urinate) at middens and males respond to this information (i.e. overmark) [13]. White rhinos (*Ceratotherium simum*) have poor eyesight and rely heavily on olfactory signals [14]. As white rhinos of all ages and sexes defecate in middens, it is possible that these middens act as information centres for male– male, female– male and female– female communication. For example, territorial males mark middens

by performing a kicking action with their back legs before and after defecation in the centre of a midden, whereas females do not perform this action and have been observed to defecate at the edge of a midden [14]. Further, middens are found throughout a white rhino territory and not localized at boundaries [16], suggesting that they are used for more than territory demarcation. Therefore, middens may act as information centres and hold records of territory ownership and the reproductive state of females in the area.

Recently, a behavioural study suggested that white rhinos recognize the sex of the depositor from dung odours [17]. However, to date no study has quantified the VOCs released from white rhino dung, nor the information that is relayed by these VOCs. To address this, we aimed to determine the information transmitted (i.e. sex, age, territorial status of males and reproductive state of females) in the dung odour profiles of wild, free-ranging white rhinos. Having achieved this, we then experimentally validated the VOC profiles by arti- ficially replicating key elements of the odours. If our odour profiles were correct, we expected them to elicit behavioural responses from wild, free-ranging territorial males (i.e. the individuals most likely to respond to the odours [18]). We expected territorial males to interpret our replicated odour of a territorial male as a potential rival (i.e. male– male communication) and thus adjust their behaviour accordingly. Specifically, we expected territorial males to increase their visitation to the midden. This would allow them to monitor the presence or absence of the novel male and provide the territory holder with an opportunity to defecate in the midden to reaffirm territory ownership [14,18]. Similarly, we expected the territorial males to increase their frequency of defecation at the midden to reaffirm this ownership. Finally, as many disputes over territorial ownership can be determined by fight- ing [14,18], we expected the territorial males to have a reduced latency in assuming a vigilance posture in response to detecting the odour of a novel territorial male. With regard to the replicated odour of an oestrous female (i.e. female– male communication), we expected territorial males to identify this as an indicator of a potential mate. Territorial males spend more time sniffing the dung of oestrous females compared with the dung of other individuals [14]. As a result, we expected the territorial males in our study to spend more time sniffing the artificial oestrous female odour compared with other odours. In addition, we expected them to increase their frequency of visitation to the midden to reaffirm the presence of our 'oestrous female'. However, as male white rhinos do not ordinarily overmark females [14], we did not expect their defecation frequency to change. Additionally, as there is no threat from the presence of an oestrous female, we did not expect a reduction in their latency in assuming a vigilance posture. Our study ulti- mately explores the VOCs used in white rhino olfactory communication and exposes the role of communal defecation. Moreover, it creates a platform for the further exploration of the theoretical and practical applications of VOCs.

2. Material and methods

(a) Collection of dung odours

We conducted our study in the 896 km^2 Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. Here we collected 150 dung odour samples from wild, free-ranging white rhinos varying in sex (adult male n ¼ 61, adult female n ¼ 46), age (adult n ¼ 107, subadult n ¼ 28, calf n ¼ 15) and state (adult territorial male n ¼ 32 and adult non-territorial male n ¼ 29, adult oestrous female n ¼ 9 and adult non-oestrous female n ¼ 37) between June 2012 and November 2014 using headspace extraction. To do this, we used a dynamic headspace extraction method [19] to collect air for 25 min from approximately 800 g (one bolus) of fresh (less than 5 min old) dung enclosed in a polyacetate bag using a micro-air sampler (Supelco PAS-500) with a realized flow rate of 150 ml min^{21}. VOCs emitted from the dung were captured in a small thermodesorption trap filled with 1 mg of Tenax and 1 mg of Carbotrap. We collected each sample from a different individual, achieved by recording variations in horn shape, skin folds and other distinguishing characteristics.

(b) Identification of characteristics

The age of each individual was categorized into calf (0 – 2 years), subadult (2 – 7 years) or adult (more than 7 years) based on body size and horn development [20]. Territorial male white rhinos are solitary and perform specific marking behaviours, including spray urination and dung kicking [18,21]. Thus, we classified adult males performing these behaviours as being territorial, and adult males not displaying these behaviours as non-territorial. We identified oestrous females via the behavioural reactions of adult males. For white rhinos, there is a consort period where a territorial male moves with an oestrous female for several days. During this time, he follows her closely, restricts her movement beyond his territory boundary and makes several mounting attempts [14]. During sampling we observed males performing these behaviours with a number of adult females, as well as having visible erections and attempting to mount these females. Moreover, we observed four of the ten sampled oestrous females mating during the study. We identified non-oestrous females as adult females without an attached adult male.

(c) Gas chromatography-mass spectrometry analysisof dung odours

We analysed thermodesorption traps using gas chromatography-mass spectrometry (GC-MS). We carried out analysis on a Bruker 450 GC with a 30 m × 0.25 mm internal diameter (film thickness 0.25 mm) Varian VF-5 ms column, connected to a Varian VF-1 ms column (11 m × 0.25 mm internal diameter, film thickness 0.25 mm) coupled to a Bruker 300 quadrupole mass spectrometer in electron-impact ionization mode at 70 eV. Thermodesorption traps were placed in a Varian 1079 injector equipped with a chro- matoprobe thermal desorption device [19]. The flow of helium carrier gas was 1 ml min^{21}. We held the injector at an initial temperature of 2508C 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 458C the temperature of the GC oven was increased to 2608C at 78C min^{21} and, after reaching 2608C, 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; Microsoft SEARCH software v. 2.0d). We verified the identification of VOCs with retention times of authentic standards and published Kovats indices wherever possible (electronic supplementary material, table S1).

(d) Implementation of artificial dung odours

After GC-MS analysis, we artificially created three dung odour treatments: (i) territorial male, (ii) oestrous female and (iii) control, comprising a subset of VOCs based on the raw data and statistical importance from this study (table 1). The top-ranked

Table 1. *The volatile compounds (aliquots) used in the artificial dung odours of territorial male, oestrous female and control 2 (common herbivore dung odours). These substances were mixed together and them 1 ml of the solution added to 1 l of water to create a mixture in which we soaked the artificial bolus.*

VOC name	functional group	territorial male treatment	oestrous female treatment	control treatment 2
phenol	benzenoid	1	1	1
acetophenone	benzenoid	1	1	1
p-cresol	benzenoid	1	1	1
m-cresol	benzenoid	1	1	1
nonanal	hydrocarbon aldehyde	1	1	—
decanal	hydrocarbon aldehyde	1	1	—
acetic acid	hydrocarbon acid	3	1	—
butyric acid	hydrocarbon acid	3	1	—
isobutyric acid	hydrocarbon acid	3	1	—
2-methylbutyric acid	hydrocarbon acid	3	1	—
(E)-caryophyllene	sesquiterpene	1	1	—

VOCs for territorial and oestrous state were nonane and 2,6-dimethylundecane, respectively. The proportion contribution of these VOCs to their respective odours was difficult to discrimi- nate per state (e.g. average proportion nonane for territorial 0.005 versus non-territorial 0.007). As a result, we were concerned about how to recreate this to accurate and sufficient levels. We then looked at the data more closely and found large variations in the proportion of hydrocarbon acids. Because the importance of acids in olfactory communication has been noted [8,22 – 24] and territorial state VOC importance included several acids, we decided to focus our attention on acids to recreate a subset of the dung odours. We selected acetic acid, for example, due to its relative proportions in both male and female odours. Specifically, acetic acid proportions displayed the largest difference between territorial and non-territorial males compared with any other acid. With regard to females, acetic acid was the only acid occurring in higher proportions in oestrous odours compared with non-oestrous odours.

We used two controls. First, the grass bolus (see below) soaked in water in case any VOCs were released by the addition of a liquid. Second, common plant-based herbivore dung odours (table 1), to test if the white rhinos would respond to any new, novel odour within the midden. We wanted to collect data in the most natural conditions possible so as to mimic natural proces- ses and thus create a realistic understanding of odours in the wild. However, in an attempt to control parameters, we deposited the artificial odours during wet-season months (November– March) where midday temperature and humidity were consistent around the average 358C and 75%, respectively. By using fresh dung odour profiles for our artificial dung odours (see below), we were able to simulate fresh dung being deposited into a midden. The baseline measures in our study represent normal behaviours without olfactory manipulation.

In order to validate the VOCs used in white rhino olfaction, we exposed free-ranging territorial males to the artificial treatments. We chose to investigate the treatments from a territorial male perspective as they should elicit the greatest responses to the odours and thus provide the most observable reactions to validate the treatments. Specifically, as territorial males would perceive a rival (i.e. territorial treatment) as a threat, they should show more of a response than either non-territorial males or adult females as they are not defending any resources [25]. Similarly, territorial males should show a greater response to the oestrous treatment over non-territorial males as they monopolize mating [25], and should thus exhibit behaviours to find and successfully mate with the perceived oestrous female. In contrast, we would not expect territorial males to change their behaviour in response to the odour of a subordinate male as a threat or the odour of a non-oestrous female as a breeding opportunity [14]. Thus, we did not generate the profiles of these individuals; if we had, we would not be able to determine whether a 'non-reaction' was because they did not to react to the odour profiles or that our artificial profiles were incorrect.

To expose a territorial male to the artificial odour, we placed an artificial dung bolus soaked in an artificial odour mixture (1 ml artificial odour solution mixed with 1 l water) into a midden. We simulated a white rhino dung bolus by using a woven ball of dried *Digitaria eriantha* grass (150 × 90 × 90 mm approximately) and soaking this bolus in the mixture of 1 ml artificial odour solution and 1 l water (i.e. artificial VOC odour profile) for 1 min prior to deposition in the midden. We used one or three aliquots of the different compounds in our artificial odour solutions (table 1) as these volumes, coupled with the sur- face area of our artificial dung boluses, provided the most natural emission of the odour profile compared with natural dung odours collected over time. To determine this, we compared the similarity between the peaks on the chromatograms from the natural and artificial dung odours. We did this for odour samples taken at 0, 6, 12, 24, 48 and 72 h after defecation. From the similarity in the changes of the peaks over this time period, we concluded that the emission rate of the quantities adminis- tered reflected natural emission rates found in the field. In an attempt to further control parameters, we used the same grass species (*D. eriantha*) for all artificial boluses. We selected ten middens, each with a different resident territorial male (i.e. n ¼ 10 territorial males) identified via variations in horn shape and size. Each male received repeated treatments at random intervals over the experimental period. We aimed for each male to receive each experimental odour four times. However, as behavioural observations were limited to three days after artificial odour depo- sition, many of the males did not visit the experimental middens during a designated odour period, and thus were not exposed to those odours. Nevertheless, in total, each male received an average two artificial territorial treatments and two artificial oestrous treatments (see table 2 for a detailed breakdown).

Artificial boluses were placed in natural locations within the middens, mirroring normal white rhino behaviour (i.e. in the centre for territorial male odour and at the edge for oestrous female odour [14]). Similarity in the changes of the peaks on the chromatograms (see above) showed that both the natural

Table 2. *Number of treatment exposures per individual territorial male.*

territorial male ID	mumber of treatment exposures			
	territorial	oestrous	control	water
M0006T	3	1	1	2
M0132T	2	2	0	0
M0142T	2	1	1	1
M0127T	4	3	1	1
M0128T	3	1	1	1
M0129T	1	3	1	1
M0131T	3	3	1	1
M0113T	3	2	1	1
M0079T	1	0	0	1
M0136T	0	3	1	1

white rhino dung odours and our synthetic odour mixtures lasted for approximately 48 h. Thus, to ensure that we covered the entire 48 h emission period, we extended our behavioural data collection beyond 48 h to 3 days (i.e. 72 h) after we depos- ited the artificial dung bolus containing the replicated VOC odour profiles into the middens. We carried out the experiment and baseline observations during the wet season of October 2014 – March 2015 and created an identification profile for each adult rhino (e.g. horn shape and size, ear notches) so that we could record individual behaviours.

To determine behavioural changes in territorial male response to the replicated VOC odour profiles, we explored four aspects of behaviour: (i) visitation frequency to the midden, (ii) defecation frequency at the midden, (iii) duration of sniffing events and (iv) latency to a vigilance posture. We used motion-triggered infra- red 'no-glow' video recording camera traps (either a Cuddeback Black Flash E3 or Cuddeback Attack Black Flash 1194 model) placed approximately 3 m from the edge of the midden. This pro- vided a sufficient field of view and allowed us to record the different behavioural reactions. We used 'no-glow' cameras as they do not emit visible light or have a flash, creating minimal dis- turbance at the midden and, therefore, allowing us to capture natural behaviour. We programmed the cameras to record 30 s videos at each trigger with a 1 s delay before becoming active again and obtained the behavioural data of the territorial males' responses from the videos.

(e) Statistical analysis

Absolute concentration is subject to variability across samples, therefore we used relative abundance of a VOC within a sample (i.e. proportion) for statistical analyses. In order to determine the characteristic odour profiles of sex, age class and territorial/ oestrous status of adult males/females, we analysed the proportion of VOCs emitted from white rhino dung using a random forests classification algorithm [26] within the R package randomForest [27]. VOC datasets contain more variables than samples; for example, headspace samples from this study contained up to 150 VOCs and each VOC acts as a single variable. As a result, we are limited in our ability to use the entire dataset for analyses. There- fore, we used random forests, a classification algorithm with features making it well suited to the analysis of VOC datasets. For example, it allows for more variables than samples, it does not overfit the data, it has high classification efficiency and it can create a minimal set of variables which can be used as group predictors. For each iteration we used default parameters for both number of permutations (ntree ¼ 500) and the number of randomly selected predictor variables at each split (mtry ¼ $\sqrt{p}$, where p ¼ number of variables) as outputs were unchanged when adjusting these parameters. We calculated a classification accuracy for each random forest using out-of-bag error rates. In order to provide an interpretation of the best predictors (i.e. VOCs) for each character- istic from the random forest, we calculated a measure of variable importance using the importance function of the randomForest package and the metric mean decrease in accuracy (MDA) [28]. The MDA is the increase in the percentage of times the outcome is misclassified when the variable is randomized. Therefore, a higher MDA means less misclassification and thus greater accuracy, and ultimately indicates higher variable importance to the classifi- cation of a characteristic. All VOCs representing undigested waste plant material were removed from the analysis [29,30]. For the age class analysis all white rhino samples were used (n ¼ 135). For sex and territorial/oestrous status, we limited our samples to only adult white rhinos (n ¼ 92).

For the behavioural responses, we calculated the visitation and defecation frequencies by the number of visits or defecations divided by the number days the camera was active. We defined sniffing events as standing still with the nose less than 20 cm above ground and nostril flares. We calculated the duration of the sniffing event as the number of seconds from nose less than 20 cm to nose more than 20 cm above ground and events were sep- arated by 2 s. We calculated the latency to assuming a vigilance posture as the number of seconds from the start of the sniffing event until vigilance posture was assumed (i.e. head up, standing still and ears rotating). If no vigilance posture was assumed, then a default 300 s was recorded. We recorded behaviours using open source behavioural coding software CowLog [31]. We com- pared each aspect of behaviour with baseline behaviours without odour manipulation and the artificial profiles with one another. As data were not normally distributed, we analysed them using non-parametric Kruskal–Wallis with a post hoc Dunn's test. We performed all statistics in RStudio v. 0.99.491 for Windows [32] and created all figures using SigmaPlot v. 8.0 for Windows.

3. Results

In our first experiment, we found that the sex, age class, male territorial and female oestrous state of an individual white rhino could be determined from the VOC profile of its dung. In total, we identified 225 VOCs emitted from the dung of white rhinos, classified within 13 functional groups (electronic supplementary material, table S1). Using a random forests classification algorithm, we identified the most important VOCs for distinguishing each characteristic. The random forest was most successful at differentiating sex (classification accuracy of 77.17%) with 2,3-dimethylundecane identified as the most important VOC for classifying this characteristic (figure 1*a*). In addition, heptanal was the most important VOC for discriminating age class (figure 1*b*), with a classification accuracy of 68.89%. With regard to defining male territorial state, we found that nonane was the most important VOC (figure 1*c*), with a classification accuracy of 55.93%. Finally, 2,6-dimethylundecane was the most important VOC for defining oestrous state in females (figure 1*d*), with a classi- fication accuracy of 72.73%. Therefore, we were able to successfully determine exact odour profiles and effectively indicate the differences between each state.

In our second experiment, during the replicated territorial male odour treatment, resident territorial males responded by significantly increasing visitation frequency to the midden

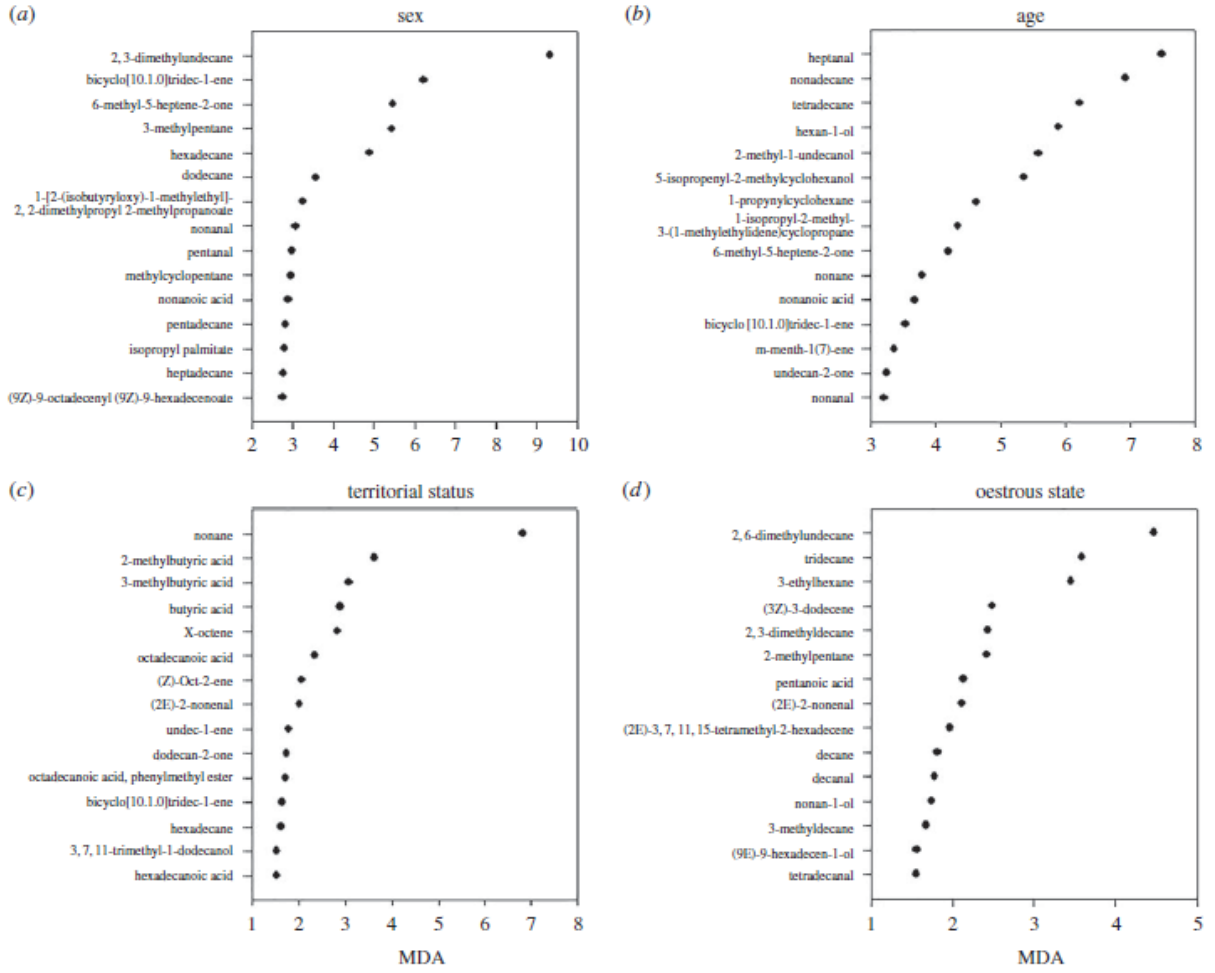

Figure 1. *The importance of volatile organic compounds (VOCs) distinguishing (a) sex, (b) age, (c) territorial state of adult males and (d) oestrous state of adult females emitted from white rhino dung. Importance was based on mean decrease in accuracy (MDA). Only the top 15 compounds are presented in the figure.*

($H_{(4)}$ ¼ 13.036, p ¼ 0.002; figure 2*a*) and decreasing latency to vigilance posture ($H_{(4)}$ ¼ 10.686, p ¼ 0.012; figure 2*b*) compared with baseline behaviour (i.e. normal behaviours with no olfactory manipulation). They did not change their frequency of defecation ($H_{(4)}$ ¼ 3.586, p ¼ 0.125; figure 2*c*) or the duration of their sniffing events ($H_{(4)}$ ¼ 6.134, p ¼ 0.458; figure 2*d*). In response to the replicated oestrous female odour treatment, territorial males significantly increased the duration of sniffing events ($H_{(4)}$ ¼ 6.134, p ¼ 0.011; figure 2*d*) and increased their visitation frequency to the midden ($H_{(4)}$ ¼ 13.036, p ¼ 0.001; figure 2*a*). The latency to vigilance posture increased (i.e. they did not assume a vigilance posture; $H_{(4)}$ ¼ 10.686, p ¼ 0.024; figure 2*b*) and they did not adjust frequency of defecation ($H_{(4)}$ ¼ 3.586, p ¼ 0.398; figure 2*c*) compared with baseline behaviour.

Comparing the territorial males' behavioural reactions with each of the artificial odour profiles, we found that the midden visitation frequency of territorial males was 15% greater in response to the artificial oestrous female odour compared with the artificial territorial male odour, although non-significant ($H_{(4)}$ ¼ 13.036, p ¼ 0.259). Furthermore, they spent significantly more time sniffing the artificial dung odour of an oestrous female ($H_{(4)}$ ¼ 6.134, p ¼ 0.021) and showed a longer latency to vigilance posture ($H_{(4)}$ ¼ 10.686, p , 0.001) compared with that of the artificial territorial male odour. There was no difference in the defecation frequency ($H_{(4)}$ ¼ 3.586, p ¼ 0.243) between the oestrous and territorial odour treatments.

Finally, territorial males did not respond to our control odours compared with baseline behaviours (control 1: visitation frequency $H_{(4)}$ ¼ 13.036, p ¼ 0.218; latency to vigilance posture $H_{(4)}$ ¼ 10.686, p ¼ 0.409; defecation frequency $H_{(4)}$ ¼ 3.586, p ¼ 0.496; duration of sniffing event $H_{(4)}$ ¼ 6.134, p ¼ 0.483; control 2: visitation frequency $H_{(4)}$ ¼ 13.036, p ¼ 0.110; latency to vigilance posture $H_{(4)}$ ¼ 10.686, p ¼ 0.417; defecation frequency $H_{(4)}$ ¼ 3.586, p ¼ 0.163; duration of sniffing event $H_{(4)}$ ¼ 6.134, p ¼ 0.248).

4. Discussion

Many mammals transmit information via their urine [5 – 8]; however, the extent to which they transmit information via their dung is unclear. Some behavioural studies have suggested that mammals can identify sex [17], age [33] or oestrous state [34] from dung odours. However, none have indicated whether a wide range of information (e.g. sex, age, territorial status and oestrous state) is transmitted in the dung odour of a single species, nor identified the VOCs that transmit this information. Here we show that white rhinos transmit information

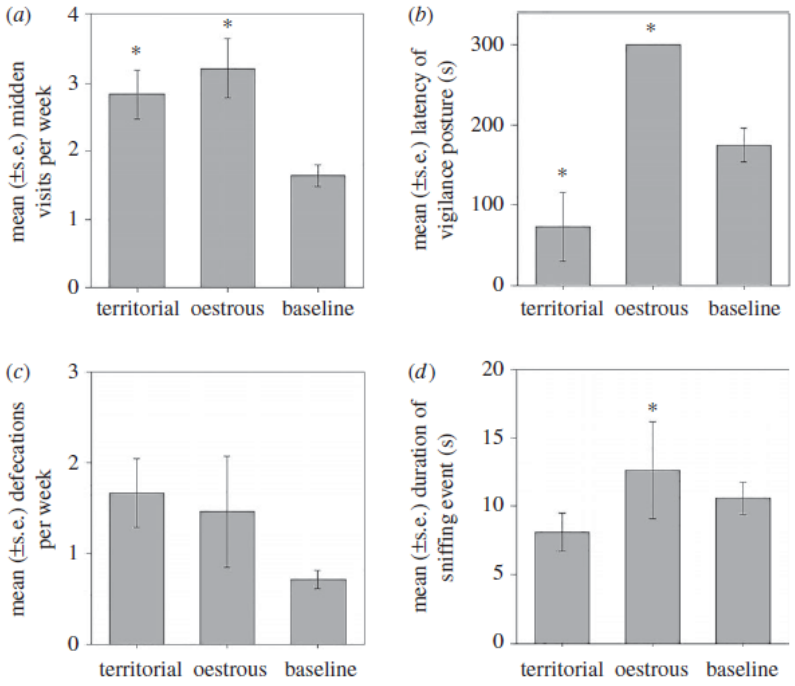

Figure 2. *Territorial male (a) midden visits per week, (b) latency of the vigilance posture, (c) midden defecations per week and (d) duration of sniffing events during odour manipulation of replicated territorial male, replicated oestrous female and no odour manipulation (baseline). Asterisks indicate significant difference to baseline.*

on sex, age, territorial status and oestrous state via the VOCs emitted from their dung. Moreover, we identify the specific VOCs responsible for transmitting this information. This is a first for a species using communal defecation sites. Finally, we produced artificial odour blends representing a territorial male and an oestrous female, comprising key VOCs from these odour profiles. We then used these artificial odours to elicit specific behaviours associated with the different odours from free-ranging territorial males.

Odour differences can be a result of genetic distinctions; for instance, the presence of an X or Y chromosome produces a unique odour signature [35]. Age class differences are prob- ably due to hormonal variations and immature physical development, where diet and gut development also influence odours [36,37]. Bacteria play an important role in mammalian olfactory communication [38]. The fermentation hypothesis proposes that the symbiotic bacteria living within scent glands break down organic material and produce VOCs that ultimately contribute to mammalian recognition cues [39]. The variation in composition and abundance of these bacterial communities then creates a unique individual odour, thereby allowing recognition by other individuals [40]. Although the fermentation hypothesis was developed for mammals that scent mark with specialized glands, it has been suggested that it could be applied to mammals that mark with faeces or urine [38]. The interaction between bacteria and hormones can also affect odours directly via their presence within glands. For example, differences in the anal gland microbiota of both male and female meerkats (*Suricata suricatta*) occur only after individuals reach sexual maturity, suggesting that reproductive hormones have a role in determining host bacterial communities [41]. Sex differences in the microbiota of adults have also been ident- ified in greater sac-winged bats (*Saccopteryx bilineata*) [42] and white-tailed deer (*Odocoileus virginianus*) [43]. This inter- action can also affect odours via the breakdown of hormone metabolites post excretion, for example microbes mediate the timed release of semiochemicals from the urine of male musth elephants (*Loxodonta africana*, *Elephas maximus*) [44]. Bacteria and hormones can also have an indirect effect on odours via behaviour modification. Higher testosterone levels can cause increased locomotor activity [45] and increased metabolic rate [46]. Both of these have subsequent effects on feeding, and therefore gut content, ultimately leading to changes in bacterial activity which could lead to differences in odours. Territorial males have significantly higher concentrations of faecal testosterone than non-territorial (sub- ordinate) adult males [12]. Therefore, for adult male white rhinos achieving territorial status, the subsequent associated increase in testosterone [12] may affect microbiota directly or indirectly. However, the random forests algorithm was least accurate at distinguishing the territorial state of males. This may be due to territory acquisition taking up to 5 years for an adult male [14]. Thus, there could be an uncertain period before adult males obtain their own territory, but are physically able to do so (i.e. higher testosterone levels). Interestingly, nonane, ranked the most important VOC, is not currently cited in relation to dominance or territory ownership. Yet some of the other VOCs identified (e.g. 2- and 3-methylbutyric acid; figure 1*c*) are the same VOCs suggested to represent dominance in other mammals [47].

Examples of female oestrous odours come primarily from urine and vaginal secretions [8,48 – 50], while those obtained from dung odours of wild animals are limited.

With regard to female white rhinos, we found a decrease, and in most cases a complete disappearance, in the proportion of several VOCs emitted during oestrus. Specifically, oestrous females emitted lower proportions of alkanes and alcohols. These same functional groups have been identified with roles in signalling reproductive state in the odours of dom- estic cow faeces [9]. However, most studies of oestrous odours have identified a higher concentration or sudden appearance of VOCs during oestrus. A potential explanation for the lower proportion of VOCs emitted from oestrous female white rhino dung could be due to the fermentation– absorption process in hindgut fermenters [51]. VOCs can be absorbed in the hindgut before they are released with faecal matter, and this can contribute significantly to host energy requirements [52,53]. White rhinos are very efficient in the absorption of VOCs in the hindgut [54], and ovulation, ges- tation and lactation are energetically costly to females [55]. As the absorption of VOCs may be under hormonal control [52], hormones may indirectly affect dung odours where oes- trous females may be absorbing higher levels of VOCs during oestrus in preparation for the subsequent strain on body condition. Further, as with males, this may be related to hormone-induced behaviour and its subsequent impact on bacteria, where females could have reduced food intake during oestrus [56].

Territory holders must manage both potential threats and mating opportunities in order to defend their territory and increase their fitness [18]. Using artificial odours comprising key elements of the complete odour profile, we showed how novel olfactory information could stimulate predicted behaviours [21]. Specifically, territorial males responded repeatedly to the replicated odour of a novel territorial male as a potential rival. They did this by increasing their vis- itation to the midden and reducing their latency in assuming a vigilance posture. Increasing midden visitation probably allows territorial males the opportunity to reassess the odour and the presence of the rival [57,58]. Shorter latency in assuming a vigilance posture, however, permits the terri- torial males to prepare for a potential aggressive encounter with another male [58]. In contrast with our expectations, the defecation frequency of territorial males did not increase in response to our artificial territorial male odour profile. We suggest that this is due to the limited availability of dung as a marking resource [1]. Despite this, overall, the behavioural responses of the territorial males to our artificial VOC profiles suggest that the key VOC elements that we identified from free-ranging white rhinos are the key VOCs that signal territorial ownership in white rhino males.

In response to our replicated oestrous female odour (i.e. a potential mate) territorial males showed repeated high levels of interest, which was unaffected by the depo- sition of dung by other individuals in the midden. In line with observations of free-ranging males responding to the dung odours of oestrous females [14], the territorial males in our study increased their duration of sniffing events of the artificial oestrous female odour. These males also increased their visitation frequency to the midden. Our manipulations did not provide an odour trail away from the midden for the males to follow [14], thus we suggest that this behaviour probably indicates a reaffirmation of the presence of our 'oestrous female'. The combination of these results suggests that, as with the scent profile of territorial males, the key VOCs we identified for oestrous females are an accurate reflection of an oestrous female odour profile.

Overall, the territorial males showed greater interest in the artificial oestrous female odour (i.e. more frequent midden visitation, more time spent sniffing) compared with our artificial territorial male odour. Males establish territories to defend high-quality resources so that they can monopolize mating opportunities [25]. Thus, the greater behavioural response to oestrous odours could indicate males looking to maximize their breeding opportunities. It is possible that the VOCs used in our odour replication study may simply indicate sex, and not specifically territorial or oestrous state. Yet territorial males do not show interest in non-oestrous females (e.g. extensive smelling of their dung), and tolerate subordinate males living within their territories (i.e. they do not see them as a threat) [14]. Thus, if our artificial odours signalled only the sex of an individual, then it would have been unlikely for the territorial males to have reacted as dramatically as they did to our artificial oestrous female odour (i.e. increased the duration of their sniffing events) and our artificial territorial male odour (i.e. reducing their latency in assuming a vigilance posture). Thus, we feel confi- dent that our artificial odours do in fact transmit information about territorial status and oestrous state.

Identifying the information transmitted in dung odours is essential for understanding how communally defecating mammalian species communicate. Despite recent progress in the field of mammalian olfactory communication, examples of specific odour profiles with subsequent bioassays in the wild are rare. Yet not all the VOCs in the odour profiles trans- mit information. To determine which VOCs are important requires confirmation of behavioural responses towards a sub- stance in a bioassay, similar to our study. Our study shows that the composition of white rhino dung odours differ with sex, age, territorial status and oestrous state. Thus, we show that dung, a waste product, is a valuable medium for transmitting biological information for male– male, female– male and potentially female– female communication. Based on these results, we propose that white rhinos use middens to deposit and extract a wealth of biological information. If correct, then this helps explain the phenomenon of communal defecation in a large number of mammalian species [1–3]. Finally, the suc- cess of our replicated odour profiles in eliciting desired behavioural responses provides a platform for further research into the theoretical and practical applications of VOCs for a wide range of mammalian species.

Ethics. The study followed local animal ethics guidelines and was granted ethics clearance from the University of KwaZulu-Natal Animal Research Ethics Committee.

Data accessibility. The data supporting this article can be obtained from the Dryad Digital Repository [59].

Authors' contributions. C.M. expanded the initial idea, collected the data and carried out the statistical analyses; A.J. helped to develop the initial project, processed GC-MS samples and produced artificial dung odours; A.M.S. created the initial project and further developed it with the co-authors. All authors discussed the results, wrote the manuscript and gave final approval for publication.

Competing interests. We declare that we have no competing interests.

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Chapter 3 : White rhinos (*Ceratotherium simum*) do not alter scent-marking behaviour in response to seasonal declines in dung odour longevity

3.1. Abstract

In order for an olfactory signal to be effective it must persist in the environment for an extended period. However, there are both biotic (e.g. microorganisms) and abiotic factors (e.g. temperature) that affect odour longevity. White rhinos of all ages and sex defecate in middens and their dung odours transmit information about sex, age, territorial and oestrous states. As these odours relay important biological information, it would be essential for an odour to persist long enough for other individuals to obtain the information. Here I examine how the dung odours of adult white rhinos (male: territorial and non-territorial, female: oestrous and non-oestrous) change over the short (hours) and long (seasons) term using headspace extraction. Additionally, I measured seasonal midden visitation and defecation frequency to link this to seasonal changes in odour longevity. I found that over a short period, male dung odours had shorter longevity than female odours. Within males, territorial odours had shorter longevity than non-territorial, while non-oestrous female odours had a shorter longevity than oestrous odours. I suggest that the elevated testosterone and progestogen metabolites, respectively, effect dung odours indirectly. Seasonally, the high temperature and humidity of the wet season caused increased emission (decreased longevity), which affected all adult dung odours. However, white rhinos did not adjust their visitation or defecation frequency during the wet season to counteract this decrease in longevity. My results highlight the limitations of utilising dung as a marking resource and provide insight into how dung odours in general may change over time.

3.2. Introduction

Many mammals use olfactory communication to aid in finding potential mates, assessing their reproductive potential and maintaining territory boundaries (Archunan and Rajagopal 2013; Barja et al. 2005; Eisenberg and Kleiman 1972). However, in order for an olfactory signal to be effective, it must persist in the environment in the absence of the sender (Eisenberg and Kleiman 1972). Olfactory signals consist of volatile organic compounds (VOCs) and the volatility of these compounds depends on factors such as their molecular weight and vapour pressure (Apps et al. 2015; Stoddart 1976). For example, VOCs with high molecular weight and low vapour pressure persist longer in the environment. The suggested ideal range for olfactory signals is a molecular weight between 50 and 300 g/mol (Wheeler 1977), where 300 g/mol is approaching the upper limit for volatility. These factors have important repercussions for the information in odours being stable over time (i.e. the heavier a VOC the longer it persists in the environment), which ultimately means a stable signal. However, an odour signal that lasts too long loses its potential to indicate the proximity of the depositor, while an odour signal that is lost

too quickly loses its ability to be effective in transferring information before another individual can encounter it. Therefore, the VOCs utilised by mammals to transfer information becomes function specific. For example, alarm signals are highly volatile (low molecular weight) so that they can dissipate once the threat has gone (Verheggen et al. 2010). Contrastingly, territorial signals tend to have low volatility (high molecular weight) so that they can persist in the environment (Hurst et al. 1998).

In addition to the molecular weight of a VOC, environmental conditions also affect odour release. High levels of humidity and temperature (e.g. hot summer rainy season compared to cool dry winters) can increase the emission of VOCs (Bossert and Wilson 1963; Nimmermark and Gustafsson 2005; Regnier and Goodwin 1977) which ultimately decreases the longevity of an odour. This was evident with Carpetan rock lizard (*Iberolacerta cyreni*) femoral gland secretions, where both the detectability and persistence were lower at higher temperatures (Martín and López 2013). With short-term (e.g. daily) and long-term (e.g. seasonal) changes occurring in both temperature and humidity, there are expected associated changes in the emission of VOCs and hence the persistence of the message over time. To counteract these variations in temperature and humidity animals could alter their behaviour to ensure effective information transfer. For example, they could increase their scent marking frequency in humid summers when conditions decease odour longevity. The scent mark frequency of black-tufted marmosets (*Callithrix penicillata*) was positively correlated with humidity (Oliveira and Macedo 2010) and aardwolves (*Proteles cristata*) also scent mark more frequently during the wet season (Marneweck et al. 2015). Many examples of increased scent marking during the wet season have been attributed breeding season (Marneweck et al. 2015; Pochron et al. 2005). However, it may be possible that there are multiple compounding factors driving seasonal increases in scent marking, including temperature and humidity.

White rhinos (*Ceratotherium simum*) of all ages and sex defecate communally in middens to communicate with conspecifics (Owen-Smith 1973). Dung odour profiles from white rhinos transmit information on sex, age, territorial status of adult males, and the oestrous state of adult females (Marneweck et al. 2017). This suggests that middens are used for both male-male and female-male communication. For males, in particular territorial males, middens are used to advertise territory ownership. Thus, it would be evolutionary advantageous for a territorial signal to persist for a long time. For instance, if an odour lasts for several days, males can spend less time remarking and more time finding mates and ultimately increasing fitness (Gosling and Roberts 2001). For females, in particular oestrous females, middens are likely used to advertise reproductive state and assess potential mates (Owen-Smith 1975). White rhinos employ a polygynous mating strategy where territorial males mate with multiple females within his territory (White et al. 2007). Female white rhinos range much larger areas than territorial males (up to 10 times the area) (Owen-Smith 1973), so in order for the female to ensure males can find her, it would be advantageous for oestrous odours to last longer than non-oestrous odours.

Territorial male white rhinos have been reported to defecate in middens on average every second day (Owen-Smith 1973), which suggests that white rhino dung odours have short longevity, although this has not been tested either *in situ* or *ex situ*. Further, studies investigating the temporal changes of odours are dominated by glandular secretions (Buesching et al. 2002; Hayes et al. 2006) and urine marks (Goodwin et al. 2012; Kwak et al. 2013). There are few studies investigating the behavioural responses to dung of increased age (Linklater et al. 2013), but there are no studies investigating detailed temporal changes of dung VOCs. Many mammals mark with dung (Brachares and Arcese 1999; Ralls and Smith 2004; Roper et al. 1993) and these temporal changes in odour profiles have important implications for behaviour.

To address this, I assessed how white rhino dung odours changed over time, both short (hours) and long-term (seasons). In addition, I explored whether white rhinos deal with environmentally driven changes behaviourally by recording both midden visitation (i.e. acquiring information) and defecation frequency (i.e. depositing information) during the different seasons (i.e. wet and dry). An increase in midden visitation during the wet season would indicate that in order to acquire relevant information on other individuals, one must make more visits before the odours disappear. An increase in defecation during the wet season would indicate that information deposited is not available for as long and needs replacing. The initial dung odours of males and females differ (Marneweck et al. 2017), therefore, their profiles of odour degradation will differ as well. As long-lasting territorial marks are beneficial, I predict that adult male (specifically territorial male) dung odours, will last the longest of all adults. Within females, I predict that oestrous odours will last longer than non-oestrous to facilitate discovery and thus breeding opportunities. To explore this, I tested the following hypotheses with regard to adult white rhinos: (1) male dung odours will last longer than female. (2) Within males, territorial dung odours will last longer than non-territorial. (3) Within females, oestrous dung odours will last longer than non-oestrous. (4) For all adults, the duration of dung odours will be reduced during the wet season, and (5) all adults will increase both visitation and defecation frequency during the wet season to counteract the reduction in odour duration.

3.3. Methods

3.3.1. Collection of dung odours

I conducted this study in the 896 km^2 Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. Here I collected 66 dung samples from different adult male (territorial and non-territorial) and female (oestrous and non-oestrous) white rhinos (Table 3.1). Each sample represents a different individual (i.e. no individual was sampled more than once). Approximately 800 g of dung was sampled per individual, either one dung bolus, or a collection of scattered dung in the case of territorial males. For each individual (n= 66) an initial sample was taken <5 minutes from defecation (Table 3.1) and each sample was subsequently resampled four more times, at intervals 6, 12, 24 and 48 hours after defecation, to create a profile of odour degradation (total n= 264). During this resampling period, the bolus remained

as one solid bolus, with the exception of territorial male scattered dung, which was left to degrade in its dispersed state. I identified individuals through variations in horn shape, skin folds, and other distinguishing characteristics. I defined adults as individuals >7 years of age, based on body size and horn development (Hillman-Smith et al. 1986). Territorial males were identified as adult males performing territorial behaviours (i.e. dung kicking, spray urinating) and non-territorial as adult males not performing these behaviours (Owen-Smith 1971; Owen-Smith 1973). Oestrous females were identified via the behavioural reactions of males (e.g. mounting), and non-oestrous identified as females without the attachment of an adult male (Owen-Smith 1973). However, as oestrous females were identified behaviourally, it is possible that I classified some oestrous females as non-oestrous. Due to the attention paid by males towards oestrous females (Owen-Smith 1973, 1988), the misclassification of females is likely to be rare. I collected samples between March and November 2014, with the wet season defined as October-March and the dry season April-September. Midday temperatures were on average 35˚C and humidity at 75% during the wet season and on average 27˚C and 56% during the dry season sampling period (Marneweck et al. unpublished data). The rainfall and conditions over these months were consistent with the ten-year average (Ezemvelo KZN Wildlife; unpublished data).

Odour samples were collected from a dung bolus using dynamic headspace extraction (Amirav and Dagan 1997). To do this I collected the air for 25 minutes from a dung bolus enclosed in a polyacetate bag using a micro-air sampler (Supelco PAS-500) with a realized flow rate of 150 ml/min. VOCs emitted from the dung were captured in a small thermodesorption trap filled with 1 mg of Tenax® and 1 mg of Carbotrap®. I limited sampling to 48 hours as my pilot study data revealed that dung odours did not last longer than three days (Appendix 2). With such short longevity, I assumed that vast changes would occur relatively quickly, therefore, I chose shorter intervals for the beginning of the sampling period. To collect odours over time, I left dung samples outside on natural substrate (short grass), unprotected, between sampling intervals to expose them to environmental conditions and for natural degradation to occur.

Table 3.1. Number and breakdown of initial dung odour samples (i.e. samples taken at 0 hours from defecation) collected over the study period.

	Male		Female		
Season	Territorial	Non-territorial	Oestrous	Non-oestrous	Total
Wet	10	11	3	11	35
Dry	11	9	2	9	31
Total	21	20	5	20	66

3.3.2. Gas chromatography-mass spectrometry analysis of dung odours

I carried out gas chromatography-mass spectrometry (GC-MS) analysis of the odour samples on a Bruker 450 GC with a 30 m x 0.25 mm internal diameter Varian VF-5ms column connected to a Varian VF-1ms column coupled to a Bruker 300 quadrupole mass spectrometer in electron-impact ionization mode at 70 eV. Thermodesorption traps were placed in a Varian 1079 injector equipped with a chromatoprobe thermal desorption device. I 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). I verified the identification of VOCs with retention times of authentic standards and published Kovats indices wherever possible (Appendix 1).

3.3.3. Collection of behavioural data

To collect data on midden visitation and defecation I setup motion-triggered video recording camera traps at ten middens, each with a different resident territorial male (identified via differences in horn shape and size), from October 2014 to August 2015. These are the same sites and cameras as in Chapter 2, but the periods of odour manipulation are excluded. This resulted in approximately five months of dry season data and five months of wet season data for each midden. The males recorded here are different to those sampled for the collection of dung odours (i.e. none of the focal males were sampled for dung odours). An average territory is 1.65 km^2 (Owen-Smith 1975), therefore, focal middens were separated by at least 2 km to ensure disconnection. Moreover, using variations in horn shape, skin folds, and other distinguishing characteristics I was able to determine that the males using the focal middens were in fact different. I recorded data on adults only and each adult was categorised into either territorial male, non-territorial male or female. It was not possible to identify oestrous state from video footage alone so I grouped all adult females together for analysis. I used infrared ‘no-glow’ camera traps (either a Cuddeback Black Flash® E3 or Cuddeback Attack Black Flash® 1194 model) placed approximately 3 m from the edge of the midden to allow for sufficient field of view. I used ‘no-glow’ cameras as they do not emit visible light or have a flash, creating minimal disturbance at the midden and therefore allowing me to capture natural behaviour. I programmed the cameras to record 30-second videos at each trigger with a 1-second delay before becoming active again and downloaded data every two weeks.

From 2403 data videos, containing over 20 hours of footage, I created an ID profile for each adult white rhino so that I could record individual visitation and defecation.

3.3.4. Statistical analysis

Absolute concentration of VOCs is subject to variability across samples so I used relative abundance of a VOC within a sample (i.e. proportion) for statistical analysis. White rhinos shift their diet from short grass species, such as *Panicum coloratum* and *Urochloa mosambicensis*, during the wet season to medium-tall grasses such as *Themeda triandra* in the dry season as a way of coping with decreasing food quality (Owen-Smith 1988). In this study, I was interested to see how the information representing biologically relevant information (i.e. sex, territorial and reproductive status) changed over time, without the influence of background noise. Therefore, I excluded any VOCs that represented undigested plant material (Gershenzon and Croteau 1991a; Ishida 2005). These included limonene, p-cresol and linalool (for a full list of VOCs, see Appendix 1).

In order to determine if there was any short-term change in dung odour per season, and the degree of change between odours at 0 hours and odours at 48 hours, I carried out an analysis of similarity (ANOSIM) based on Bray-Curtis similarities of square-root transformed proportions for each sex (male, female), male state (territorial, non-territorial) and female state (oestrous, non-oestrous). R values close to 1 indicate high separation (i.e. very different) while R values close to 0 indicate no separation (i.e. not different). Further, I ran a similarity percentage (SIMPER) test to assess short-term change in VOC composition across the wet and dry season for each sex (male, female), male state (territorial, non-territorial) and female state (oestrous, non-oestrous). From the SIMPER test, I recorded the top three VOC contributors to each odour profile during both seasons.

To test factors effecting individual VOC contribution I ran a linear mixed-effects model using the R package nlme (Pinheiro et al. 2015). I set the proportion contribution of the VOC as the response variable and season, time (i.e. hours from defecation), VOC functional group, plus their interactions, as fixed factors, and sample ID as a random factor. This was repeated for each sex (male, female), male state (territorial, non-territorial) and female state (oestrous, non-oestrous). To investigate factors effecting the number of VOCs emitted (i.e. emission) I ran another linear mixed-effects model. I set the number of VOCs emitted as the response variable, season and time plus their interaction as fixed factors and sample ID as a random factor. This was repeated for each sex (male, female), male state (territorial, non-territorial) and female state (oestrous, non-oestrous).

I calculated individual visitation and defecation frequency by the number of visits or defecations divided by the number 30 day periods (range= 5-9 30-day periods). To assess seasonal differences in midden visitation and defecation frequency I ran a linear mixed-effects model. I set visitation or defecation frequency as the response variable, season as the fixed factor and rhino ID as a random factor. I performed ANOSIM and SIMPER tests in Primer version 6.1.15 and linear mixed-effects models in

RStudio version 0.99.491. All figures were created using RStudio, with MDS plots created using the vegan package (Oksanen et al. 2015).

3.4. Results

3.4.1. Odour profiles

3.4.1.1. Sex

Male dung odours changed significantly over both the wet and dry season (wet Global R=0.300, p=0.001; dry Global R=0.215, p=0.001). A moderate degree of change occurred between 0 and 48 hours during both seasons, but degree of change was higher during the wet season (wet R=0.642, p=0.001, Fig. 3.1a; dry R=0.522, p=0.001, Fig. 3.1b). Female dung odours also changed significantly over both seasons (wet Global R=0.202, p=0.001; dry Global R=0.051, p=0.046). A moderate degree of change occurred between 0 and 48 hours during the wet season (R=0.475, p=0.001, Fig. 3.2a). However, there was a very low degree of change between 0 to 48 hours during the dry season (R=0.097, p=0.040, Fig. 3.2b).

The odour composition of male dung was effected by a three-way interaction between season, time, and VOC functional group ($F_{28,1200}$=1.767, p=0.008). As environmental conditions became hotter and more humid, initial male odours were comprised of mostly hydrocarbon alkanes and acids, while aged dung odours were dominated by hydrocarbon aldehydes such as nonanal and decanal (Fig. 3.3a, Table 3.2). During the wet season, male dung odours were characterised by the increase of acids between 6-12 hours, including acetic and butyric, and aldehydes between 12-48 hours from defecation (Table 3.2). Further, during the hot and humid months, there was also a decrease in complexity over time (i.e. fewer VOC functional groups present, Fig. 3.3a). As conditions became cooler and drier, the dung odour composition and complexity remained relatively stable over the 48-hour time period (Fig. 3.3b). During the dry season, male dung odours were also characterised by the increase of acetic and butyric acids between 6-12 hours from defecation (Table 3.2).

The odour composition of female dung was effected by an interaction between time and VOC functional group ($F_{28,694}$=7.005, p<0.001). Alkanes showed an initial decrease but then re-established and increased over time, whereas acids increased up until 12 hours and then decreased (Fig. 3.4a,b). Several acids, including acetic, butyric, and 2-methylbutyric, remained prominent over 6-12 hours, and the alkane tridecane remained a large contributor to the odour over the 48-hour sampling period (Table 3.3). In contrast to male dung odours, season had no effect on the odour composition of female dung ($F_{1,18}$=1.723, p=0.206) and odours sustained their complexity (i.e. number of VOC functional groups present) over time.

The number of VOCs emitted from male dung was effected by an interaction between time and season ($F_{4,149}$=16.461, p<0.001). The initial number of VOCs emitted was high during the wet season but decreased over the 48-hour sampling period (Fig. 3.3a). In contrast, the initial number of VOCs was lower during the dry season and these persisted over the 48-hour sampling period (Fig. 3.3b). The

number of VOCs emitted from female dung was effected by both time ($F_{4,91}$=20.223, p<0.001) and season ($F_{1,23}$=10.692, p=0.003), but no interaction ($F_{4,91}$=1.710, p=0.155). The number of VOCs emitted decreased over the 48-hour time period, and this decrease occurred faster during the wet season (Fig. 3.4a,b).

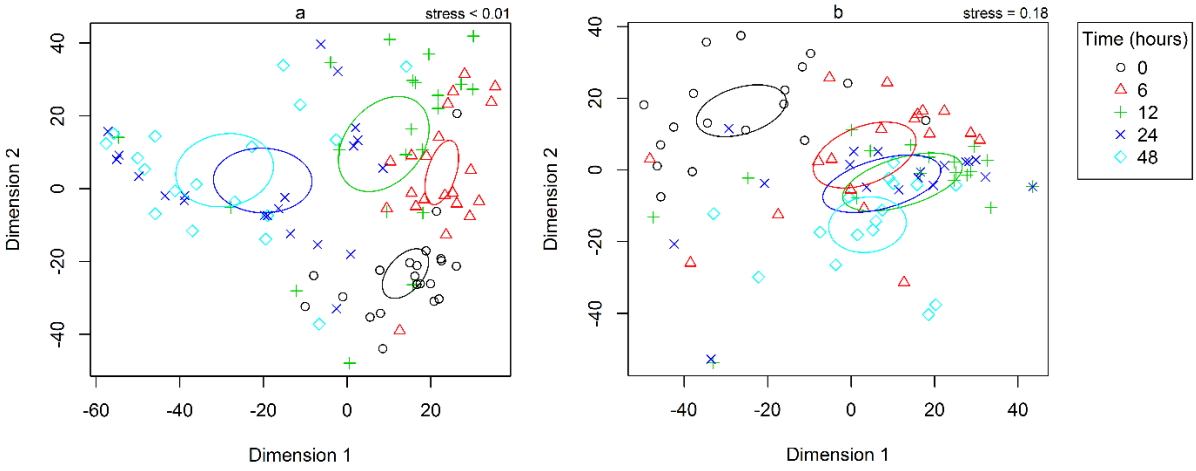

Figure 3.1. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of VOCs emitted from male dung over 48 hours during (a) wet and (b) dry season. Encompassing circles represent 95% confidence intervals.

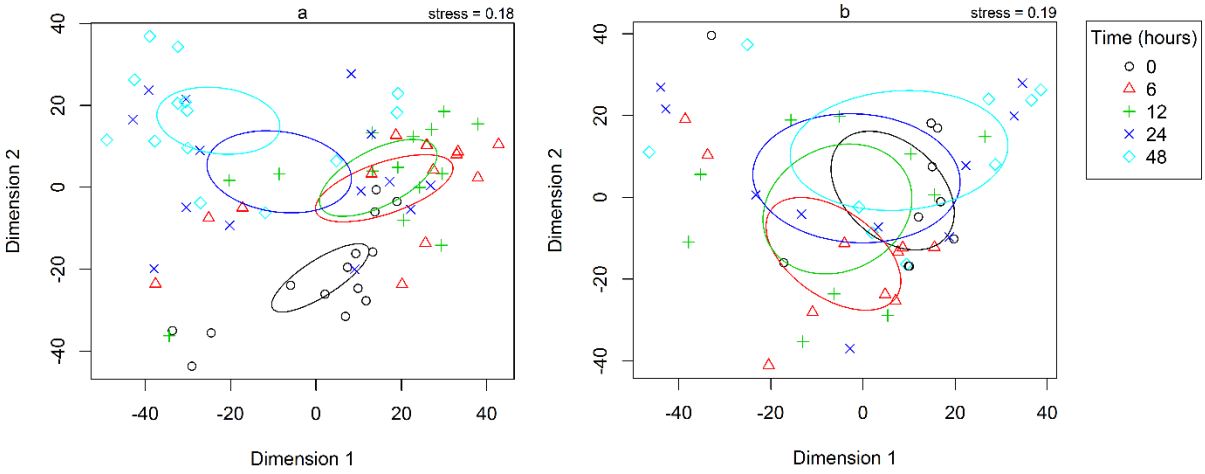

Figure 3.2. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of VOCs emitted from female dung over 48 hours during (a) wet and (b) dry season. Encompassing circles represent 95% confidence intervals.

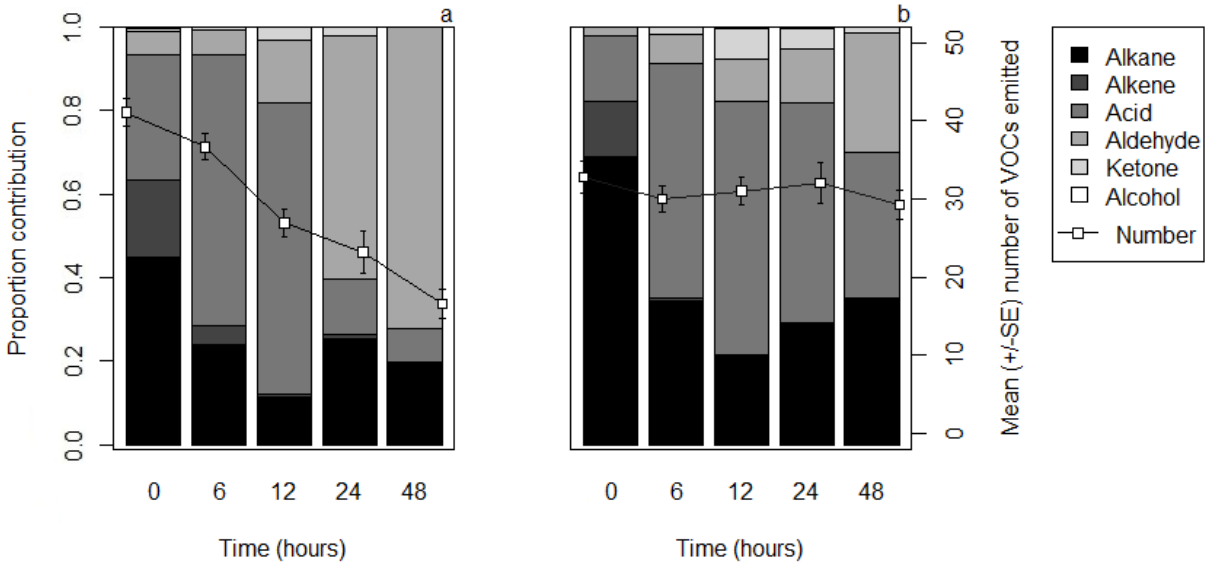

Figure 3.3. The odour composition and mean number of VOCs emitted from male over 48 hours during the (a) wet and (b) dry season.

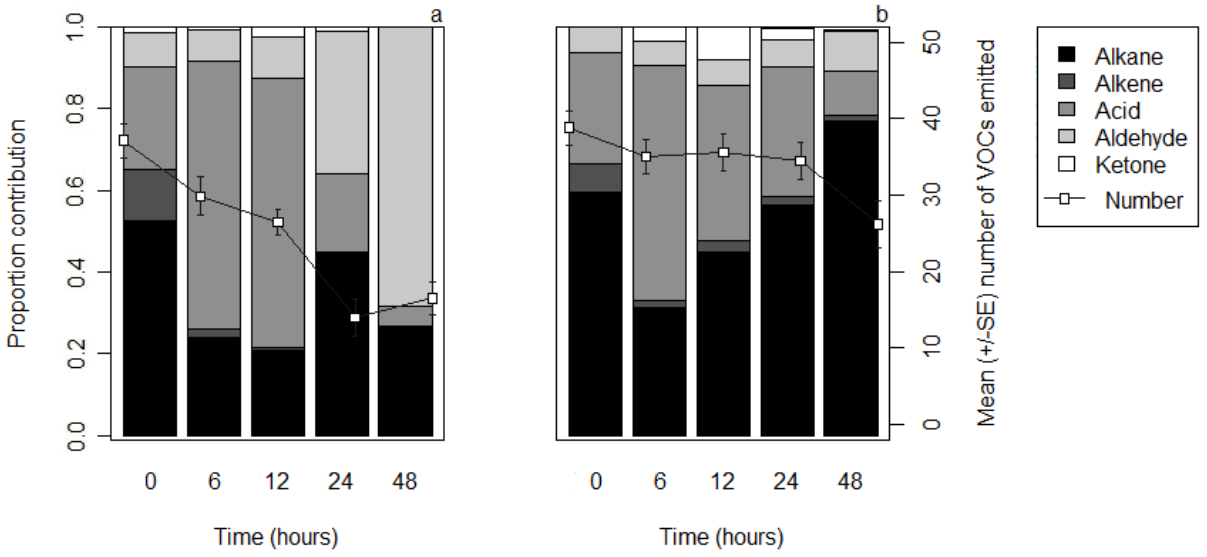

Figure 3.4. The odour composition and mean number of VOCs emitted from female over 48 hours during the (a) wet and (b) dry season.

Table 3.2. List of the top three VOCs contributing to male dung odours over the 48-hour sampling period during the wet and the dry season.

Time (hours from defecation)	Ranked VOC contributors	Wet season	Dry season
0	1	Tridecane	Nonane
	2	Nonane	Tridecane
	3	Acetic acid	4-Methyldecane
6	1	Butyric acid	Acetic acid
	2	2-Methylbutyric acid	Tridecane
	3	Acetic acid	2-Methylbutyric acid
12	1	Butyric acid	Acetic acid
	2	Acetic acid	Butyric acid
	3	3-Methylbutyric acid	2-Methylbutyric acid
24	1	Nonanal	Acetic acid
	2	Decanal	Butyric acid
	3	Hexadecane	Tridecane
48	1	Nonanal	Nonanal
	2	Decanal	Decanal
	3	Nonane	Tridecane

Table 3.3. List of the top three VOCs contributing to female dung odours over the 48-hour sampling period during the wet and the dry season.

Time (hours from defecation)	Ranked VOC contributors	Wet season	Dry season
0	1	Tridecane	Tridecane
	2	Nonane	Acetic acid
	3	Acetic acid	Nonane
6	1	Acetic acid	Acetic acid
	2	Tridecane	Tridecane
	3	Butyric acid	2-Methylbutyric acid
12	1	Acetic acid	Tridecane
	2	Butyric acid	Acetic acid
	3	2-Methylbutyric acid	4-Methyldecane
24	1	Tridecane	Tridecane
	2	Nonanal	2,6-Dimethylundecane
	3	Decanal	4-Methyldecane
48	1	Nonanal	Tridecane
	2	Decanal	Hexadecane
	3	Tridecane	4-Methyldecane

3.4.1.2. Males

Territorial male dung odours changed significantly during both the wet and the dry season sampling period (wet Global R=0.449, p=0.001; dry Global R=0.264, p=0.001). A high degree of change occurred between 0 and 48 hours, most noticeably during the wet season (wet R=0.953, p=0.001, Fig. 3.5a; dry R=0.492, p=0.001, Fig. 3.5b). Specifically, during the wet season significant changes occurred between 0 and 6 hours (R=0.473, p=0.001) and again between 12 and 24 hours (R=0.161, p=0.041). While no significant changes occurred between 6 and 12 hours (R=0.075, p=0.075) or 24 and 48 hours (R=0.004, p=0.418) during the wet season. During the dry season, significant changes occurred between 0 and 6 hours (R=0.328, p=0.002), 6 and 12 hours (R=0.096, p=0.043) and 24 and 48 hours (R=0.153, p=0.018). There was no significant change in odours between 12 and 24 hours (R=-0.016, p=0.570.

Non-territorial male dung odours also changed significantly during both seasons (wet Global R=0.250, p=0.001; dry Global R=0.092, p=0.027). However, only a moderate degree of change occurred between 0 and 48 hours, and even less so during the dry season (wet R=0.536, p=0.001, Fig. 3.6a; dry R=0.232, p=0.011, Fig. 3.6b). Specifically, during the wet season significant changes occurred between 0 and 6 hours (R=0.153, p=0.026) and 12 and 24 hours (R=0.229, p=0.006). With no significant changes occurring between 6 and 12 hours (R=0.06, p=0.115) or 24 and 48 hours (R=-0.039, p=0.663). During

the dry season, significant changes occurred between 0 and 6 hours only (R=0.220, p=0.014), with no significant changes between 6 and 12 hours (R=-0.068, p=0.954), 12 and 24 hours (R=-0.090, p=0.962), or 24 and 48 hours (R=-0.106, p=0.966).

The odour composition of territorial dung was effected by a three-way interaction between season, time (i.e. hours from defecation) and VOC functional group ($F_{28,639}$=1.823, p=0.006). As environmental conditions became hotter and more humid, initial odours were comprised of mostly hydrocarbon alkanes and acids, while aged dung odours were dominated by hydrocarbon aldehydes (Fig. 3.7a). During the wet season, territorial dung odours were characterised by the increase of 2- and 3-methylbutyric acids between 0-6 hours, and the aldehydes nonanal and decanal between 12-24 hours from defecation (Table 3.4). Further, during the hot and humid months, there was also a decrease in complexity over time (i.e. fewer VOC functional groups present, Fig. 3.7a). As conditions became cooler and drier, the dung odour composition and complexity remained relatively stable over the 48-hour time period (Fig. 3.7b). During the dry season, territorial dung odours were characterized by the increase of the alkanes 4-methyldecane and tridecane between 0-6 hours, and acetic, 2- and 3-methylbutyric, and butyric acids between 12-48 hours from defecation (Table 3.4).

The odour composition of non-territorial male dung was effected by a two-way interaction between time (i.e. hours after defecation) and VOC functional group ($F_{28,522}$=2.046, p=0.001). Similar to the initial odour of territorial dung in the wet season, the initial odour of non-territorial dung included a majority of hydrocarbon alkanes, acids and alkenes. Dissimilar to territorial dung, the odour of aged non-territorial dung contained a large proportion of hydrocarbon alkanes, and odours showed a decrease in complexity over time. Moreover, season had no effect on the odour composition of non-territorial male dung ($F_{1,14}$=1.651, p=0.220, Fig. 3.8a,b). Odours were characterised by tridecane at 0-6 hours, and butyric and acetic acids at 6-12 hours from defecation (Table 3.5).

The number of VOCs emitted from territorial dung was effected by a significant interaction between time (i.e. hours from defecation) and season ($F_{4,76}$=20.020, p<0.001). The initial number of VOCs emitted was high during the wet season but decreased over the 48-hour sampling period (Fig. 3.7a). In contrast, the initial number of VOCs was lower in the dry season and these persisted over the 48-hour sampling period (Fig. 3.7b). Similar to territorial males, the number of VOCs emitted from non-territorial male dung was also effected by a significant interaction between time (i.e. hours after defecation) and season ($F_{4,65}$=2.597, p=0.044). During the wet season, the number of VOCs emitted decreased over the 48-hour sampling period (Fig. 3.8a), but these remained stable during the dry season (Fig. 3.8b).

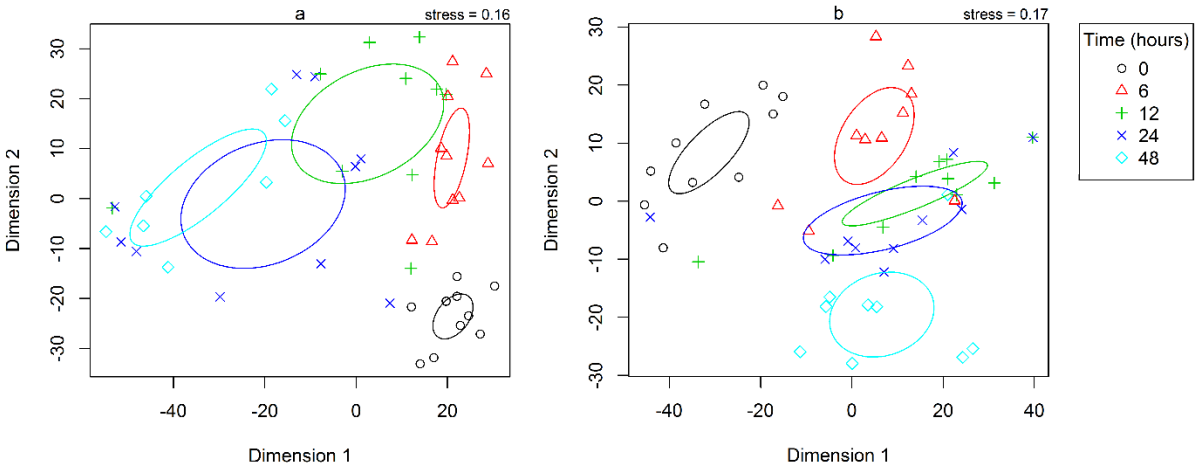

Figure 3.5. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of VOCs emitted from territorial male dung over 48 hours during (a) wet and (b) dry season. Encompassing circles represent 95% confidence intervals.

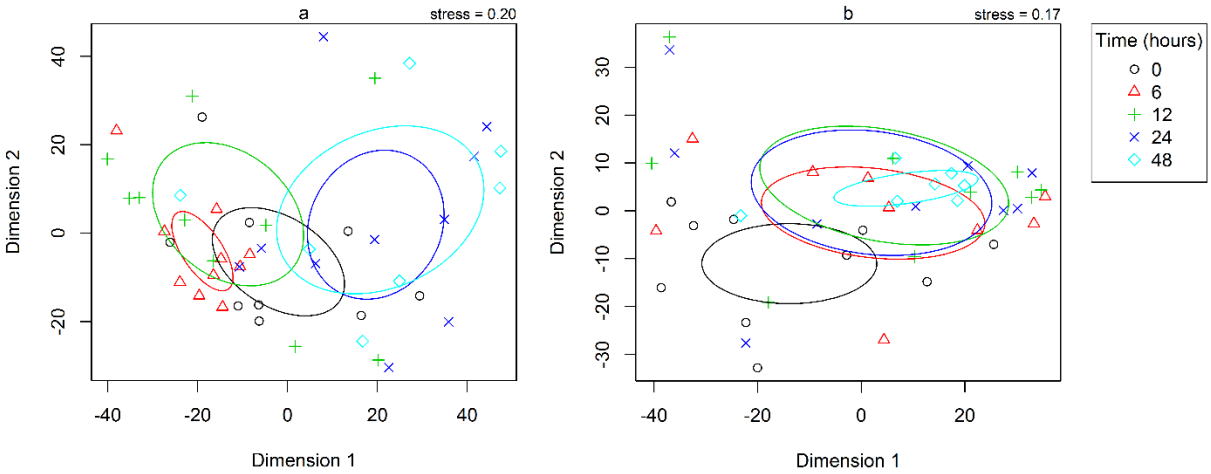

Figure 3.6. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of VOCs emitted from non-territorial male dung over 48 hours during (a) wet and (b) dry season. Encompassing circles represent 95% confidence intervals.

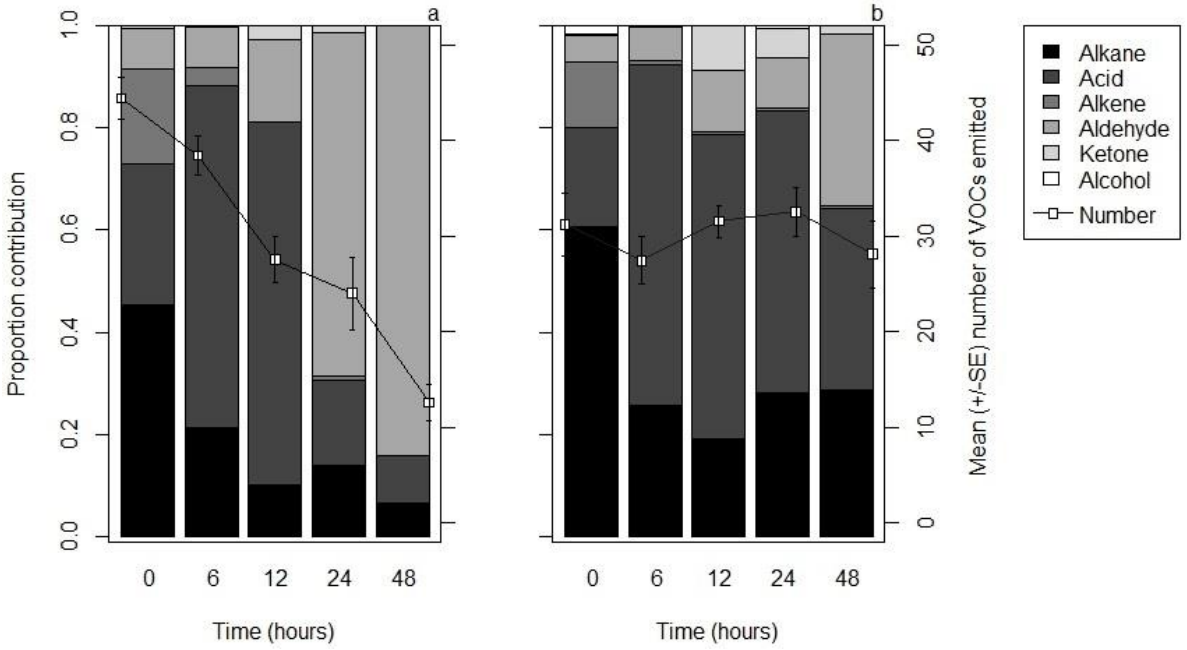

Figure 3.7. The odour composition and mean number of VOCs emitted from territorial male dung over 48 hours during the (a) wet and (b) dry season.

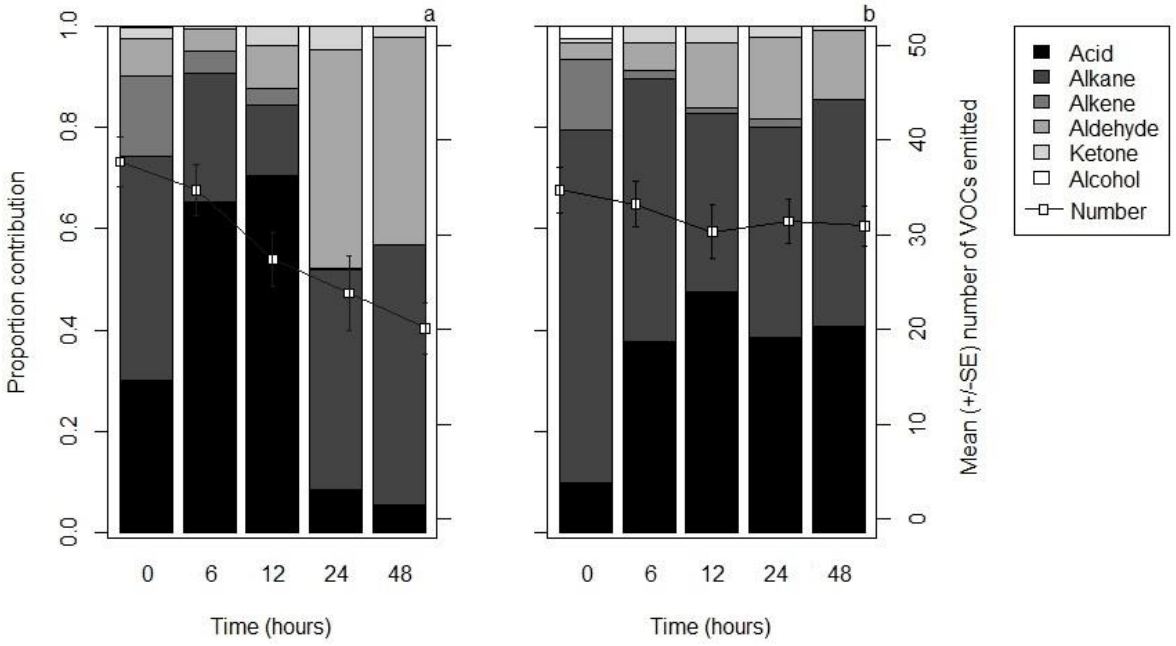

Figure 3.8. The odour composition and mean number of VOCs emitted from non-territorial male over 48 hours during the (a) wet and (b) dry season.

Table 3.4. List of the top three VOCs contributing to territorial male dung odours over the 48-hour sampling period during the wet and the dry season.

Time (hours from defecation)	Ranked VOC contributors	Wet season	Dry season
0	1	Tridecane	Tridecane
	2	Nonane	Nonane
	3	2-Methylbutyric acid	4-Methyldecane
6	1	2-Methylbutyric acid	Acetic acid
	2	Acetic acid	2-Methylbutyric acid
	3	3-Methylbutyric acid	Tridecane
12	1	Butyric acid	Acetic acid
	2	Acetic acid	Butyric acid
	3	Nonanal	2-Methylbutyric acid
24	1	Nonanal	Acetic acid
	2	Decanal	Butyric acid
	3	Acetic acid	Tridecane
48	1	Nonanal	Nonanal
	2	Decanal	Decanal
	3	Hexadecane	3-Methylbutyric acid

Table 3.5. List of the top three VOCs contributing to non-territorial male dung odours over time during the wet and the dry season.

Time (hours from defecation)	Ranked VOC contributors	Wet season	Dry season
0	1	Tridecane	Tridecane
	2	(3E)-3-Decene	Nonane
	3	Nonane	2,6,10-Trimethyldodecane
6	1	Butyric acid	Tridecane
	2	Tridecane	4-Methyldecane
	3	2-Methylbutyric acid	Nonane
12	1	Butyric acid	Tridecane
	2	Acetic acid	Acetic acid
	3	3-Methylbutyric acid	Butyric acid
24	1	Nonanal	Tridecane
	2	Decanal	Decanal
	3	Hexadecane	Hexadecane
48	1	Nonanal	Tridecane
	2	Nonane	Butyric acid
	3	Decanal	Acetic acid

3.4.1.3. Females

The odour of oestrous female dung did not change significantly during the wet (0 to 6 hours R=0.200, p=0.214; 6 to 12 hours R=-0.148, p=0.700; 12 to 24 hours R=0.037, p=0.500; 24 to 48 hours R=0.407, p=0.200; Fig. 3.9a) or the dry season sampling periods (0 to 6 hours R=0.071, p=0.333; 6 to 12 hours R=-0.250, p=1.00; 12 to 24 hours R=1.00, p=0.333; 24 to 48 hours R=0.500, p=0.333; Fig. 3.9b). In contrast, the odour of non-oestrous female dung changed significantly during both seasons (wet Global R=0.192, p=0.001; dry Global R=0.247, p=0.001). Moreover, there was a moderate degree of change in the odour of non-oestrous dung between 0 and 48 hours during both seasons (wet R=0.444, p=0.001, Fig. 3.10a; dry R=0.482, p=0.002, Fig. 3.10b). Specifically, during the wet season the odour of non-oestrous dung changed significantly between 12 and 24 hours (R=0.162, p=0.023), but no other time period (0 to 6 hours R=0.142, p=0.057; 6 to 12 hours R=-0.001, p=0.433; 24 to 48 hours (R=-0.048, p=0.719; Fig. 3.10a). During the dry season, the odour of non-oestrous dung changed on between 0 and 6 hours (R=0.253, p=0.006), but not between 6 to 12 hours (R=-0.028, p=0.704), 12 to 24 hours (R=-0.045, p=0.749) or 24 to 48 hours (R=-0.071, p=0.857; Fig. 3.10b).

The odour composition of oestrous female dung was effected by a two-way interaction between time (i.e. hours from defecation) and VOC functional group ($F_{28,156}$=4.575, p<0.001). Alkanes showed

an initial decrease but then re-established and increased over time, whereas acids increased up until 12 hours and then decreased (Fig. 3.11a,b). Several acids, including 2-methylbutyric and butyric, remained prominent during 6-12 hours (Table 3.6). Season had no effect on the odour composition ($F_{1,3}$=0.571, p=0.505) and odours sustained their complexity (i.e. number of VOC functional groups present) over the 48-hour sampling period.

The odour composition of non-oestrous female dung was also effected by a two-way interaction between time (i.e. hours from defecation) and VOC functional group ($F_{28,538}$=4.193, p<0.001). Like oestrous dung, alkanes showed an initial decrease, but then re-established and increased over time, whereas acids increased up until 12 hours after dung deposition and then decreased (Fig. 3.12a,b). Acetic acid remained a significant contributor for 0-12 hours (Table 3.7). Moreover, season had no effect on the odour composition of non-oestrous female dung ($F_{1,13}$=1.039, p=0.327) and odours sustained their complexity (i.e. VOC functional group contribution) over the 48-hour sampling period.

The number of VOCs emitted from oestrous female dung was effected by time ($F_{4,16}$=4.444, p=0.013), where the number of VOCs emitted decreased over the 48-hour sampling period (Fig. 3.11a,b). In contrast, season did not affect the number of VOCs emitted ($F_{1,3}$=2.016, p=0.251, Fig. 3.11a,b). The number of VOCs emitted from non-oestrous female dung was also effected by time ($F_{4,75}$=15.673, p<0.001, Fig. 3.12a,b), where the number of VOCs emitted decreased over the 48-hour time period. Season, however, did affect the number of VOCs emitted from non-oestrous female dung ($F_{1,18}$=7.566, p=0.013, Fig. 3.12a,b), where the number of VOCs emitted decreased faster during the wet season sampling period.

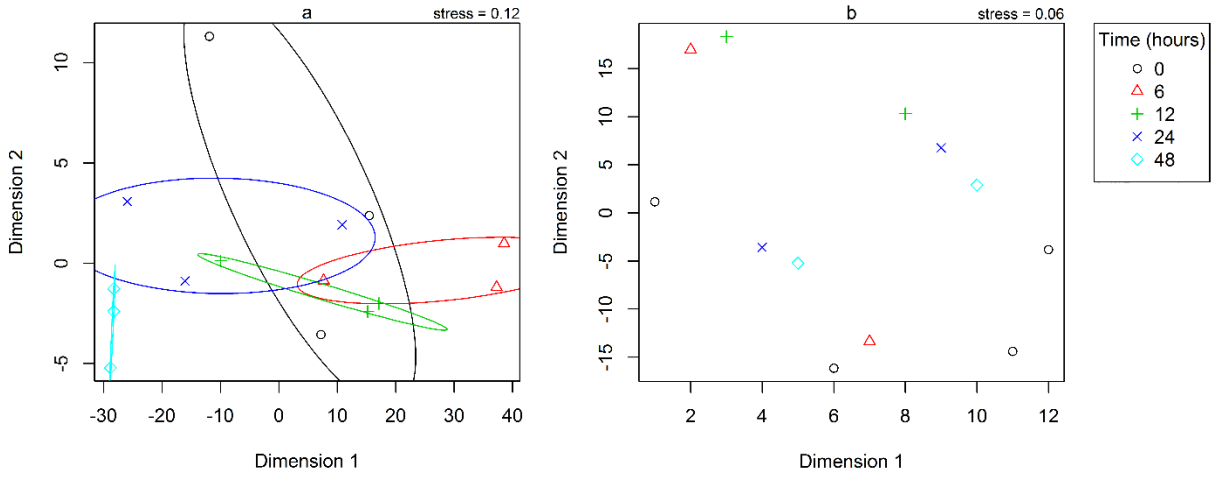

Figure 3.9. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of volatile compounds emitted from oestrous female dung over 48 hours during (a) wet and (b) dry season. Encompassing circles represent 95% confidence intervals (the lack of confidence intervals on (b) are due to small sample size).

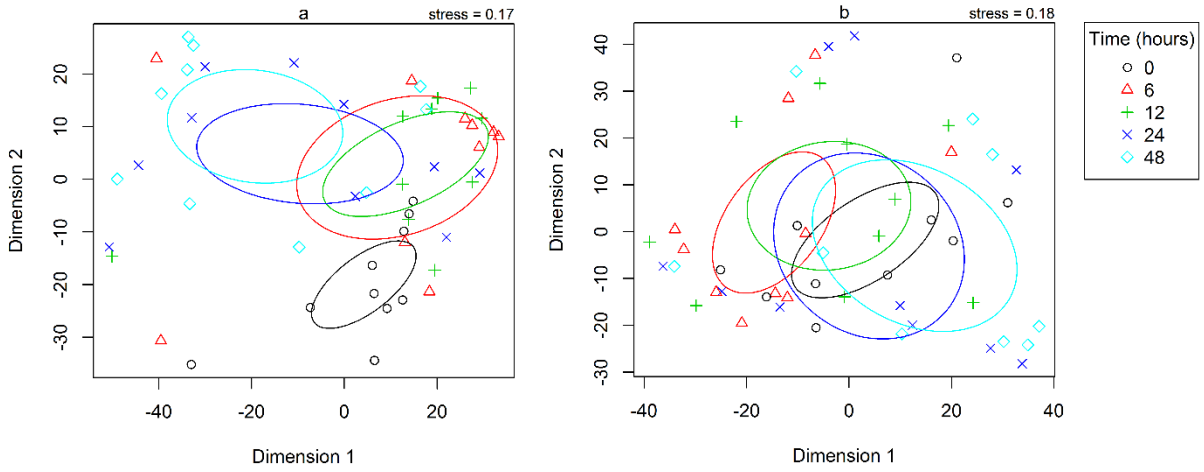

Figure 3.10. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of volatile compounds emitted from non-oestrous female dung over 48 hours during (a) wet and (b) dry season. Encompassing circles represent 95% confidence intervals.

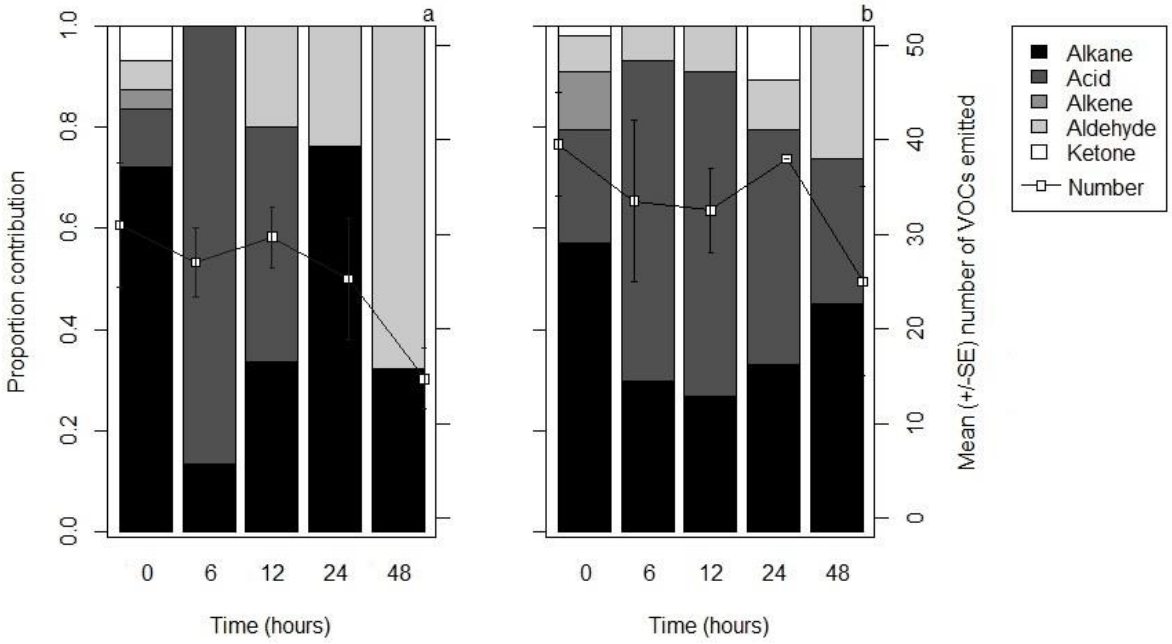

Figure 3.11. The odour composition and mean number of VOCs emitted from oestrous female over 48 hours during the (a) wet and (b) dry season.

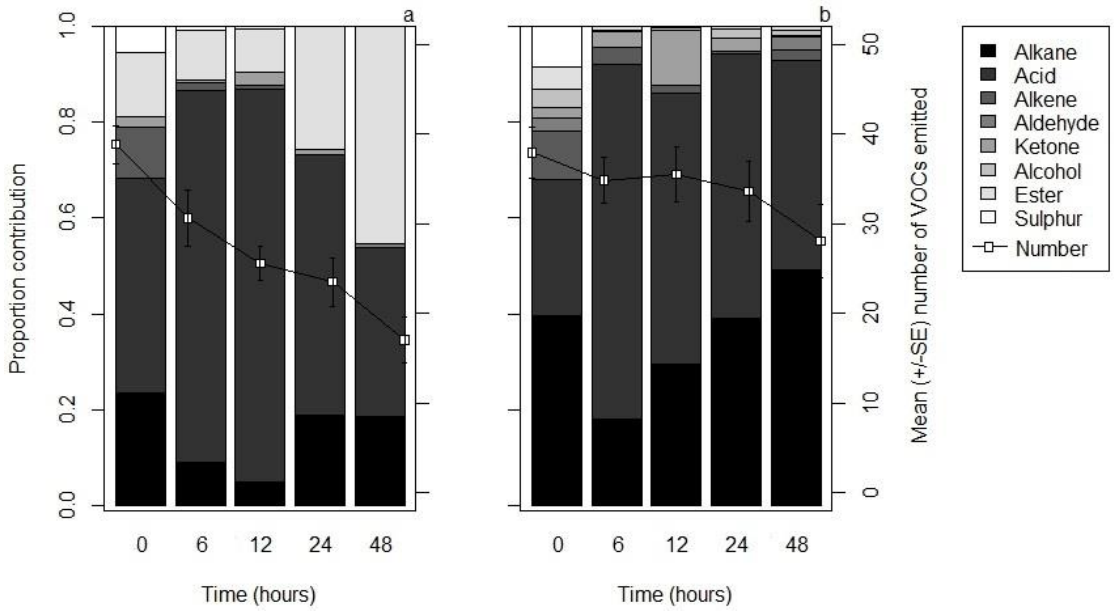

Figure 3.12. The odour composition and mean number of VOCs emitted from non-oestrous female over 48 hours during the (a) wet and (b) dry season.

Table 3.6. List of top three VOCs contributing to oestrous female dung odours over time during the wet and the dry season.

Time (hours from defecation)	Ranked VOC contributors	Wet season	Dry season
0	1	Tridecane	Nonane
	2	Nonane	Tridecane
	3	Hexadecane	Acetic acid
6	1	Butyric acid	2-Methylbutyric acid
	2	Acetic acid	Butyric acid
	3	2-Methylbutyric acid	3-Methylbutyric acid
12	1	Tridecane	Butyric acid
	2	2-Methylbutyric acid	Acetic acid
	3	3-Methylbutyric acid	2-Methylbutyric acid
24	1	Tridecane	Tridecane
	2	Decanal	2-Methylbutyric acid
	3	Nonane	Butyric acid
48	1	Nonanal	Tridecane
	2	Decanal	Acetic acid
	3	Decane	Nonanal

Table 3.7. List of top three VOCs contributing to non-oestrous female dung odours over time during the wet and the dry season.

Time (hours from defecation)	Ranked VOC contributors	Wet season	Dry season
0	1	Acetic acid	Nonane
	2	Nonane	3-(Allyldisulfanyl)-1-propene
	3	Octadecanoic acid, phenylmethyl ester	Acetic acid
6	1	Acetic acid	Acetic acid
	2	Butyric acid	Propanoic acid
	3	Pentanoic acid	2-Methylbutyric acid
12	1	Acetic acid	Acetic acid
	2	Butyric acid	2-Methylbutyric acid
	3	2-Methylbutyric acid	3-Methylbutyric acid
24	1	Octanoic acid	Undecanoic acid
	2	Octadecanoic acid, phenylmethyl ester	Dodecane
	3	Dodecane	2-Methylbutyric acid
48	1	Octadecanoic acid, phenylmethyl ester	Dodecanyl acetate
	2	Octanoic acid	Dodecane
	3	Dodecane	Octanoic acid

Table 3.8. Listed volatile organic compounds and their chemical properties

VOC name	VOC functional group	Molecular weight (g/mol)	Vapor pressure at 25˚C (mm/Hg)
Acetic acid	Acid	60.05	15.70
3-(Allyldisulfanyl)-1-propene	Alkene	146.28	0.98
Butyric acid	Acid	88.11	1.65
Decanal	Aldehyde	156.27	0.21
(3E)-3-Decene	Alkene	140.27	2.1
2,6-Dimethylundecane	Alkane	184.36	0.18
Dodecane	Alkane	170.34	0.14
Dodecanyl acetate	Alkane	228.38	$9E^{-03}$
Hexadecane	Alkane	226.45	$5E^{-03}$
2-Methylbutyric acid	Acid	102.13	0.55
3-Methylbutyric acid	Acid	102.13	0.55
4-Methyldecane	Alkane	156.31	0.87
Nonanal	Aldehyde	142.24	0.53
Nonane	Alkane	128.26	4.63
Octadecanoic acid	Acid	284.48	$8E^{-6}$
Octadecanoic acid, phenylmethyl ester	Ester	360.58	$2.4E^{-08}$
Pentanoic acid	Acid	102.13	0.45
Propanoic acid	Acid	74.08	4.23
Tridecane	Alkane	184.36	0.06
2,6,10-Trimethyldodecane	Alkane	212.42	0.04
Undecanoic acid	Acid	186.29	$2E^{-03}$

3.4.2. Seasonal midden use

In contrast to expectations, season did not affect the midden visitation (t=-1.024, p=0.719, Fig. 3.13a) or defecation frequency (t=-0.363, p=0.643, Fig. 3.13b) of male or female adult white rhinos.

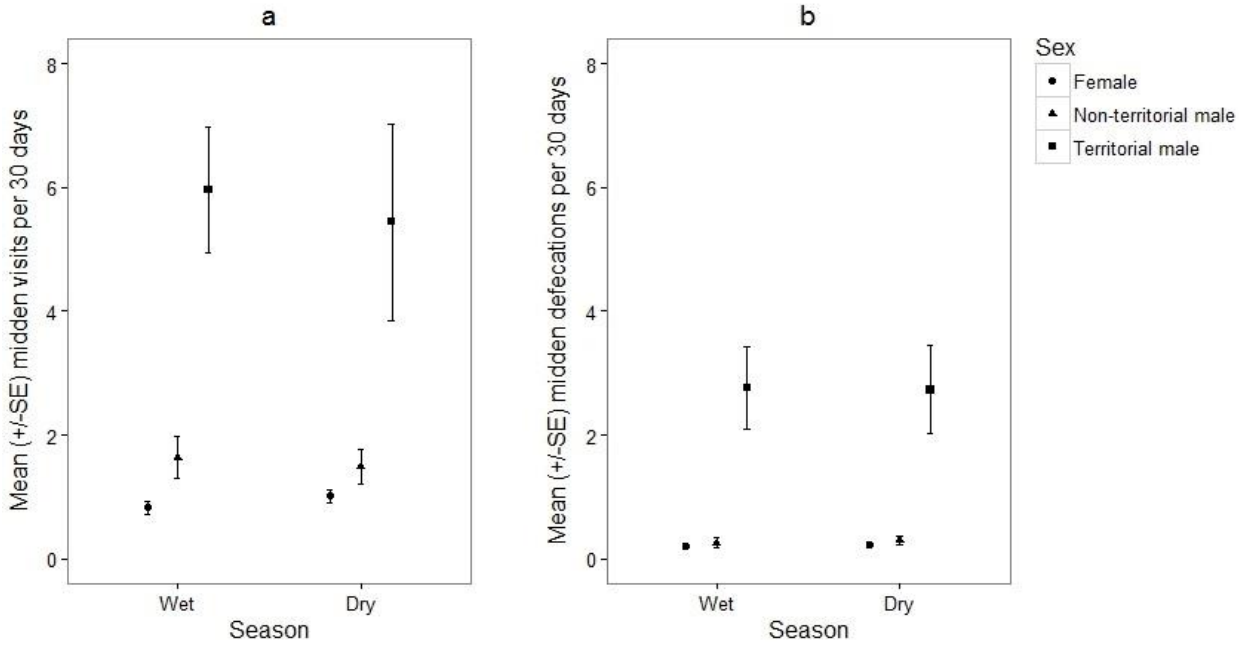

Figure 3.13. Seasonal midden (a) visitation and (b) defecation of adult white rhinos.

3.5. Discussion

The longevity of olfactory signals has implications for fitness. For example, if an odour lasts for several days then males can spend less time remarking territories and more time finding mates (Gosling and Roberts 2001). However, environmental conditions effect VOC emission beyond an animal's control, thus an individual may need to alter their behaviour seasonally to counteract this. The results of this study show that male dung odours persist for less time than female dung odours (i.e. male odours changed more rapidly). Within males, territorial dung odours surprisingly persisted for less time than non-territorial (i.e. territorial odours changed more rapidly). Within females, oestrous dung odours persisted for more time than non-oestrous (i.e. non-oestrous odours changed more rapidly). Finally, the dung odour longevity of all individuals decreased during the wet season, but this did not elicit a change in the white rhinos' defecation frequency or midden visitation.

As the initial dung odour of males and females differs (Marneweck et al. 2017), I expected that their profiles of degradation would also differ. Male dung odours contain more hydrocarbon aldehydes than female odours, and female dung odours contain more hydrocarbon alkanes than male odours (Marneweck et al. 2017). Aldehydes are of a lower molecular weight than alkanes and therefore more susceptible to emission. This ultimately causes decreased longevity for male dung odours compared to the females' odour.

In line with my predictions, the dung odour of territorial males changed rapidly during the wet season. This was likely the result of the higher temperatures and humidity levels, which causes an increase in VOC emission (Nimmermark and Gustafsson 2005; Regnier and Goodwin 1977). However, these drastic changes during the wet season are not apparent in the short-term change of dung odours

from non-territorial males and further, territorial male dung odours show the most complex relationship between season, time (i.e. hours after defecation) and VOC functional group. Therefore, there must be biotic factors driving the changes of territorial male dung odours.

When comparing males, territorial males have higher concentrations of faecal testosterone metabolites than non-territorial males (Rachlow et al. 1998), which likely indirectly accounts for the differences in initial dung odours. The role of bacteria in mammals that use specialised scent glands has been widely documented (see review by Archie and Theis (2011)). Microbes flourish because the glands provide a warm, moist and nutrient rich habitat for them to grow. The fermentation hypothesis proposes that the symbiotic bacteria living within scent glands break down organic material and produce VOCs that ultimately contribute to mammalian recognition cues (Albone and Eglinton 1974). The variation in composition and abundance of these bacterial communities then creates a unique individual scent thereby allowing recognition by other individuals (Gorman 1976).

Although the fermentation hypothesis was developed for mammals that scent mark with specialised glands, it has been suggested that it could be applied to mammals that mark with faeces or urine (Archie and Theis 2011). Anal gland microbiota of both male and female meerkats (*Suricata suricatta*) differ only after individuals reach sexual maturity, suggesting that reproductive hormones likely play a key role in determining host bacterial communities (Leclaire et al. 2014). Sex differences in the microbiota of adults has also been identified in greater sac-winged bats (*Saccopteryx bilineata*) (Voigt et al. 2005) and white-tailed deer (*Odocoileus virginianus*) (Alexy et al. 2003). Therefore, for adult male white rhinos achieving territorial status, the subsequent associated increase in testosterone (Rachlow et al. 1998) may affect microbiota directly or indirectly via changes in the faecal matter. Moreover, territorial males have higher faecal testosterone metabolite concentrations during the wet season compared to the dry season, coinciding with the seasonal peak in white rhino breeding (Kretzschmar et al. 2004; Owen-Smith 1973). As a result, the rapid change in territorial male dung odour during the wet season may be associated with their highest levels of faecal testosterone metabolites combined with changes due to higher temperatures and humidity levels.

Hydrocarbon acids are characteristic of male dung odours and dung from non-territorial adult males emits lower proportions of these acids than territorial male dung (Marneweck et al. 2017). These volatile acids are of low molecular weight, meaning that they are quicker to emit (Stoddart 1976). If non-territorial males emit less of these lighter VOCs then their dung odour will be more stable over time in comparison to territorial male dung odour. My results support this, as I found non-territorial dung odours were more stable compared to territorial odours. Further, non-territorial males have significantly less faecal testosterone metabolites than territorial males (Rachlow et al. 1998) and, although this has not been investigated, it is likely that there are no seasonal fluctuations in faecal testosterone metabolites because non-territorial males are not competing for mates as territorial males monopolize mating opportunities (Owen-Smith 1971; Owen-Smith 1973). With this in mind, I suggest that the lack of mating opportunities accounts for the decreased seasonal change in non-territorial male dung odours

compared to territorial dung odours. However, the small changes observed (i.e. the decrease in VOCs emitted over time during the wet season) can likely be attributed to abiotic factors such as temperature and humidity (Nimmermark and Gustafsson 2005; Regnier and Goodwin 1977).

With regard to females, the fermentation hypothesis may also explain differences in female odours. Indirectly, the hormone activity of the female oestrous cycle could affect dung odours and the more stable oestrous odours may be associated with their highest levels of faecal progestogen metabolites. The presence of more progestogen metabolites may affect the microbiota directly or indirectly by changing the environment of the dung. Subsequently, these bacteria will produce VOCs that contribute to the dung odours. Oestrous odours last longer than non-oestrous and this may be due to the type of VOCs created by the bacteria. For example, if the bacterial breakdown causes the emission of VOCs of higher molecular weight (less volatile) then this will contribute to a more stable odour over time. A stable odour would mean that information on their reproductive state would be transmitted for a longer period, increasing the potential for detection by males and ultimately increasing reproductive success. However, the pattern of stable odours may be the result of the low sample size that I obtained for oestrous females.

The dung odour of non-oestrous females showed similar emission patterns to those from oestrous females. It is possible that the hormonal differences between oestrous and non-oestrous (i.e. progestogen) are much less apparent than the hormonal differences between territorial and non-territorial males (i.e. testosterone), making the odours more similar. The seasonal changes observed in non-oestrous dung odours (i.e. the decrease in VOCs emitted over time during the wet season) can likely be attributed to abiotic factors such as temperature and humidity (Nimmermark and Gustafsson 2005; Regnier and Goodwin 1977). However, as I identified oestrous females via behavioural observations, it is possible that some females identified as non-oestrous could have been in oestrus. Thus, it is possible that the similarity of the oestrous and non-oestrous emission patterns are the result of the misclassification of some females. As males tend to identify and follow oestrous females closely (Owen-Smith 1973, 1988), it is unlikely that a large number of oestrous females were misidentified.

The behavioural function of middens is different for male and female white rhinos. Adult males, specifically territorial males, use middens to advertise territory ownership and assess information on female reproductive state in order to find mates. Territorial signals that last a long time would be beneficial for fitness as less time and energy would need to be invested in marking (Gosling and Roberts 2001). However, I found that territorial male dung odours underwent rapid changes, most notably, during the wet season coinciding with the seasonal peak in breeding activity. Because the longevity of territorial male dung odours is shorter during the wet season, I expected territorial males to increase their midden visitation and defecation frequency during this time in order to keep signals constant and assess other odours before they disappear, especially considering the peak in breeding during this time. This, however, was not the case. Contrary to my expectations, adult white rhinos did not seasonally adjust their midden visitation or defecation frequency. I suggest that this is due to dung being a limited

resource (Brachares and Arcese 1999) and that white rhinos are already defecating at the highest frequency possible for their physiology. Territorial males already defecate smaller volumes than other adults to increase their marking frequency (Chapter 4), and the only way to increase this would be to limit dung even further. However, this would likely cause an even faster loss of information due to the surface area to volume ratio available for emission. Thus, males may be defecating with optimal volume and frequency for their odour signals.

With regard to visitation, territorial males may also be visiting middens at the highest possible frequency as well. With 30 middens reported within one territory (Owen-Smith 1975), males must visit many middens to assess other individuals and to mark their territory. Chapter 2 showed that territorial males did increase their visitation after detecting both a rival male and an oestrous female. Thus, they are prepared to alter this frequency if a situation occurs (such as territory intrusion or detecting an oestrous female), but their baseline visitation frequency appears optimal for depositing and acquiring information throughout their territory.

Vast changes occur relatively quickly within white rhino dung odours. As white rhinos can recognise sex, age, territorial and oestrous state from the odour of fresh dung (Marneweck et al. 2017), it is likely that they are also able to determine the age of the dung from odour changes and thus respond accordingly. For instance, identifying the odour of an oestrous female over 48 hours later may allow reproductive males to decide on the cost-benefit of trying to locate the female (i.e. a time threshold after which the costs of pursuing the female depositor outweigh the potential benefits and/or possibility of locating her). These results provide the first detailed insight into the odour of dung marks over time, yet highlight the short longevity of these odours for white rhinos, and therefore the limitations of marking with dung. Furthermore, my study provides a relevant paradigm to understand the role of scent marks in species utilising communal marking sites, specifically, the detailed information that dung marks carry has been under-appreciated. Studies investigating marking strategies, seasonal changes, and their population-level fitness effects should strive to understand both short-term and long-term changes in odour profiles that are driving decision-making in the individuals both depositing and interpreting such odours.

3.6. References

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Chapter 4 : The role of middens in white rhino (*Ceratotherium simum*) olfactory communication

4.1. Abstract

Many mammals use olfactory communication to demarcate territories and to find potential mates. White rhinos transmit information about their sex, age, territorial status (males) and oestrous state (females) via dung odours. Moreover, as white rhinos defecate communally in middens, it has been suggested that these middens may act as information centres. However, it is uncertain which individuals are primarily transmitting information in these middens or who this information is intended for. Using video recording camera traps, I investigated the behaviour of white rhinos at middens. Middens were utilised predominately by adults, although they did not defecate each time they visited. Territorial adult males visited and defecated more than any other individuals. Adult males (both territorial and non-territorial) investigated dung piles more than any other age or sex, and the majority of these piles belonged to territorial males and adult females (i.e. male-male and female-male communication). Adult females investigated the dung of non-territorial males more than adult males did, and also investigated the dung of other females as much as males did (male-female and female-female communication). In addition to olfactory signals, there was a spatial aspect to defecating in the middens, where territorial males defecated in the centre of the midden, while other individuals defecated around the periphery. Lastly, territorial males regulated their dung output, with a higher defecation frequency and smaller dung volume than any other adult. Ultimately, my results indicate that middens act as information centres, where the primary function seems to be for territorial males to transmit and obtain information (male-male and female-male communication). However, in addition, non-territorial males can assess female reproductive state, while females may be assessing the quality of all the males and even the number of other females that use a midden (male-female and female-female communication).

4.2. Introduction

Many mammals use olfactory cues to communicate a wide range of information including kin recognition (Stoffel et al. 2015), reproductive status (Archunan and Rajagopal 2013) and territory ownership (Barja et al. 2005). This information can be transmitted via scent glands (Cross et al. 2014; Vaglio et al. 2016), urine (Archunan and Rajagopal 2013; Kimura 2001) and/or dung (Karthikeyan et al. 2013; Marneweck et al. 2017). As many mammals defecate communally in middens (Brown and Macdonald 1985; Roper et al. 1993; Sneddon 1991) it has been suggested that middens may act as information centres (Eisenberg and Kleiman 1972; Owen-Smith 1973). Middens can be found in the approximate centre of a territory or home range (e.g. swift fox *Vulpes velox* (Darden et al. 2008)), along the boundary (e.g. oribi *Ourebia ourebi* (Brachares and Arcese 1999)), or scattered throughout a territory

(e.g. white rhinos *Ceratotherium simum* (Kretzschmar et al. 2001; Owen-Smith 1973)). Further, the location of a midden has implications for its function. For instance, middens at the edge of a territory are likely used for territorial marking, whereas middens in the centre may be used for social group communication (Darden et al. 2008; Roper et al. 1993).

Middens of several ungulate species are utilised by both sexes, for example, dik-diks *Madoqua kirkii* (Hendrichs and Hendrichs 1971), klipspringers *Oreotragus oreotragus* (Dunbar and Dunbar 1974), bushbucks *Tragelaphus scriptus* (Wronski et al. 2006), and Arabian gazelles *Gazella arabica* (Wronski et al. 2013). Although these species utilise middens, their mating strategies differ. Specifically, dik-diks and klipspringers are facultatively monogamous (Brotherton and Manser 1997; Roberts and Dunbar 2000), whereas bushbucks and Arabian gazelles are polygynous (Wronski 2005; Wronski et al. 2013). However, even though species share a mating strategy, how they utilise middens can differ. For example, polygynous bushbucks use middens for inter-sexual communication (i.e. male-female communication) (Wronski et al. 2006), while polygynous Arabian gazelle middens have a dual function of both male territorial defence (i.e. male-male communication) and within female group communication (i.e. female-female communication) (Wronski et al. 2013).

White rhinos are the most social of the rhinoceros species and individuals of both sexes defecate in middens (Owen-Smith 1973). As white rhinos transmit information about their sex, age, male territorial status (territorial vs. non-territorial) and female oestrous state in the odour of their dung (Marneweck et al. 2017), it is likely that these middens act as information centres. It is unclear, however, if these middens are utilised equally by the different age and sex classes, or whether the information deposited is meant for specific individuals or rather a number of individuals. Middens are found throughout a territory (Kretzschmar et al. 2001), but are concentrated around frequented paths, water holes, and territory boundaries (Owen-Smith 1975), and thus likely used for territorial marking. Therefore, middens are likely utilised most frequently by territorial males. White rhinos employ a polygynous mating strategy where adult males defend a territory and monopolise mating opportunities with multiple females (White et al. 2007). They do this by defending small territories (average 1.65 km^2) that are part of larger, overlapping, female home ranges (average 11.6 km^2) (Owen-Smith 1973, 1975). Thus, one female home range incorporates a number of territories.

The key information transmitted by white rhinos in their dung odours (i.e. territory ownership and oestrous state) is related to breeding opportunities (Marneweck et al. 2017). Therefore, it is likely that adults will utilise middens the most. Territorial males should use middens to both advertise territory ownership and search for mates (Brachares and Arcese 1999; Wronski et al. 2006). Although it was originally thought that territorial adult males monopolized mating, sneaky copulations by non-territorial males can occur (Guerier 2012), suggesting that non-territorial males could also use middens to search for mates. Non-territorial males can be divided into two categories, those living within a territory but not challenging the territorial male for ownership (i.e. subordinate bulls), and those that are passing through a territory with the hope of challenging a territorial male in order to gain a territory (Owen-

Smith 1973). These males may show different behaviour, for example, frequent visiting males may investigate female dung looking for potential mating opportunities, whereas infrequent males may investigate the territorial male's dung in order to assess his condition.

Both sexes utilise middens so it is likely that females also obtain information as well as deposit it. Females do not maintain exclusive home ranges, or compete for food or mates with other females (Owen-Smith 1973), but they may use middens to assess male quality, especially if mating occurs outside of territory ownership.

In addition to just defecating in middens, Owen-Smith (1973) observed that different individuals seemed to defecate in specific locations within the midden. Specifically, territorial males defecated in the centre of middens, while adult females and sub-adults tended to defecate around the periphery. If this is the case, then it would seem that there is not only an olfactory component to dung, but also a spatial component that helps with information transfer. In line with this, as territorial males defecate at a number of middens within their territory, and thus provide information across a large area (i.e. average territory size in iMfolozi 1.65 km^2; Owen-Smith 1975), males should regulate their dung output, relative to non-territorial males and adult females, in order to increase marking events, as reported in oribi (Brachares and Arcese 1999).

With this in mind, I made the following predictions; (1) adult white rhinos would utilise middens more than sub-adults or calves. (2) Territorial males would visit and defecate in middens more frequently than other adults (i.e. non-territorial male or adult female), and (3) territorial males would spend more time investigating (i.e. sniffing) dung within the middens compared to other adults (e.g. non-territorial male or female). (4) Frequent non-territorial visitors to the midden (i.e. subordinate and non-challenging males) would investigate (i.e. sniff) dung from the territorial male and adult females. (5) Infrequent non-territorial visitors (i.e. challenging males) would sniff territorial male dung. (6) Adult females would utilise middens to advertise reproductive state, and (7) females would investigate (i.e. sniff) the dung of territorial males more often than they investigate the dung of other individuals. (8) Females would not be interested in the dung of other females. (9) Territorial males would regulate their dung output, relative to non-territorial males and adult females, to increase marking events. Finally, (10) only the territorial male would defecate in the centre of the midden.

4.3. Methods

4.3.1. Behavioural data collection

I conducted this study in the southwestern portion of the 896 km^2 Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. To record midden visitation and use, I setup motion-triggered video recording camera traps at ten middens, each with a different resident territorial male (identified via differences in horn shape and size) from November 2014 to August 2015. These are the same sites and cameras as in Chapter 2, but with all odour manipulation periods excluded. This resulted in approximately five months of dry season data and five months of wet season data for each midden. The average territory size of a

white rhino is 1.65 km^2 (Owen-Smith 1975), therefore, focal middens were separated by at least 2 km to help ensure separation. Video footage of the males utilising these middens indicated that the middens were in fact in separate territories (see below). I used infrared 'no-glow' camera traps (either a Cuddeback Black Flash® E3 or Cuddeback Attack Black Flash® 1194 model) placed approximately 3 metres from the edge of the midden to allow for sufficient field of view. I used 'no-glow' cameras as they do not emit visible light or have a flash, creating minimal disturbance at the midden and therefore allowing me to capture natural behaviour. I programmed the cameras to record 30-second videos at each trigger with a 1-second delay before becoming active again and downloaded data every two weeks.

I recorded data on all the individuals that visited the middens. From 2403 data videos containing over 20 hours of footage, I created an ID profile for each white rhino so that I could record individual visitation, defecation and olfactory investigation. When individuals sniffed specific dung piles, I determined the age and sex of the white rhino that deposited the dung, by reviewing previous video footage. Adults were identified as individuals >7 years, sub-adults as 2-7 years, and calves <2 years, based on body size and horn development (Hillman-Smith et al. 1986). Territorial males were identified as adult males performing territorial behaviours (i.e. dung kicking, spray urinating) at the middens and non-territorial as adult males not performing these behaviours (Owen-Smith 1971; Owen-Smith 1973). The oestrous state of adult female white rhinos can be determined by the behaviours of territorial males. When a female is in oestrus, territorial males move with her and try to prevent her from leaving his territory (Owen-Smith 1973). Moreover, the male will attempt to rest his chin on her flank and eventually mount her (Owen-Smith 1973). Unfortunately, despite observing territorial males following oestrous females (Marneweck pers. obs.), I did not record these sorts of behaviours at the middens with the camera traps. As a result, I could not determine oestrous state and thus grouped all adult females together for analysis.

4.3.2. Statistical analysis

I calculated individual visitation and defecation frequency by dividing the number of visits or defecations by the number of days the camera was active (range= 172-282 days). To assess differences in midden visitation and defection frequency from each age and sex, I ran a linear mixed-effects model using the R package nlme (Pinheiro et al. 2015). Visitation or defecation frequency was set as the response variable, age and sex were set as fixed factors and rhino ID as a random factor. I repeated this with adult state as the fixed factor (i.e. territorial male, non-territorial male or female).

For analysis of information acquisition and deposition, I recorded each midden visit (n= 1675) with a yes or no regarding olfactory investigation (i.e. acquiring information) and defecation (i.e. depositing information). I defined olfactory investigation as standing still and sniffing a dung pile (nose <20 cm from ground with nostril flares). To investigate which age and sex deposited or acquired information most often during their visits I ran a generalized linear mixed-effects model with a binomial distribution using the R package lme4 (Bates et al. 2015). The number of visits containing defecation or

investigation was set as the response variable, age and sex were set as fixed factors and rhino ID as a random factor. I repeated this with adult state as the fixed factor (i.e. territorial male, non-territorial male or female).

After each olfactory investigation event, I looked back into the video log to identify the depositor of the dung pile. I was able to identify the depositor of 593 of 772 (77%) of the sniffed dung piles. To assess which adult dung piles were investigated by each adult state (i.e. territorial male, non-territorial male or female), I ran a generalized linear mixed-effects model with a binomial distribution. State of the investigator was set as the response variable, sex of the dung owner was set as the fixed factor and rhino ID as a random factor. I repeated this with state of the dung owner as a fixed factor.

To compare the weight of dung deposited I collected 40 whole dung samples from adult white rhinos (territorial males n= 12, non-territorial males n= 10, oestrous females n= 4, non-oestrous females n= 14) between January and May 2015. I followed individuals on foot until defecation occurred in order to record territorial or oestrous state. Territorial males were identified as adult males performed dung kicking, and non-territorial males as adult males not performing this behaviour (Owen-Smith 1971; Owen-Smith 1973). Oestrous females were identified by the attachment of an adult male, and non-oestrous females as adult females without the attachment of a male (Owen-Smith 1973). To collect the whole dung from territorial males, I collected all scattered pieces of the kicked dung boluses. As dung was fresh (<5 minutes from defecation), the temperature, colour and moisture of the dung made it easily distinguishable from other dung piles in the midden. I collected samples from different individuals, achieved by recording variations in horn shape and size, and other distinguishing characteristics. I spread and dried each dung pile in direct sunlight for 72 hours and then weighed the dry contents. As data were not normally distributed, I performed a Kruskal-Wallis with post hoc Dunn's test to explore any differences in dung weight.

Finally, from 433 observed defecations, I recorded the location of each dung pile according to each midden's dimensions and categorised them into one of four locations; centre, midrange, edge or outside the midden. Middens are often ellipses, thus the average diameter of the widest part was 7.7 m (range= 5-10 m) and the narrowest part 5.5 m (range= 3-8 m). The average area of the middens was 34.1 m^2 (range= 15.7-50.3 m^2). For small middens (<30 m^2), I classified the centre as a 1.5 m radius from the centre most point, the midrange as a 1.5-3 m radius from the centre most point, the edge as a 3-4.5 m radius from the centre most point, and anything further than 1 m from the edge of the midden as being outside. For medium sized middens (30-40 m^2), I classified the centre as a 2.5 m radius around the centre most point, the midrange as a 2.5-5 m radius from the centre point, edge as 5-7.5 m from the centre point, and anything over 1 m from the edge as outside the midden. For large middens (>40 m^2), I classified the centre as a 3 m radius around the centre most point, midrange as 3-6 m from the centre point, edge as 6-9 m from the centre point, and anything over 1 m from the edge as outside.

To assess the effect of age and sex on chosen defecation location, I ran a linear mixed-effects model. Location of defecation was set as the response variable, age and sex were set as fixed factors and

rhino ID as a random factor. I repeated this for the fixed factor of adult state (i.e. territorial male, non-territorial male or female). All statistical analyses and figures were created using RStudio version 0.99.903 for Windows.

4.4. Results

4.4.1. Visitation frequency

Age had a significant effect on visitation frequency, where adults visited 41% and 44% more often than sub-adults and calves, respectively (t=-2.741, p=0.007 and t=-3.830, p<0.001, Fig. 4.1a). Males visited 47% more often than females (t=4.869, p<0.001, Fig. 4.1b). Within adults, territorial males visited 71% and 84% more often than non-territorial males and females respectively (z=6.430, p<0.001 and z=8.677, p<0.001, Fig. 4.1c), while non-territorial males visited 44% more often than females (z=2.442, p=0.037, Fig. 4.1c).

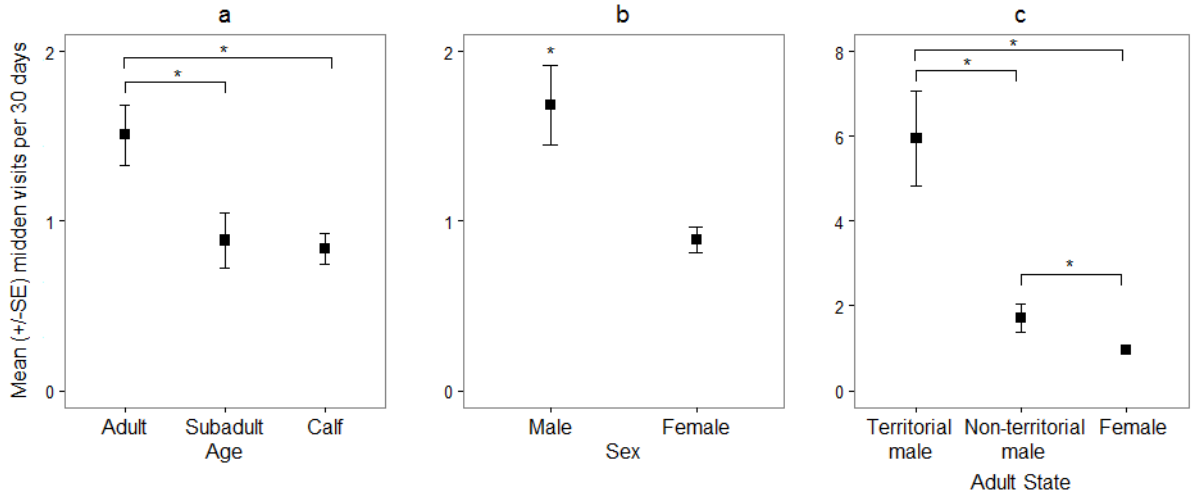

Figure 4.1. Midden visitation frequency of white rhinos of each (a) age, (b) sex and (c) adult state. * indicates significance.

Table 4.1. Total number of individuals visiting each midden during the study.

	Number of adult individuals visiting over sample period			
Midden ID	Territorial male	Non-territorial male	Female	Total
M0006	1	5	8	14
M0079	1	1	4	6
M0113	1	2	5	8
M0127	1	4	10	15
M0128	1	2	7	10
M0129	1	4	11	16
M0131	1	3	11	15
M0132	1	3	5	9
M0136	1	6	11	18
M0142	1	2	7	10

4.4.2. Defecation frequency

Age had a significant effect on defecation frequency when visiting the middens, where adults defecated 50% and 68% more often in the middens than sub-adults and calves respectively (t=-2.213, p=0.028 and t=-3.421, p<0.001, Fig. 4.2a). Males defecated 65% more often than females (t=4.119, p<0.001, Fig. 4.2b) and, within adults, territorial males defecated 89% and 92% more often than non-territorial males and females (z=9.201, p<0.001 and z=10.523, p<0.001; Fig. 4.2c). There was no difference between the defecation frequency of non-territorial males and females (z=0.607, p=0.811, Fig. 4.2c).

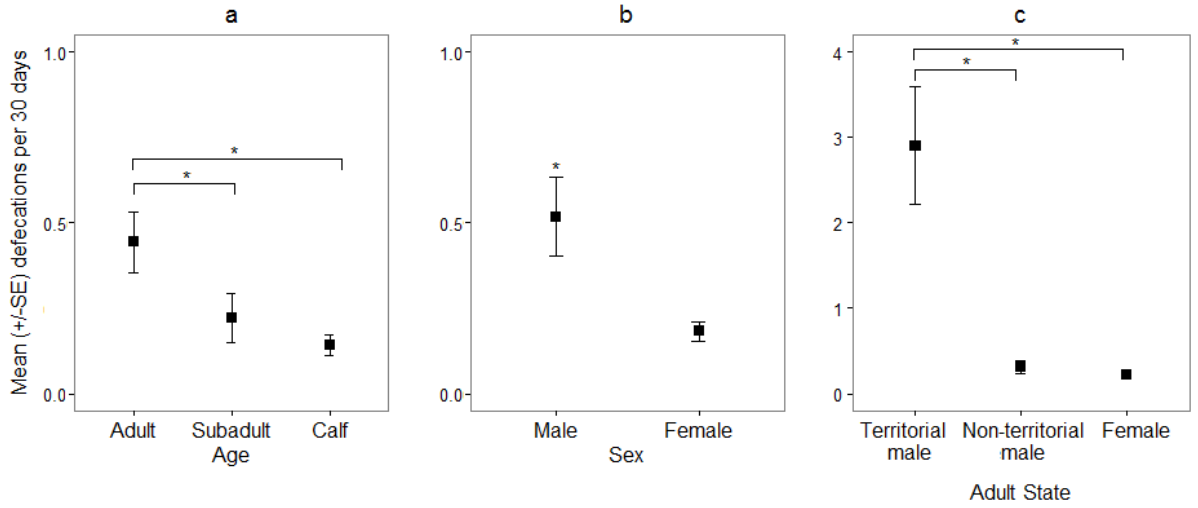

Figure 4.2. Defecation frequency of white rhinos of each (a) age, (b) sex and (c) adult state. * indicates significance.

4.4.3. Information acquisition

Age had a significant effect on the proportion of midden visits involving olfactory investigation of dung, where adults sniffed dung more often than calves (z=-7.063, p<0.001, Fig. 4.3a) and sub-adults (z=-2.677, p=0.007, Fig. 4.3a). Males investigated dung more often than females (z=4.019, p<0.001, Fig. 4.3b), and within adults, females investigated dung less often than territorial and non-territorial males (z=4.808, p<0.001 and z=3.919, p<0.001, Fig. 4.3c). Territorial and non-territorial males spent the same proportion of midden visits investigating dung (z=1.829, p=0.157, Fig. 4.3c).

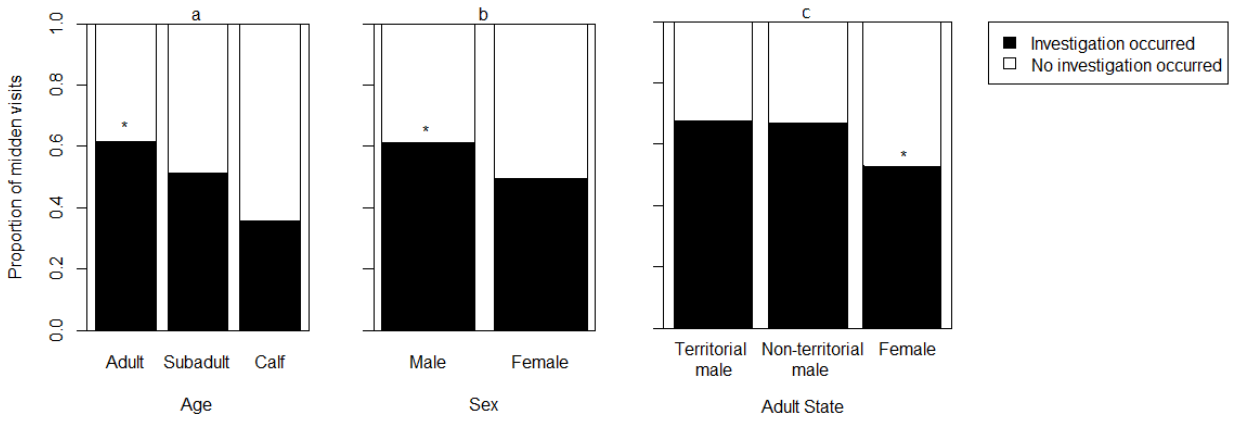

Figure 4.3. Proportion of midden visits involving olfactory investigation by white rhinos of each (a) age, (b) sex and (c) adult state. * indicates significance.

4.4.4. Information deposition

As with investigation, age had a significant effect on the proportion of midden visits where individuals defecated, where adults defecated significantly more than calves (z=-2.729, p=0.006, Fig. 4.4a), but similar to sub-adults (z=0.139, p=0.889; Fig. 4.4a). Males defecated more often than females (z=2.013, p=0.044, Fig. 4.4b) and, within adults, territorial males defecated more often than non-territorial males and females (z=2.613, p=0.024 and z=2.945, p=0.009, Fig. 4.4c). However, the proportion of visits where non-territorial males and females defecated was similar (z=0.218, p=0.974, Fig. 4.4c).

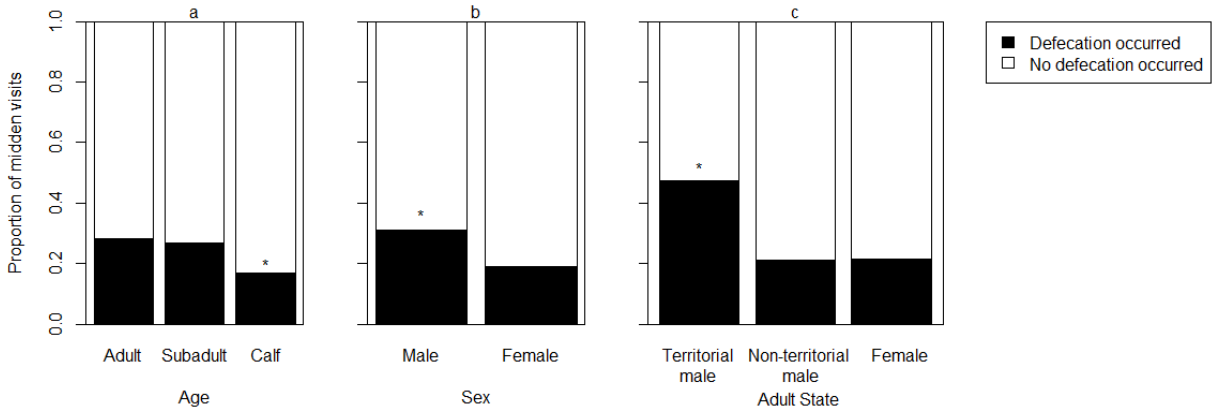

Figure 4.4. Proportion of midden visits involving defecation by white rhinos of each (a) age, (b) sex and (c) adult state. * indicates significance.

4.4.5. Identification of investigated dung piles

There was no difference in the sex of the dung investigated by the different adult classes (z=-0.661, p=0.785, Fig. 4.5a). However, all adults showed a preference to investigating male dung over female dung (Fig. 4.5a). Non-territorial male dung piles were investigated more by females than by territorial males (z=2.455, p=0.037, Fig. 4.5b), while 53% of the dung sniffed by territorial males was their own (Fig. 4.5b). With regard to non-territorial males, there was no difference in the dung piles investigated by resident or visiting subordinate males (z=0.701, p=0.483, Fig. 4.5c).

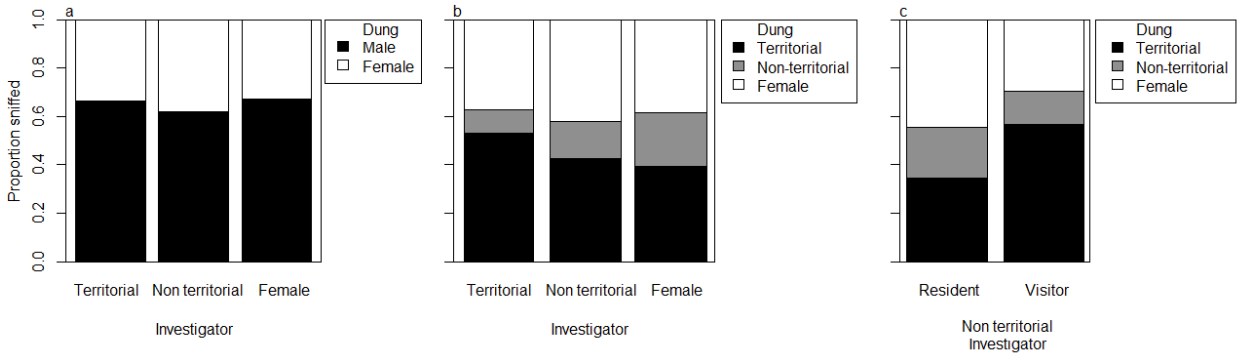

Figure 4.5. Dung piles identified by (a) sex, (b) adult state that were investigated by adults and (c) dung piles investigated by non-territorial males.

4.4.6. Dung weight

Sex had no effect on dung weight ($H_{(1)}$=1.464, p=0.226, Fig. 4.6a). However, when I compared adult state, I found that the dung piles from territorial males were significantly lighter than dung piles from non-territorial males ($H_{(3)}$=5.804, p=0.027, Fig. 4.6b), oestrous females ($H_{(3)}$=5.804, p=0.028, Fig. 4.6b) and non-oestrous females ($H_{(3)}$=5.804, p=0.047, Fig. 4.6b). The dung piles from non-territorial males, oestrous females and non-oestrous females were all similar (non-territorial vs oestrous $H_{(3)}$=5.804,

p=0.319, Fig. 4.6b; non-territorial vs non-oestrous $H_{(3)}$=5.804, p=0.345, Fig. 4.6b; oestrous vs non-oestrous $H_{(3)}$=5.804, p=0.217, Fig. 4.6b).

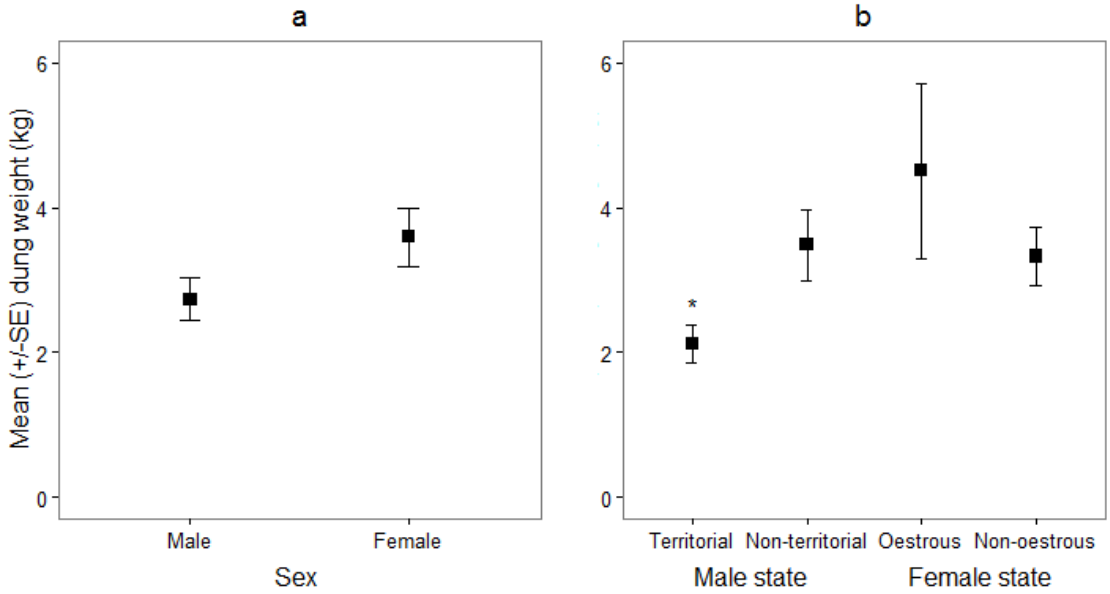

Figure 4.6. Mean dry dung weight from adult white rhinos of each (a) sex, (b) adult state. * indicates significance.

4.4.7. Defecation location

Age had a significant effect on the chosen location of defecation, where adult location was significantly different to both sub-adult (t=2.355, p=0.020, Fig. 4.7a) and calf (t=4.255, p<0.001, Fig. 4.7a) location. The majority of adult defecations (reflecting primarily males as their defecation frequency is higher; section 4b) occurred in the centre of the midden, while sub-adults and calves tended to defecate around the edge of the midden (Fig. 4.7a). Sex also affected defecation location, where males defecated most often in the centre and females most frequently at the edge (t=-5.587, p<0.001, Fig. 4.7b). Further, within adults, territorial males defecated exclusively in the centre, compared to non-territorial males who only defecated in the centre 50% of the time and females mostly at the edge of the midden (t=-3.751, p<0.001 and t=4.124, p<0.001, Fig. 4.7c).

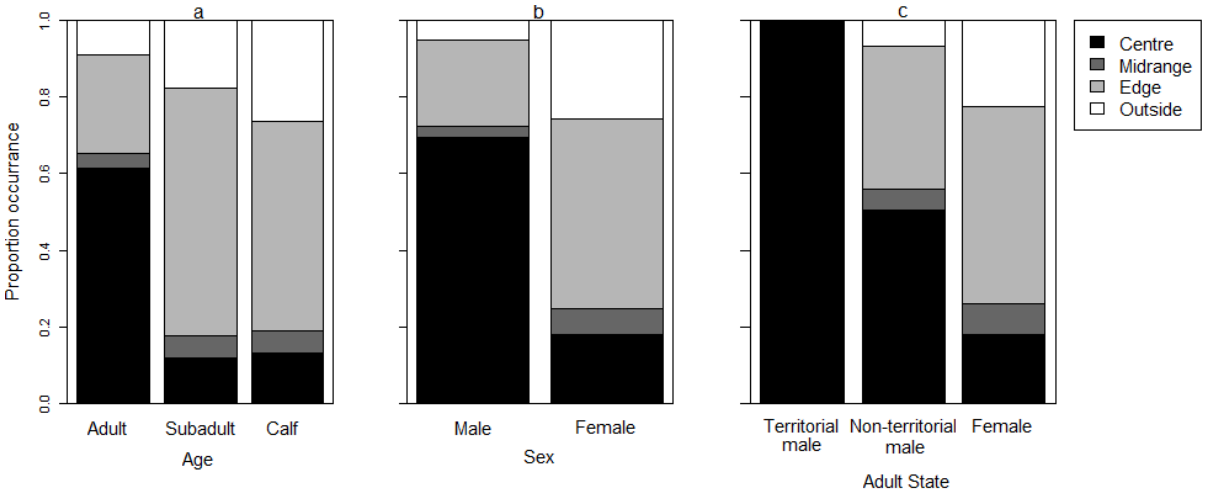

Figure 4.7. Occurrence of defecation location within middens for white rhinos of each (a) age, (b) sex and (c) adult state.

4.5. Discussion

Olfactory communication can be considered one of the least reliable forms of communication as the direction and duration of information transfer, and thus the probability of being detected by a desired target, is greatly influenced by wind, heat and other factors (Alberts 1992; Bossert 1968; Nimmermark and Gustafsson 2005). However, some mammals ensure detection of olfactory signals by utilising communal marking sites such as middens (Darden et al. 2008; Eppley et al. 2016; Rodgers et al. 2015). White rhinos defecate communally in middens, but it is unclear why or how frequently individuals use these sites. As characteristics of sex, age, and territorial and oestrous state are identifiable via dung odours (Marneweck et al. 2017), it is likely that middens act as information centres for white rhinos. As predicted, adult white rhinos (the likely breeders) were the ones that utilised the middens the most (visited, defecated and sniffed the dung of other individuals). Yet, of the different adult classes, territorial males visited and defecated in middens and olfactorily investigated (i.e. sniffed) the dung of individuals most of all. Both non-territorial males and adult females also sniffed dung in these middens suggesting that middens act as information centres for a wide range of individuals and not just territorial males. In addition to obtaining information, territorial males showed dung regulation by defecating smaller volumes more frequently than other adults (i.e. non-territorial male or female). Additionally, they defecated exclusively in the centre of middens, while other individuals defecated around the periphery, providing a spatial aspect to the distribution of dung odours.

The extensive visitation and use of middens by territorial males suggests that middens are important for these individuals. This is in line with a polygynous mating system, where males deposit information for territorial defence and acquire information on female reproductive state. As white rhino males defecate in middens along territorial boundaries, and throughout the territory, it is likely that they use olfactory cues to indicate territorial ownership (Owen-Smith 1975). This can be directed at rival males (i.e. male-male communication) and/or towards adult females (potential mates) visiting the

territory (i.e. male-female communication). Moreover, the high visitation frequency to the middens, coupled with greater investigation (sniffing), suggests that territorial males are acquiring the most information. This would be important for territorial defence (e.g. detecting an intruding rival male) and for increasing their fitness (i.e. locating a visiting oestrous female). However, all the territorial males also sniffed their own dung when visiting the middens. This was unexpected, but they may do this to assess the strength of their own dung signals as a way to determine whether they need to remark. Examples of this in the literature are limited. During an experiment to assess if European badgers (*Meles meles*) could discriminate between self, neighbour and alien odours, individuals did also spend time investigating their own faecal marks (Palphramand and White 2007). I suggest that this may often be overlooked as studies focus on the investigation of conspecifics related to increasing fitness.

Surprisingly, I found that non-territorial males also spent a considerable amount of time sniffing the dung of both adult females and territorial males. Thus, they likely obtain information on the reproductive state of these females as well. Although territorial males defend an area, and should therefore monopolise mating opportunities (Owen-Smith 1973), subordinate males can obtain sneaky copulations (Guerier 2012). Thus, it makes sense that non-territorial males would be interested in the reproductive state of females in the area. As non-territorial males living within the territory (i.e. subordinate bulls) do not challenge the territorial male for the territory (Owen-Smith 1973), the investigation of a territorial male's dung likely provides information on how recently he was in the area. However, for visiting males, sniffing the dung of the territorial male may provide insight into his body condition (Gosling and Roberts 2001; Rajagopal et al. 2010), and thus help assess whether he can be challenged for the territory.

For females, defecating in middens is a way to advertise oestrous state (i.e. female-male communication). However, females did not just deposit information, but also obtained information when visiting middens. Specifically, adult females investigated dung piles of both territorial and non-territorial males. By investigating the territorial male's dung, a female may be able to assess his quality (Charpentier et al. 2008; Johansson and Jones 2007; Kavaliers and Colwell 1995). This would then help her to decide whether to breed with him or not. However, as non-territorial males sometimes breed (Guerier 2012), it is possible that females may be assessing the quality of all the males that have defecated in the midden and thus not limiting her options to the territory owners. This would make sense as the benefits of multiple copulations in improving reproductive success have been shown (Jennions and Petrie 2000), and thus females may be adopting a strategy that ultimately increases their fitness. Further, it may be possible that oestrous females may use middens to assess male quality and hence avoid territories of sub-optimal males when in oestrus. However, as the oestrous state of females in this study was unknown, this would require further study.

In addition to investigating the dung of adult males, females surprisingly also sniffed the dung of other adult females. In fact, they did this as often as they sniffed territorial male dung, suggesting the possibility of female-female communication. The question is, however, what are they communicating

and/or what information are they interested in? Adult females live in home ranges that extensively overlap with the home ranges of other females (Owen-Smith 1973; Owen-Smith 1975). Thus, it is highly unlikely that females use olfactory signals to demarcate home range boundaries. Moreover, as male white rhinos do not provide any parental care (Owen-Smith 1973), adult females are unlikely to compete over mates. It could simply be that adult females keep track of the other females whose home ranges overlap, and thus know who is close by. A by-product of this, is that the presence of dung from a range of females within middens may transmit information on the local density of adult females, which dispersing individuals may use to determine where to settle (Shrader and Owen-Smith 2002).

Within adults, territorial males defecated the most frequently in middens, likely to reaffirm territory ownership. As they have a number of middens both along the boundaries and within their territory, it makes sense for these males to reduce the volume of dung per deposit and increase the frequency of defecation. By limiting dung volume per defecation event, this allows them to regulate their dung output, a strategy also utilised by several other communally defecating, territorial ungulates (Brachares and Arcese 1999; Lunt and Mhlanga 2011; Sun et al. 1994). Ultimately, dung is a limited resource, therefore territorial males utilising dung for olfactory communication across their territory must manage their dung output to ensure effective distribution. However, reduced dung volume likely limits signal strength and duration (Chapter 5). Nevertheless, due to the nature of territorial dung kicking, it is possible that part of the territorial males' defecation was not collected. This would mean that the weight of territorial male defecations was underestimated. However, due to the immediate collection of dung (<5 minutes), and the ease of identifying fresh dung due to heat and colour, it is unlikely that large portions of dung were not collected and thus affecting the results.

One factor that may increase signal detection and thus enhance detectability is the spatial arrangement of dung within middens. The location of a scent mark has importance in several species. For example, giant pandas (*Ailuropoda melanoleuca*) spend more time investigating odours placed higher from the ground because that indicates body size and associated competitive ability (White et al. 2002). Further, female dwarf mongooses (*Helogale parvula*) spend more time investigating scent marks placed higher from the ground even when they did not differ chemically (Sharpe 2015). This suggests that the location of a scent mark is as important as the scent profile itself.

For white rhinos, many individuals defecate in a midden. By defecating in specific areas, white rhinos add a spatial component to their olfactory signals which may increase detectability and strengthen information on identity. Yet, middens located at a territory boundary may be utilised differently to those located in the core of a territory (Owen-Smith 1973). Further, the number of overlapping female home ranges could also have an effect on midden use by territorial males. With males potentially frequenting middens utilised by a large number of females more than middens only utilised by a few females. Table 4.1 shows that the range of over-lapping females in this study varies considerably (range= 4-11 individual adult females). However, to determine whether midden use is driven by location within a territory and over overlapping female home ranges would require further study.

Overall, the results of my study show that territorial male white rhinos used middens the most frequently. This suggests that an important function of these middens is for information transfer from (male-male and potentially male-female communication) and to territorial males (female-male and male-male communication). However, as both non-territorial males and adult females deposited and obtained information from middens, it is likely that these individuals also use middens as information sources. Ultimately, these results provide the first conclusive evidence for the role of middens as communal information centres in white rhino olfactory communication.

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Chapter 5 : White rhino males (*Ceratotherium simum*) amplify olfactory signals but reduce odour duration via dung kicking

5.1. Abstract

Many mammals enhance their olfactory signals visually by depositing them in conspicuous locations such as well-travelled paths. However, it is also possible to enhance the odour itself through behaviours aimed at modifying odour emission rates. White rhinos, irrespective of age or sex, defecate communally in middens. While defecating, territorial males kick sharply with their back feet, which disperses their dung. Despite being a ubiquitous trait of territorial male white rhinos, the reason behind this behaviour is unclear. I hypothesised that the purpose of dung kicking was for olfactory signal amplification (OSA) in terms of an increased emission of volatile compounds (i.e. increased signal strength). Using dung collected from non-territorial adult males (because it was not possible to get un-kicked territorial male dung), I show that the dispersal of male white rhino dung causes OSA by increasing the emission of hydrocarbon acids. Because the dung odours of territorial and non-territorial males differ only quantitatively, it is likely that the same patterns will occur for territorial male dung odours following dung dispersal. However, despite the benefits of OSA, dung dispersal carried a cost of decreased odour longevity. Thus, signal detectability is temporally reduced. Yet, territorial males likely counteract this by defecating in middens during peak visitation times by other individuals. As a result, I suggest that dung kicking by territorial males amplifies signal strength, such that their dung odours are the most prominent and easily detectable by individuals visiting the middens. This then would better signal territorial ownership to both potential rivals and potential mates.

5.2. Introduction

Olfactory signals communicate a wide range of information including territory ownership, reproductive state, and group membership (Archunan and Rajagopal 2013; Barja et al. 2005; Theis et al. 2012). Information in these signals is transmitted via volatile organic compounds (VOCs) emitted from urine, faeces and/or specialised glandular secretions (Archunan and Rajagopal 2013; Cross et al. 2014; Karthikeyan et al. 2013). As VOCs disperse from their source, their concentration decreases with increased distance. Therefore, the active space of an olfactory signal is the area around an odour source where the VOCs are at sufficient concentration to produce a behavioural reaction from a receiver (Elkinton and Cardé 1984).

As the dispersal of olfactory signals is influenced by environmental factors such as heat and wind (Alberts 1992; Bossert 1968; Nimmermark and Gustafsson 2005), a key challenge for animals using olfactory communication is to increase the likelihood that individuals will detect these signals (Gosling and Roberts 2001). This can be achieved via visual enhancement, for example, individuals can

increase detectability by depositing scent marks in heavily utilised areas. Wolves (*Canis lupus*) do this by leaving faecal marks on regularly used paths and crossroads (Barja et al. 2004). In addition, the specific location of scent marks can also provide key information. Elevated scent marks provide visual enhancement of the scent mark, but this also provides information on the depositors body size and thus competitive ability (Alberts 1992). Further, placing scent marks at elevated locations may also effect odour dispersal and thus increase the active space (Alberts 1992; Gorman and Mills 1984). For instance, black backed jackals (*Canis mesomelas*) defecate on top of rocks and large herbivore dung piles, increasing the height and therefore the active space of their faecal marks (Hayward and Hayward 2010). Thus, strategic placement can offer olfactory signal amplification (OSA) as well as visual enhancement.

Increasing the duration of the odour is another form of OSA. For example, lipids found in the urine marks of lions (*Panthera leo*) and tigers (*Panthera tigris*) slow down the release of VOCs and therefore increase longevity of the odour (Andersen and Vulpius 1999; Asa 1993; Burger et al. 2008). However, many species do not possess physiological mechanisms that regulate odour release, and thus rely on adjusting their behaviour to increase signal detection (e.g. strategic placement of scent marks). Although behavioural OSA has been suggested, studies investigating the effect of specific behaviours on odour release are limited. Moreover, herbivores in particular remain understudied in this regard. For instance, beira (*Dorcatragus megalotis*) and steenbok (*Raphicerus campestris*) perform ground scraping as part of their defecation sequence, but the reason for such behaviour is unknown (Giotto et al. 2008; Walther 1990).

White rhinos (*Ceratotherium simum*) have poor eyesight and rely heavily on olfactory communication. Olfactory signals are concentrated in communal dung heaps (i.e. middens) by individuals of all ages and sex (Owen-Smith (1973), Marneweck et al. 2017, Chapter 3). These middens are located at strategic locations including territory boundaries, well-travelled rhino paths, and next to water sources (Owen-Smith 1973), which increases encounter probability. The primary function of middens seems to be for territorial males to advertise territory ownership (male-male communication) and find reproductive females (female-male communication) (Chapter 4). However, secondary functions may include non-territorial males locating reproductive females for sneaking copulations, and/or for females to assess male quality (male-female communication) (Chapter 4).

At a fine scale, individual white rhinos defecate in specific locations within a midden. Specifically, territorial males defecate in the centre, while non-territorial males, adult females, and younger animals defecate around the midden edge (Owen-Smith (1973), Chapter 4). This fine-scale placement may increase the detectability of the signals from these different individuals (Chapter 4). Yet, within these middens, territorial males are the only individuals that scatter their dung, which they do with backward kicking motions of their hind feet (Owen-Smith 1971). To date, it is unclear why they do this. As one of the main functions of middens is communication from territorial males (i.e. male-male and male-female communication), it is possible that these males kick their dung to enhance their olfactory signals (i.e. OSA). To explore this, I tested whether the increase in dung surface area caused

by kicking resulted in an increase in VOC emission, and thus facilitated OSA. However, an increase in odour amplification could lead to a decrease in longevity. Plants are able to produce VOCs (Lerdau et al. 1997) thus, their VOC emission is limitless. However, odour sources such as scent marks do not produce VOCs, rather, they emit them as the result of the organic matter present and bacterial breakdown (Archie and Theis 2011). This results in the VOC emission being finite. Therefore, if an individual were to increase the emission of their scent mark via OSA, the overall longevity would be reduced. If this was the case, then dung kicking by territorial males could constitute a strategy of increasing VOC emission at the expense of odour duration. To counteract the reduced longevity, territorial males could adjust their behaviour such that they deposit dung at optimal times to ensure detection before odour depletion (i.e. during times of high midden use by other individuals). To explore this, I tested the following hypotheses: (1) the dispersal of dung will cause an increase in VOC emission, (2) the increase in VOC emission will ultimately decrease odour longevity, and (3) territorial males defecate primarily during periods of high visitation by other individuals to increase olfactory signal detectability.

5.3. Methods

5.3.1. Collection of dung odours

I conducted this study in the 896 km^2 Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. Here I collected fresh (<5 minutes) dung samples (one dung bolus per sample) from 19 non-territorial (subordinate) adult male white rhinos (i.e. 19 dung samples in total, one per male). I did this by following these individuals on foot until they defecated. I used dung from non-territorial adult males as a surrogate for territorial males as it is not possible to obtain intact (i.e. whole and un-kicked) dung boluses from territorial males. This is because territorial males kick their dung before, during, and after defecating, which breaks up the boluses (Owen-Smith 1973). I felt confident in using non-territorial adult male dung as a surrogate, because the VOCs emitted from territorial and non-territorial male dung differ only quantitatively, with territorial males emitting higher proportions of hydrocarbon acids (Marneweck et al. 2017). This is likely due to higher concentrations of faecal testosterone found in territorial male dung (Rachlow and Berkeley 1998). Specifically, the proportion of acids emitted by territorial dung is 69% higher than those emitted from non-territorial dung (Marneweck et al. unpublished data). Therefore, I expected the same pattern of emission in territorial dung odours, just on a larger scale. Specifically, I would expect the dispersal of dung to cause an increase in acid emission from territorial dung compared to that emitted from dispersed non-territorial dung. Moreover, the classification algorithm used in Chapter 2 had low success in identifying the territorial state of males from dung odours, highlighting the similarities. Consequently, I am confident that by using non-territorial dung, I will gain key insight into the pattern of OSA of territorial male dung odours.

To determine if breaking up of the bolus resulted in OSA, I first collected odour samples (see below) from ten intact boluses. I then broke down the remainder of the samples (n= 9) by hand into approximately 3 x 3 x 6 cm pieces (similar size to what is observed in the wild; Marneweck pers. obs.)

to mimic the kicking action of territorial males. Once the sample was dispersed, I collected odour samples by combining all the dispersed pieces of the bolus. I collected odour samples from all the boluses (n= 19) at intervals of 0, 24 and 48 hours from time of defecation. In between sampling intervals, I left the dung outside on natural substrate (short grass), unprotected, to allow natural degradation to occur. I collected the dung and odour samples between May and August 2014 (i.e. dry season).

Odour samples were collected using headspace extraction. To do this, I used a dynamic headspace method (Amirav and Dagan 1997) to collect the air for 25 minutes from a dung bolus enclosed in a polyacetate bag using a micro-air sampler (Supelco PAS-500) with a realized flow rate of 150 ml/min. VOCs emitted from the dung were captured in a small thermodesorption trap filled with 1 mg of Tenax® and 1 mg of Carbotrap®. I collected each sample from a different non-territorial male, identified by variations in horn shape, skin folds, and other distinguishing characteristics. I defined adults as individuals >7 years of age (Hillman-Smith et al. 1986) and non-territorial males as adult males not performing territorial specific behaviours (i.e. dung kicking, spray urinating) (Owen-Smith 1971; Owen-Smith 1973).

5.3.2. Gas chromatography-mass spectrometry analysis of dung odours

I carried out gas chromatography-mass spectrometry (GC-MS) analysis of the odour samples on a Bruker 450 GC with a 30 m x 0.25 mm internal diameter Varian VF-5ms column, connected to a Varian VF-1ms column coupled to a Bruker 300 quadrupole mass spectrometer in electron-impact ionization mode at 70 eV. Thermodesorption traps were placed in a Varian 1079 injector equipped with a chromatoprobe thermal desorption device. I 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). I verified the identification of VOCs with retention times of authentic standards and published Kovats indices wherever possible (Appendix 1).

5.3.3. Collection of behavioural data

I collected behavioural data between April and September 2015 (i.e. dry season) to correlate with the odour data collection period. I setup motion-triggered video recording camera traps at ten middens, each frequented by a different resident territorial male (identified via differences in horn shape and size). These are the same sites and cameras as used in Chapter 2, just without the experimental manipulation. An average territory is 1.65 km^2 (Owen-Smith 1975), therefore, focal middens were separated by at least 2 km to help ensure separation. Further, video recordings confirmed that the middens were utilised by different territorial males. To record behavioural data I used infrared ‘no-glow’ camera traps (either a Cuddeback Black Flash® E3 or Cuddeback Attack Black Flash® 1194 model). I used these cameras as they do not emit visible light or have a flash, creating minimal disturbance at the midden and therefore allowing me to capture natural behaviour. Cameras were placed approximately 3 metres from the edge of the midden to allow for sufficient field of view. I programmed the cameras to record 30-second videos

at each trigger with a 1-second delay before becoming active again and downloaded. Data were downloaded every two weeks and I created an ID profile for each adult white rhino so that I could record individual visitation and defecation.

From video footage I recorded data on all adults, identified as individuals >7 years based on body size and horn development (Hillman-Smith et al. 1986). I identified territorial males as adult males performing territorial behaviours (i.e. dung kicking, spray urinating) and non-territorial as adult males not performing these behaviours (Owen-Smith 1971; Owen-Smith 1973). Although the oestrous state of white rhino females can be determined by observing the behaviours of territorial males (i.e. following, mounting etc.) (Owen-Smith 1973), I was unable to record these behaviours on the cameras and thus unable to identify female oestrous state. As a result, I grouped all adult females together for analysis.

5.3.4. Statistical analysis

As absolute concentration is subject to variability across samples, I used relative abundance of a VOC within a sample (i.e. proportion) for statistical analysis. I excluded any VOCs that represented undigested plant material (Gershenzon and Croteau 1991b; Ishida 2005) to reduce background noise. I ran an analysis of similarity (ANOSIM) to explore the variation in the dung odours of both intact and dispersed dung. R values close to 1 indicate high separation (i.e. different) while R values close to 0 indicate no separation (i.e. similar). I then ran a similarity percentage (SIMPER) test to determine the major VOC contributors to the odours over the sampling period. To assess if odours of intact and dispersed dung were different over the 48-hour sampling period, I conducted a permANOVA with posthoc pairwise comparisons. Lastly, to investigate if time (i.e. hours from defecation) or treatment (i.e. intact or dispersed) had an effect on the number of VOCs emitted from the non-territorial male dung, I ran a linear mixed-effects model using the R package nlme (Pinheiro et al. 2015). The number of VOCs emitted was set as the response variable, time and treatment, plus their interaction, were set as fixed factors, and rhino ID as a random factor.

The exact time of each visit and defecation was recorded for all adults and categorised into one of six four-hour time periods (10:00-13:59, 14:00-17:59, 18:00-21:59, 22:00-01:59, 02:00-05:59 or 06:00-09:59). These time periods were based on the active periods recorded by Owen-Smith (1973), where white rhinos are often found resting during the heat of the day. I calculated the number of visits and defecations per individual during each time period as a proportion of their total number of visit and defecations. To assess differences in the time of midden visit or defecation from individuals of different adult state (i.e. territorial male, non-territorial male and female) I ran a linear mixed-effects model. Proportion of visits per time period was set as the response variable, state and time period as fixed factors, and rhino ID as a random factor. This was repeated for the proportion of defecations per time period. I performed permANOVA, ANOSIM and SIMPER tests in Primer 6 and performed linear mixed-effects models in RStudio version 0.99.491 for Windows. I created all figures using RStudio, with MDS plots created using the vegan package (Oksanen et al. 2015).

5.4. Results

5.4.1. Odour

The odour of dispersed dung was significantly different to the odour of intact dung at 0 hours (t=1.453, p=0.027) and at 24 hours after defecation (t=1.455, p=0.044). However, after 48 hours the odour of dispersed and intact dung did not differ (t=1.350, p=0.104). Breaking up the bolus caused a higher degree of change in dung odours over the sampling period (intact; R=0.255, p=0.001, Fig. 5.1a; dispersed; R=0.401, p=0.001, Fig. 5.1b). Specifically, at 0 hours, dispersal caused an increase in the proportion of hydrocarbon acids and alkanes, and a decrease in the proportion of hydrocarbon alkenes (Fig. 5.2a, Table 5.1, Table 5.2). At 24 hours after dispersal, there were decreases in the proportions of hydrocarbon alkanes and aldehydes, and an increase in the proportion of hydrocarbon acids compared to the intact dung bolus (Fig. 5.2b, Table 5.1, Table 5.2). The number of VOCs emitted from the dung was effected by the two-way interaction between time (i.e. hours after defecation) and treatment (i.e. intact or dispersed) ($F_{2,29}$=6.196, p=0.006) where the dispersal of dung caused an increase in the number of VOCs emitted at 0 hours, had no effect at 24 hours, and caused a decrease in the number of VOCs emitted at 48 hours (Fig. 5.2a,b,c). Thus, VOCs were amplified by breaking up the bolus, but the duration of emission was reduced.

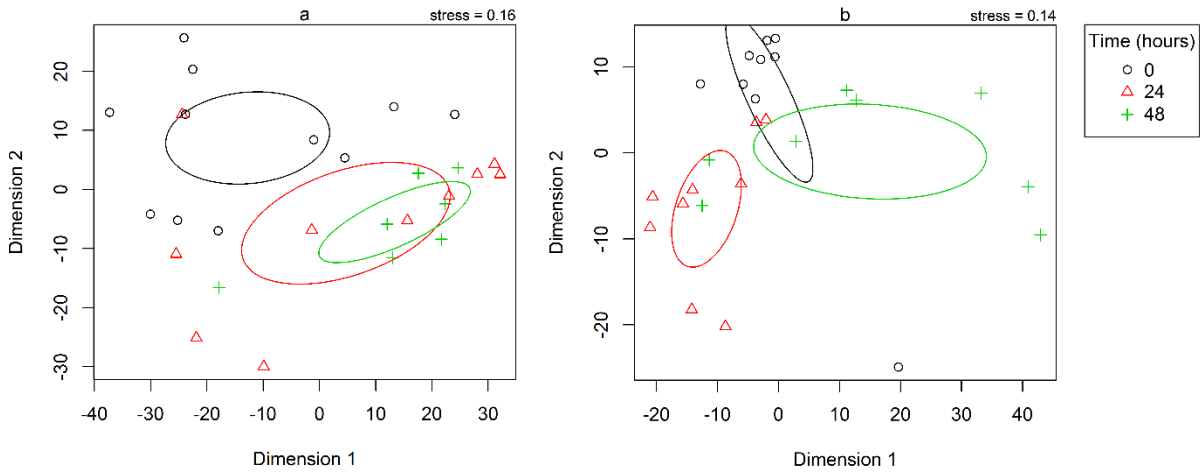

Figure 5.1. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of VOCs emitted from male dung (a) intact and (b) dispersed over 48 hours. Encompassing circles represent 95% confidence intervals.

Table 5.1. List of the top three VOCs contributing to non-territorial male dung odours, intact and dispersed, over 48 hours.

Time (hours)	Ranked VOC contribution	Intact	Dispersed
0	1	Tridecane	Tridecane
	2	Nonane	2,6-Dimethylundecane
	3	(E)-Oct-2-ene	Nonane
24	1	Decanal	Acetic acid
	2	Tridecane	Tridecane
	3	Hexadecane	Butyric acid
48	1	Tridecane	Tridecane
	2	Butyric acid	Decanal
	3	Acetic acid	Hexadecane

Table 5.2. List of volatile organic compounds and their chemical properties.

VOC name	VOC functional group	Molecular weight (g/mol)	Vapor pressure at 25˚C (mm/Hg)
Acetic acid	Hydrocarbon acid	60.05	15.70
Butyric acid	Hydrocarbon acid	88.11	1.65
Decanal	Hydrocarbon aldehyde	156.27	0.10
2,6-Dimethylundecane	Hydrocarbon alkane	184.22	0.18
Hexadecane	Hydrocarbon alkane	226.44	$5E^{-03}$
Nonane	Hydrocarbon alkane	128.26	4.63
(E)-Oct-2-ene	Hydrocarbon alkene	112.13	16.40
Tridecane	Hydrocarbon alkane	184.36	0.06

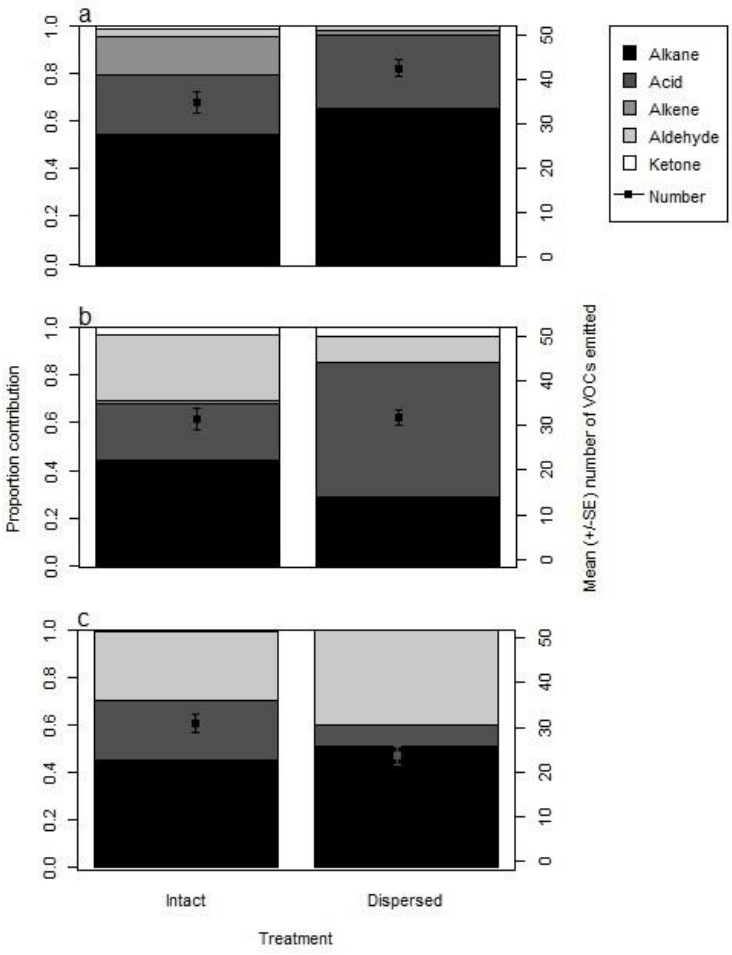

Figure 5.2. The odour composition (indicated by stacked bars) and mean number of VOCs emitted (indicated by point and error bars) from non-territorial male dung, intact and dispersed, at (a) 0 hours, (b) 24 hours and (c) 48 hours after deposition.

5.4.2. Behaviour

Adult state (i.e. territorial male, non-territorial male or female) had no effect on the proportion of midden visits ($F_{2,95}$=0.000, p=1.000) or defecations ($F_{2,529}$=2.892, p=0.056). However, state almost significantly affected the proportion of defecations, where territorial males tended to defecate at higher proportions during time period 10:00-13:59 than other adults. The time of day significantly affected both proportion of visits ($F_{5,485}$=17.151, p<0.001, Fig. 5.3a) and defecations ($F_{5,529}$=7.787, p<0.001, Fig. 5.3b) of all adults, where adults both visited and defecated most often between 14:00-17:59, 18:00-212:59 and 22:00-01:59.

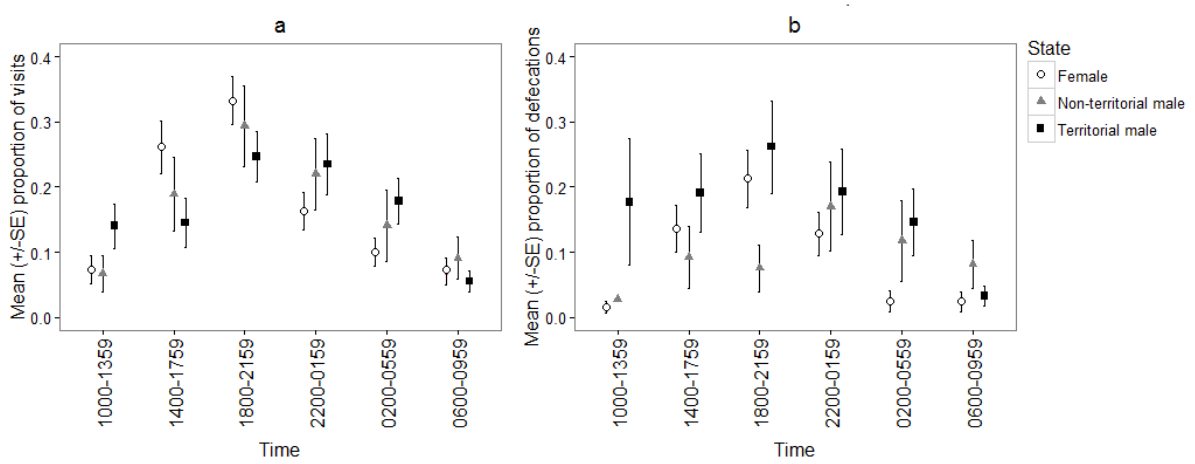

Figure 5.3. Mean proportion of midden (a) visits and (b) defecations by adults per time period.

5.5. Discussion

Olfactory signal amplification (OSA) increases signal detectability by either increasing the VOC emission (signal strength) or the extending VOC emission (signal longevity) (Alberts 1992; Hayward and Hayward 2010; Piñeiro and Barja 2012). When defecating in communal middens, territorial male white rhinos are the only individuals to kick their dung (Owen-Smith 1973). It is possible that by doing this, these males amplify the olfactory signals released from the dung. Using non-territorial male dung as a surrogate, due to its similar VOC profile to territorial male dung, coupled with the difficulty in obtaining un-kicked territorial male dung, I found that dispersal of the dung increased VOC emission (signal strength), but reduced signal duration. However, it is likely that this reduction does not affect signal detectability as territorial males tend to defecate in middens at times of high visitation by other individuals.

Factors affecting the volatility of a VOC include its molecular weight and vapour pressure (Stoddart 1976). Further, a key factor affecting VOC emission is the surface area from which these VOC are emitted (Alberts 1992). In this study, the VOCs with the lowest molecular weights and highest vapour pressures were the ones to show increased emission after the dung was dispersed (i.e. increased surface area) and the most rapid changes between sampling periods. Hydrocarbon acids (acetic and

butyric acid) were larger contributors to the odour sooner when the dung was dispersed compared to when the bolus remained intact. These compounds are of the lowest molecular weight and have the highest vapour of the VOCs identified pressures (Table 5.2) making them the most susceptible to emission. Moreover, these VOCs are associated with territoriality in white rhinos, where territorial male dung emits higher proportions than non-territorial males (Marneweck et al. 2017). With the predicted pattern of 69% larger proportion acid emission for territorial male dung, this means that the hydrocarbon acids would be even larger contributors to the odour and potentially dominate the odour at 0-24 hours after dispersal. As these VOCs are important to signalling territorial status, and their increased emission would mean quicker depletion, I would expect that the odour of territorial signals would be very strong but the longevity even further reduced as a result of dung kicking.

Territorial male dung odours are different to those from non-territorial males (Marneweck et al. 2017). As territorial males have higher levels of faecal testosterone than non-territorial (Rachlow et al. 1998), this may affect odours indirectly via bacteria. The role of bacteria in mammals that use specialised scent glands has been widely documented (Archie and Theis 2011) and although the fermentation hypothesis was developed for mammals that scent mark with specialised glands, it has been suggested that it could be applied to mammals that mark with dung (Archie and Theis 2011). Bacteria, present either within the gut or within the dung, can break down organic matter or produce VOCs that contribute to odour cues (Albone and Eglinton 1974; Gorman 1976). Differences in the anal gland microbiota of meerkats (*Suricata suricatta*) occurs only after individuals reach sexual maturity, suggesting that reproductive hormones have a role in determining host bacterial communities (Leclaire et al. 2014). Therefore, for adult male white rhinos, by achieving territorial status and its subsequent associated increase in testosterone (Rachlow et al. 1998), this may affect microbiota directly (altering the bacteria within the digestive system) or indirectly via the environment (providing specific organic matter for bacteria). Thus, the bacterial activity may be associated with the breakdown of testosterone and therefore the emission of specific VOCs. If the VOCs produced by bacteria include hydrocarbon acids, it is possible that by dispersing dung, males could create an impression of higher testosterone levels by increasing the volatile acid content of the odour. As increased testosterone levels are associated with greater competitive ability (Zielinski and Vandenbergh 1993), the kicking action of the dung could create a signal of a larger and more aggressive male, which has reproductive and intimidation benefits for females and non-territorial males respectively.

The results from this study invites the question, if dung from a territorial male was not dispersed, would the odour be same as that from a non-territorial male? It was not possible to collect intact dung from territorial males, nor is it possible to collect dispersed dung and store it as a whole bolus, as odours are affected immediately (i.e. at 0 hours the odour of dispersed non-territorial dung was different). There are two alternative hypotheses that could address this question, (1) territorial males have higher testosterone than non-territorial males which would affect their dung odours. If this was the case then territorial male dung odour would be different to that of non-territorial even if it remained intact. In

contrast, (2) it may be that the physical act of dispersal is the reason for the difference in territorial and non-territorial male dung odours. Where this is an interesting questions, addressing it is beyond the scope of this study. Thus, further investigation is required.

The dispersal of dung increases the surface area, which consequently increases the area available for the emission of VOCs. Because the VOCs available for emission are limited, as scent marks do not produce VOCs as plants do (Archie and Theis 2011; Lerdau et al. 1997), increased odour emission rates can lead to reduced longevity, which is what I found. In the case of male white rhino dung, a greater number of VOCs were emitted earlier, followed by a quicker depletion, compared to the more stable release of VOCs from intact dung. Therefore, there was a trade-off between the intensity of the signal and its duration. These results suggest that the kicking action associated with scent marking in territorial white rhinos is for OSA, but carries the cost of reduced signal longevity. However, the fact that all territorial males kick their dung suggests that the benefits of signal amplification outweigh the costs of reduced longevity. Moreover, behavioural adjustments by territorial white rhinos may help counteract reduced signal longevity.

White rhino territories are relatively small, on average 1.65 km^2 (Owen-Smith 1975), which would suggest that regular re-marking of middens could be achieved over a short period of time. If this was the case, then the impact of reduced signal longevity could be minimised and the probability of individuals visiting the midden detecting the olfactory signals increased. However, I found that territorial males only defecated in the same midden on average once every ten days (Chapter 4). Compounded with this, white rhino dung odours only last for a couple of days (Chapter 3). Although signals change over time, they are still recognisable as territorial (i.e. still different to non-territorial odours after 48 hours) (Chapter 3). Thus, it would seem that there are long periods where the territorial male's signal will be present but very weak. Dung is a limited resource, thus the likely reason that territorial males are not increasing the rate at which they remark middens may be due to the fact that they are defecating at the maximum frequency permitted by their physiology. Moreover, it seems that males attempt to maximise the spatial distribution of their dung by limiting how much they deposit at any one midden (Chapter 4). Thus, it is likely that they are unable to increase their marking frequency.

However, one way that territorial males could increase the probability of their signals being detected by other rhinos, is to defecate strategically so that the amplified signal strength is highest during the time period when the majority of individuals visit the midden. This would increase detectability and thus counteract the issue of reduced signal duration. I found that territorial males did in fact defecate both before and during times of high visitation from other individuals. Therefore, this suggests that territorial males cope with the reduced signal duration after their signal amplification, by responding with a strategy to increase detectability.

Many species using dung as a scent marking source also perform over-marking strategies, with regard to both territorial defence and mate guarding (Brachares and Arcese 1999; Jordan et al. 2011; Vogt et al. 2014). With this in mind, it is possible that the function of dung kicking by territorial male

white rhinos may be for both odour amplification and over-marking. However, I was unable to obtain an adequate sample size (i.e. n = 6) to test this hypothesis. As a result, it requires further study. It is possible that the dung of territorial males may have amplified odours in comparison to non-territorial males, irrespective of it being dispersed. However, as it is not possible to collect whole dung (i.e. non-dispersed) from territorial males, I was unable to tease apart amplified odours from dispersal.

The results of this study identify the purpose of dung kicking in territorial male white rhinos. Specifically, territorial males use both OSA and strategic temporal placement of signals at a large scale (utilising middens at specific times) as well as a small scale (placement within a midden, Chapter 4). Ultimately, by kicking dung, males increase detection by other individuals visiting the middens by increasing signal strength. Consequently, providing distinctive and unambiguous signals to both intimidate males (potential rivals) and show competitive ability to females (potential mates). Moreover, these results also provide insight into the possible reasons behind the scraping behaviours observed from other communally defecating ungulates.

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Chapter 6 : Conclusions and implications

6.1. Conclusions

The broad aim of my PhD study was to better understand white rhino (*Ceratotherium simum*) olfactory communication. Specifically, which volatile organic compounds (VOCs) are emitted from dung and whether they communicate specific information. In addition, I was interested in the role of communal defecation sites (middens) in white rhino olfactory communication and how these megaherbivores utilised them to access information. To achieve this, I carried out a four studies to incorporate several aspects of white rhino olfactory communication. Initially, I analysed dung odour profiles relaying them to specific characteristics and tested this with an in-situ bioassay (Chapter 2). I then looked at how these dung odours change over time, and their potential implications on behaviour (Chapter 3). Following this, I carried out a behavioural study to investigate who was picking up and/or leaving information at middens (Chapter 4). Lastly, I conducted an experiment to explore the reason for territorial male dung kicking (Chapter 5).

Studies of olfactory communication using wild animals are rare, but those with bioassays in the field are even more so. Chapter 2 is therefore an original and novel contribution to behavioural ecology specifically with regards to animal communication. The results from this chapter show that white rhinos can identify specific characteristics via dung odours, and adjust their behaviour accordingly in response. Moreover, this shows that dung is a valuable medium for transmitting biological information. In addition to just identifying key VOCs, I identified specific odour profiles, creating a ranked list of volatile organic compound (VOC) importance, so that odours could be reproduced. The methods used provide a platform for the investigation of dung odours in other communally defecating and olfactory orientated species and have implications for the future application of VOCs in wildlife management and conservation.

The results of Chapter 3 confirm that the dung odours identified in Chapter 2 have short longevity (2 days), even less so during the wet season. This study is the first to record detailed odour degradation from dung signals and therefore provides a platform for the further investigation of other species utilising dung as a marking source and their longevity. This chapter also highlights the limitations of utilising a metabolic waste product for marking, as marking frequency is limited and white rhinos were unable to adjust frequency during summer months of decreased odour duration.

Chapter 4 revealed a primary function of middens, where territorial males use middens to advertise territory ownership (male-male communication) and to assess potential mates (female-male communication). Potential secondary functions were for non-territorial males to assess the reproductive status of females (female-male communication), and for adult females to assess potential mates (male-female communication) and possibility keep track of other females in the area (female-female communication). This reveals a novel result that white rhinos, although adopting a polygynous mating system, may be sexually promiscuous and only behaviourally polygynous. These results highlight the

importance of understanding how a species utilises middens and how that helps us to understand their social structure and mating system.

The experiment in Chapter 5 demonstrates how ritualised behaviours at middens can cause olfactory amplification, creating the illusion of higher competitive ability. These results are novel, as the purpose of this behaviour was unknown. This also has implications for the behaviours performed by other mammals, for example, the scraping behaviours performed by other ungulates at middens (e.g. oribi, suni, and steenbok). These results also show that territorial male white rhinos face a trade-off, as amplifying the dung odour causes decreased longevity, but they seem to overcome this by defecating at times of high midden visitation by other adults to increase detectability.

Ultimately, the results from these studies support many hypotheses developed previously by Owen-Smith (1973), for example, that white rhino middens act as information centres for individuals in the area, and show the importance of middens to white rhino behavioural ecology. They highlight that a waste product such as dung can be effective at transferring valuable biological information, although with limited longevity. They also indicate the complexity of the system, with several forms of communication occurring (male-male, female-male, male-female and female-female). Further, the combined results of the different studies (i.e. chapters) provide a model for understanding similar behaviours in other communally defecating and/or olfactory orientated species. Lastly, they provide a platform to develop the use of VOCs in the management and conservation of endangered species.

6.2. Further research

It is possible that, along with characteristics of age, sex and state, there is individual recognition via dung odours in white rhinos. If so, then even greater information would be transmitted via dung. Sadly, the exploration of individual odours was beyond the scope of this study, but an important avenue of further research with further implication for white rhino olfactory communication. In addition, further research should take into account the location of a midden (e.g. territory boundary vs. core of a territory) and the number of overlapping female home ranges to investigate the potential effects they may have on midden behaviour and usage (Chapter 4).

The results from Chapter 4 highlight the possibility of female-female communication occurring in white rhinos. This was an unexpected, as the purpose of this form of female communication is unclear. For example, 1) females do not defend exclusive home ranges from other females, 2) they do not form or move in groups with other adult females, and 3) as males do not provide parental care, females do not complete for males (Owen-Smith 1973). It is possible that females keep track of the individuals whose home ranges overlap with theirs, but it is unclear what the purpose of this would be or why this would be important. With the above points in mind, it would be interesting to explore (using behavioural reactions) the degree to which females recognise specific individuals from their dung odours. Coupled with this, it would be interesting to investigate the olfactory communication behaviour of female white rhinos within their home ranges to determine the purpose behind female-female communication.

The results from chapter 5 also invite further questions. It is not clear whether the act of dung kicking causes territorial male dung to smell differently to that from non-territorial males, or whether territorial male dung would smell differently even if it remained intact. These two alternate hypotheses require further investigation. Moreover, the potential function of over-marking is another avenue of further research.

6.3. Implications

The results of my overall study indicates the importance of communal defecation and has unlocked the door to the complex world of olfaction in white rhinos (and likely a wide range of mammals). Consequently, my results provide a platform for further research into the theoretical and practical applications of VOCs for a wide range of mammals. The methods used here should provide a baseline for future research on olfactory communication. Specifically, how to analyse odours over time, which is currently under-studied. This study also shows how future studies should also aim to include both chemical and behavioural aspects to their research on olfactory communication, in order to create a rounded understanding. This is applicable to any species utilising any odour source for communication.

Further research should include other ungulates utilising middens, especially those which are difficult to study, such as suni antelope (*Neotragus moschatus*), due to their solitary lifestyle and the dense habitat within which they live. Additionally, by understanding the VOCs responsible for conveying specific information, we can synthetically reproduce them for conservation benefit (see below) and begin to explore olfactory manipulation armed with greater knowledge. There are direct implications for rhino species as well as other olfactory orientated species.

6.3.1. Scent broadcasting

Scent broadcasting refers to the depositing of an odour source (e.g. dung) in an area with the aim of manipulating behaviour. The success of reintroductions relies on the familiarity of the area and the response of resident individuals (Campbell-Palmer and Rosell 2011). Post-release mortality is high in black rhinos (*Diceros bicornis*) due to aggression from resident individuals and even dehydration/starvation after moving vast distances in suboptimal habitat (Emslie et al. 2009). Scent broadcasting for black rhinos was explored by Linklater et al. (2006) with the aim of reducing post-release translocation risk and mortality. Interestingly, individuals released into an area containing their own dung moved farther than those released without scent broadcasting. Chapter 2 provides a framework, where by repeating the methods described with black rhino dung odours, we can better understand the messages transmitted and develop an effective tool for management. For example, if we reproduce the dung odour of a non-territorial male black rhino, and distribute the odour around a resident territorial male, he may be expecting the arrival of a new individual and show decreased aggression. Further, we may be able to restrict movement of the relocated individual by depositing the odour of a territorial male as a boundary, or by depositing the odour of an oestrous female to encourage him to stay

in a specific area. Ultimately, by increasing our knowledge on the information transmitted via dung we can target specific information carriers to increase relocation success for rhinos and provide an effective management tool to encourage relocated individuals to stay within a new area and reduce conflict with conspecifics (Swaisgood 2007).

6.3.2. Increasing captive reproduction

White rhinos do not suffer post-release mortality as black rhinos do, but they do have poor reproductive success in captivity. Captive breeding is an important conservation strategy but the elimination of mate choice is a problem for many captive species, including white rhinos, where males require competition for the stimulation of sexual behaviour and females require mate choice for oestrous cycling to be initiated (Lindemann 1982; Owen-Smith 1988). Using the key VOCs, I identified in Chapter 2, the dung odour of a territorial male(s) white rhino can be reproduced. The results in Chapter 2 showed that territorial males responded to these odours, so by implanting these odours into white rhino enclosures we can create the illusion of multiple males and stimulate both sexes. Ultimately, this may increase the likelihood of copulations and successful breeding and thus provide an important conservation tool.

6.3.3. Manipulating movements

Scent broadcasting is a tool that can be developed for any olfactory species. Using Chapter 2 as a model, we can successfully identify and validate specific signals and use them for conservation benefit. Potentially, this has applications for manipulating movements. For example, to encourage movement along corridors to promote genetic flow. Further, Jackson et al. (2012) were able to restrict the movement of African wild dogs (*Lycaon pictus*) using translocated dung and urine. By identifying the key VOCs emitted from these odour sources we can replicate and reproduce them for distribution on a large scale and, ultimately, reduce conflict with humans by restricting movements to within protected areas. This potential use of natural cues to modify behaviour and reduce conflict may offer a cheaper and more effective option to translocations and mitigate potential poaching opportunities.

6.4. References

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Appendix 1: All identified volatile organic compounds from white rhino dung

Table A1. Volatile organic compounds distinguished from all samples of white rhino dung odour. Compound identification criteria and notes: a = comparison of MS with published data; b = comparison of MS and retention time with published data; c = comparison of MS and retention time with authentic standard.

Compound name	**Functional group**	**Retention time**	**CAS #**	**KOVA TS index**
Acetic acid[c]	Hydrocarbon acid	1.681	64-19-7	645
Propanoic acid[b]	Hydrocarbon acid	2.514	79-09-4	710
Isobutyric acid[b]	Hydrocarbon acid	3.396	79-31-2	765
Butyric acid[b]	Hydrocarbon acid	4.019	107-92-6	789
3-Methylbutyric acid[b]	Hydrocarbon acid	4.976	503-74-2	848
2-Methylbutyric acid[b]	Hydrocarbon acid	5.397	116-53-0	863
Pentanoic acid[b]	Hydrocarbon acid	5.800	109-52-4	841
Butylacetic acid[b]	Hydrocarbon acid	8.038	1070-83-3	952
Butylethylacetic acid[b]	Hydrocarbon acid	11.129	149-57-5	1123
Nonanoic acid[b]	Hydrocarbon acid	14.126	112-05-0	1273
Undecanoic acid[b]	Hydrocarbon acid	19.561	112-37-8	1561
Hexadecanoic acid[b]	Hydrocarbon acid	25.602	112-39-0	1870
Octadecanoic acid[b]	Hydrocarbon acid	28.380	57-11-4	2171
Octanoic acid[a]	Hydrocarbon acid	29.666	124-07-2	-
Butan-1-ol[b]	Hydrocarbon alcohol	2.272	71-36-3	653
3-Methyl-2-butanol[a]	Hydrocarbon alcohol	2.764	598-75-4	-
3-Methyl-1-butanol[b]	Hydrocarbon alcohol	3.159	123-51-3	737
Pentan-1-ol[b]	Hydrocarbon alcohol	3.665	71-41-0	766
(3E)-3-Hexen-1-ol[b]	Hydrocarbon alcohol	5.238	544-12-7	845
Hexan-1-ol[b]	Hydrocarbon alcohol	5.637	111-27-3	860
6-Methyl-3-heptanol[b]	Hydrocarbon alcohol	8.352	589-98-0	991
(2E)-2-Nonen-1-ol[a]	Hydrocarbon alcohol	9.695	31502-14-4	-
3-Nonanol[a]	Hydrocarbon alcohol	9.909	624-51-1	-
Nonan-2-ol[b]	Hydrocarbon alcohol	10.939	628-99-9	1100
Undecan-1-ol[a]	Hydrocarbon alcohol	11.005	112-42-5	-
Nonan-1-ol[b]	Hydrocarbon alcohol	12.432	112-42-5	1169

2-Methyl-1-undecanol[a]	Hydrocarbon alcohol	15.881	10522-26-6	-
2-Butyl-1-octanol[a]	Hydrocarbon alcohol	16.220	3913-02-8	-
(9E)-9-Hexadecen-1-ol[a]	Hydrocarbon alcohol	27.377	64437-47-4	-
Pentanal[b]	Hydrocarbon aldehyde	2.645	110-62-3	693
Hexanal[c]	Hydrocarbon aldehyde	4.234	66-25-1	776
(2E)-2-Hexanal[b]	Hydrocarbon aldehyde	5.253	6728-26-3	850
Heptanal[c]	Hydrocarbon aldehyde	6.256	111-71-7	901
(2Z)-2-Heptenal[b]	Hydrocarbon aldehyde	7.445	57266-86-1	952
Octanal[c]	Hydrocarbon aldehyde	8.449	124-13-0	1003
(2E)-2-Nonanal[a]	Hydrocarbon aldehyde	9.912	18829-56-6	-
Nonanal[c]	Hydrocarbon aldehyde	10.976	124-19-6	1103
Decanal[c]	Hydrocarbon aldehyde	13.166	112-31-2	1205
Tetradecanal[b]	Hydrocarbon aldehyde	20.409	124-25-4	1574
(9Z)-9-Octadecenal[b]	Hydrocarbon aldehyde	27.830	2423-10-1	-
(9Z)-9-Hexadecenal[b]	Hydrocarbon aldehyde	30.430	56219-04-6	-
2-Methylpentane[b]	Hydrocarbon alkane	1.882	107-83-5	560
3-Methylpentane[b]	Hydrocarbon alkane	1.962	96-14-0	570
Hexane[c]	Hydrocarbon alkane	1.927	110-54-3	600
Methylcyclopentane[b]	Hydrocarbon alkane	2.265	96-37-7	635
3-Ethylhexane[c]	Hydrocarbon alkane	4.180	619-99-8	772.9
2,4-Dimethylheptane[b]	Hydrocarbon alkane	4.649	2213-23-2	820
4-Methyloctane[b]	Hydrocarbon alkane	5.475	2216-34-4	862.85
Nonane[c]	Hydrocarbon alkane	6.411	111-84-2	900
Decane[c]	Hydrocarbon alkane	8.658	124-18-5	1000
4-Methyldecane[b]	Hydrocarbon alkane	9.004	2847-72-5	1059
6-Methyloctadecane[a]	Hydrocarbon alkane	11.506	10544-96-4	-
3-Methyldecane[a]	Hydrocarbon alkane	12.340	13151-34-3	-
2-Methylundecane[b]	Hydrocarbon alkane	12.194	7045-71-8	1163
2,3-Dimethyldecane[a]	Hydrocarbon alkane	12.497	17312-44-6	-
Undecane[c]	Hydrocarbon alkane	13.099	629-59-4	1100
Dodecane[c]	Hydrocarbon alkane	13.222	112-40-3	1200
2,6-Dimethylundecane[b]	Hydrocarbon alkane	13.376	17301-23-4	1213
Pentadecane[a]	Hydrocarbon alkane	14.120	629-62-9	-
2,3-Dimethylundecane[a]	Hydrocarbon alkane	14.432	17312-77-5	-
2-Methyldecane[a]	Hydrocarbon alkane	14.347	6975-98-0	-

Nonadecane[a]	Hydrocarbon alkane	14.517	629-92-5	-
Tridecane[c]	Hydrocarbon alkane	15.217	629-50-5	1300
2,6,10-Trimethyldodecane[a]	Hydrocarbon alkane	18.233	3891-98-3	-
Pentadecane[c]	Hydrocarbon alkane	18.935	629-62-9	-
Hexadecane[c]	Hydrocarbon alkane	20.636	544-76-3	-
Heptadecane[c]	Hydrocarbon alkane	22.288	629-78-7	-
Octadecane[c]	Hydrocarbon alkane	23.858	593-45-3	-
Isopropyl Myristate[b]	Hydrocarbon alkane	23.649	110-27-0	1827
Isopropyl Palmitate[b]	Hydrocarbon alkane	27.008	142-91-6	1981
Tetracosane[b]	Hydrocarbon alkane	31.307	646-31-1	2400
Pentacosane[b]	Hydrocarbon alkane	32.684	630-02-4	2500
Hexacosane[b]	Hydrocarbon alkane	34.319	630-01-3	2600
(E)-Oct-2-ene[b]	Hydrocarbon alkene	4.319	111-67-1	810
(Z)-Oct-2-ene[b]	Hydrocarbon alkene	4.379	7642-04-8	815
X-Octene	Hydrocarbon alkene	4.485	-	-
X-Octene	Hydrocarbon alkene	4.601	-	-
(3E)-1,3-Octadiene[b]	Hydrocarbon alkene	4.784	1002-33-1	827
Non-1-ene[a]	Hydrocarbon alkene	6.215	124-11-8	-
(3E)-3-Nonene[a]	Hydrocarbon alkene	6.228	20063-77-8	-
(4E)-2,4-Dimethyl-2,4-heptadiene[a]	Hydrocarbon alkene	5.850	74421-05-9	-
(3E)-3-Decene [a]	Hydrocarbon alkene	7.773	19150-21-1	-
Undec-1-ene[a]	Hydrocarbon alkene	10.734	821-95-4	-
(3Z)-3-Dodecene[a]	Hydrocarbon alkene	13.055	7239-23-8	-
Tridecene-1[b]	Hydrocarbon alkene	15.060	2437-56-1	1291
Acetic acid, butyl ester[b]	Hydrocarbon ester	4.515	123-86-4	813
Octadecanoic acid, phenylmethyl ester[c]	Hydrocarbon ester	10.932	637-55-8	-
Butyl n-hexanoate[b]	Hydrocarbon ester	12.825	626-82-4	1186
Dodecanyl acetate[b]	Hydrocarbon ester	20.290	122-66-3	1606
Pentan-2-one[b]	Hydrocarbon ketone	2.432	107-87-9	690
Hexan-2-one[b]	Hydrocarbon ketone	4.029	591-78-6	7-90
5-Methylhexan-2-one[a]	Hydrocarbon ketone	5.991	110-12-3	
1-Octen-3-one[b]	Hydrocarbon ketone	8.112	4312-99-6	980
beta-Nonanone[b]	Hydrocarbon ketone	10.644	821-55-6	1090

Undecan-2-one[b]	Hydrocarbon ketone	14.953	112-12-9	1292
Dodecan-2-one[a]	Hydrocarbon ketone	14.608	6175-49-1	-
Toluene[c]	Benzenoid	3.726	108-88-3	762
alpha-Methyltoluene[b]	Benzenoid	5.561	100-41-4	855
1,4-Dimethyl benzene[c]	Benzenoid	5.696	106-42-3	862
Styrene[c]	Benzenoid	6.224	100-42-5	895
4-Ethylbenzoic acid, cyclopentyl ester[a]	Benzenoid	6.522		-
1-Ethyl-3-methylbenzene[b]	Benzenoid	7.622	98-82-8	958
Benzaldehyde[c]	Benzenoid	7.769	100-52-7	947
Carbamic acid, methyl-, phenyl ester[a]	Benzenoid	7.975	1943-79-9	-
Trimethyl benzene[b]	Benzenoid	8.110	95-63-6	990
Acetophenone[c]	Benzenoid	10.137	98-86-2	1062
p-Cresol[c]	Benzenoid	10.717	106-44-5	1077
m-Cresol[c]	Benzenoid	10.525	108-39-4	-
1-Isopropenyl-2-methylbenzene[a]	Benzenoid	10.492	7399-49-7	-
Phenylethyl Alcohol[c]	Benzenoid	11.180	60-12-8	1121
Benzenepropanol[b]	Benzenoid	13.401	122-97-4	1237
1-Isopropyl-2-methoxy-4-methylbenzene[a]	Benzenoid	13.721	1076-56-8	-
3-Propylphenol[a]	Benzenoid	14.195	621-27-2	-
Benzaldehyde, 3-hydroxy-4-methoxy[a]	Benzenoid	16.978	621-59-0	-
Diethyltoluamide[a]	Benzenoid	20.208	134-62-3	-
5-Isopropenyl-2-methylcyclohexanol[a]	Miscellaneous	7.457	18675-33-7	-
(3E)-2,6-Dimethyl-1,3,7-octatriene[a]	Miscellaneous	7.898	6876-07-9	-
1-Propynylcyclohexane[a]	Miscellaneous	7.930	18736-95-3	-
(2E)-3,7-Dimethyl-2-octene[a]	Miscellaneous	7.951	-	-
5,7-Dimethyl-1,6-octadiene[a]	Miscellaneous	8.152	85006-04-8	-
1-Methyl-3-{2-methyl-2-propenyl)cyclopentane[a]	Miscellaneous	8.113	75873-00-6	-
6-Methyl-5-heptene-2-one[b]	Miscellaneous	8.232	110-93-0	984

(6E)-2,6-Dimethyl-2,6-octadiene[b]	Miscellaneous	8.332	2609-23-6	1004
1,2-Dimethyl-1-cyclooctene[a]	Miscellaneous	8.335	54299-96-6	-
1-Isopropyl-2-methyl-3-(1-methylethylidene)cyclopropane[a]	Miscellaneous	8.451	24524-52-5	-
(6Z)-2,6-Dimethyl-2,6-octadiene[a]	Miscellaneous	8.636	2492-22-0	-
(3E)-3-Ethyl-2-methyl-1,3-heptadiene[a]	Miscellaneous	8.562	61142-35-6	-
Pentylidenecyclopentane[a]	Miscellaneous	8.869	53366-55-5	-
(3E)-3-Ethyl-2,5-dimethyl-1,3-hexadiene [a]	Miscellaneous	8.922	62338-07-2	-
2-Ethylhexan-1-ol[a]	Miscellaneous	9.060	104-76-7	-
m-Menth-3(8)-ene[a]	Miscellaneous	9.218	13828-34-7	-
m-Menth-1(7)-ene[a]	Miscellaneous	9.223	13837-71-3	-
(3-Methylbutylidene)cyclopentane[a]	Miscellaneous	9.253	53366-51-1	-
(5E)-4-Methyl-1,5-heptadiene[a]	Miscellaneous	9.813	998-94-7	-
(6E)-2,6-Dimethyl-2,6-octadiene[b]	Miscellaneous	9.780	2792-39-4	990
3-Ethyl-1,5-octadiene[a]	Miscellaneous	9.872	-	-
1-Isopropenyl-2-methylcyclohexane[a]	Miscellaneous	10.003	15193-25-6	-
(2E,6E)-4-Methyl-2,6-octadiene[a]	Miscellaneous	10.078	74498-94-5	-
4,8-Dimethyl-1,7-nonadiene[a]	Miscellaneous	10.099	62108-28-5	-
2,5-Dimethyloctahydropentalene[a]	Miscellaneous	9.973	28588-55-8	-
6,6-Dimethylbicyclo[3.1.1]hept-2-ene-2-carbaldehyde[b]	Miscellaneous	11.088	564-94-3	1151
2-Ethylcyclohexanone[a]	Miscellaneous	11.410	4423-94-3	-
1,2-Dimethyl-1,3-cyclopentadiene[a]	Miscellaneous	11.691	4784-86-5	-
4-Trifluoroacetoxytridecane[a]	Miscellaneous	12.404	-	-
p-Menthan-3-one[a]	Miscellaneous	12.415	1196-31-2	-

1-Methyl-4-(1-hydroxy-1-methylethyl) benzene[b]	Miscellaneous	12.828	1197-01-9	1186
alpha-lonene[a]	Miscellaneous	13.468	475-03-6	-
Hexyl 2-methylbutanoate[b]	Miscellaneous	13.792	10032-15-2	1234
Guanidineacetic acid[a]	Miscellaneous	13.845	352-97-6	-
6,7-Dodecanedione[a]	Miscellaneous	13.845	13757-90-9	-
Quinoline[a]	Miscellaneous	13.984	91-22-5	-
p-Mentha-6,8-dien-2-one[a]	Miscellaneous	14.050	2244-16-8	-
p-Menth-1-en-3-one[a]	Miscellaneous	14.278	89-81-6	-
(3E)-4-(2-Hydroxy-2,6,6-trimethylcyclohexyl)-3-buten-2-one[a]	Miscellaneous	15.679	55955-46-9	-
Bicyclo[10.1.0]tridec-1-ene[a]	Miscellaneous	17.092	54766-91-5	-
(5E)-2,3,5,8-Tetramethyl-1,5,9-decatriene[a]	Miscellaneous	17.128	230646-72-7	-
1,5,9-Undecatriene, 2,6,10-trimethyl-[a]	Miscellaneous	17.161	62951-96-6	-
6,11-Dimethyl-2,6,10-dodecatrien-1-ol[a]	Miscellaneous	17.642	-	-
(5E)-6,10-Dimethyl-5,9-undecadien-2-one[a]	Miscellaneous	17.959	3796-70-1	1445
2-Hexyl-1-decanol[a]	Miscellaneous	18.126	2425-77-6	-
(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrienal[a]	Miscellaneous	19.137	66408-55-7	-
(6E)-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol [a]	Miscellaneous	19.597	7212-44-4	1561
2,3,6-Trimethylnaphthalene[a]	Miscellaneous	19.943	829-26-5	1548
1-[2-(Isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate [a]	Miscellaneous	20.472	74381-40-1	-
3,7,11-Trimethyl-1-dodecanol[a]	Miscellaneous	23.169	6750-34-1	-
6,10,14-Trimethyl-2-pentadecanone[a]	Miscellaneous	24.441	502-69-2	1848
(2E)-3,7,11,15-Tetramethyl-2-hexadecene[b]	Miscellaneous	24.487	14237-73-1	-

1,2-Benzenedicarboxylic acid, dibutyl ester[b]	Miscellaneous	25.569	84-74-2	1897
1-(Octyloxy)octane[a]	Miscellaneous	26.179	629-82-3	-
(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol[b]	Miscellaneous	27.708	150-86-7	2112
2-Ethylhexyl (2E)-3-(4-methoxyphenyl)-2-propenoate[a]	Miscellaneous	28.855	5466-77-3	-
(9Z)-9-Octadecenyl (9Z)-9-hexadecenoate [a]	Miscellaneous	32.353	22393-98-2	-
1,7,7-Trimethyltricyclo[2.2.1.0,2,6]heptane[a]	Monoterpene	6.983	508-32-7	924
2,7-Dimethyl-1,7-octadiene[a]	Monoterpene	7.069	59840-10-7	-
alpha-Pinene[c]	Monoterpene	7.264	80-56-8	932
3,7-Dimethyl-1,6-octadiene[a]	Monoterpene	7.395	10281-55-7	-
3,7-Dimethyl-1,6-octadiene[a]	Monoterpene	7.541	2436-90-0	-
Camphene[b]	Monoterpene	7.615	79-92-5	946
beta-Pinene[c]	Monoterpene	8.275	127-91-3	980
alpha-Terpinolene[a]	Monoterpene	8.722	586-62-9	-
2-Carene[a]	Monoterpene	8.764	554-61-0	-
alpha-Phellandrene[b]	Monoterpene	8.898	99-83-2	1007
alpha-Terpine[c]	Monoterpene	9.110	99-86-5	-
Cymene[a]	Monoterpene	9.257	527-84-4	-
Limonene[c]	Monoterpene	9.405	1461-27-4	1039
3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene[a]	Monoterpene	9.939	13466-78-9	-
gamma-Terpinen[a]	Monoterpene	10.031	99-85-4	-
Geranial[a]	Monoterpene	10.371	141-27-5	-
Linalool[c]	Monoterpene	10.637	78-70-6	1096
p-Mentha-1,4(8)-diene[a]	Monoterpene	10.668	586-62-9	-
1,7,7-Trimethylbicyclo [2.2.1]heptan-2-ol[b]	Monoterpene	12.591	507-70-0	1174
Indole[c]	Nitrogen compound	14.670	120-72-9	1295
Benzyl nitrile[a]	Nitrogen compound	14.822	140-29-4	-
3-Methyl-1H-indole[c]	Nitrogen compound	16.846	83-34-1	-

Allo-Aromadendrene[a]	Sesquiterpene	16.312	25246-27-9	-
alpha-Longipinene[b]	Sesquiterpene	16.418	5989-08-2	1353
2,3-Dimethyldodecane[a]	Sesquiterpene	16.441	6117-98-2	-
Farnesane[a]	Sesquiterpene	16.666	3891-98-3	-
(+)-Cyclosativene[a]	Sesquiterpene	16.733	22469-52-9	-
gamma-Muurolene[a]	Sesquiterpene	16.813	30021-74-0	-
alpha-Copaene[a]	Sesquiterpene	16.817	3856-25-5	1392
alpha-Bourbonene[a]	Sesquiterpene	16.997	-	-
beta-Elemene[a]	Sesquiterpene	17.074	515-13-9	-
4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene[a]	Sesquiterpene	17.431	118-65-0	1413
alpha-Santalene[a]	Sesquiterpene	17.220	512-61-8	1420
beta-Caryophyllene[c]	Sesquiterpene	17.715	87-44-5	1440
Germacrene D[a]	Sesquiterpene	17.841	23986-74-5	1464
2,6-Dimethyl-6-(4-methyl-3-pentenyl)bicyclo[3.1.1]hept-2-ene[a]	Sesquiterpene	17.459	17699-05-7	1436
beta-Gurjunene[a]	Sesquiterpene	17.914	17334-55-3	1427.7
alpha-Caryophyllene[a]	Sesquiterpene	18.357	6753-98-6	1459
Aromadendrene[a]	Sesquiterpene	18.458	109119-91-7	1460
alpha-Muurolene[b]	Sesquiterpene	18.619	10208-80-7	-
alpha-Amorphene[a]	Sesquiterpene	18.650	483-75-0	1468
beta-Farnesene[a]	Sesquiterpene	18.788	18794-84-8	1458
1-Methyl-4-(5-methyl-1-methylene-4-hexenyl)-1-cyclohexene[a]	Sesquiterpene	18.748	495-61-4	1501.2
Calamenene[a]	Sesquiterpene	19.059	483-77-2	1516
delta-Cadinene[a]	Sesquiterpene	19.400	483-76-1	-
alpha-Panasinsen[a]	Sesquiterpene	19.495	56633-28-4	-
alpha-Calacorene[a]	Sesquiterpene	19.814	21391-99-1	-
(Methyldisulfanyl)methane[a]	Sulphur compound	3.322	624-92-0	745
Isopropyl isothiocyanate[a]	Sulphur compound	-	2253-73-8	-
3-(Allylsulfanyl)-1-propene[a]	Sulphur compound	5.441	592-88-1	860.3
1-Isothiocyanato-2-methylpropane[a]	Sulphur compound	7.353	591-82-2	-

Thiacyclopentan-2-one[a]	Sulphur compound	8.406	1003-10-7	-
3-(Allyldisulfanyl)-1-propene[a]	Sulphur compound	10.250	2179-57-9	1085

Appendix 2: Pilot study investigating longevity of dung odours

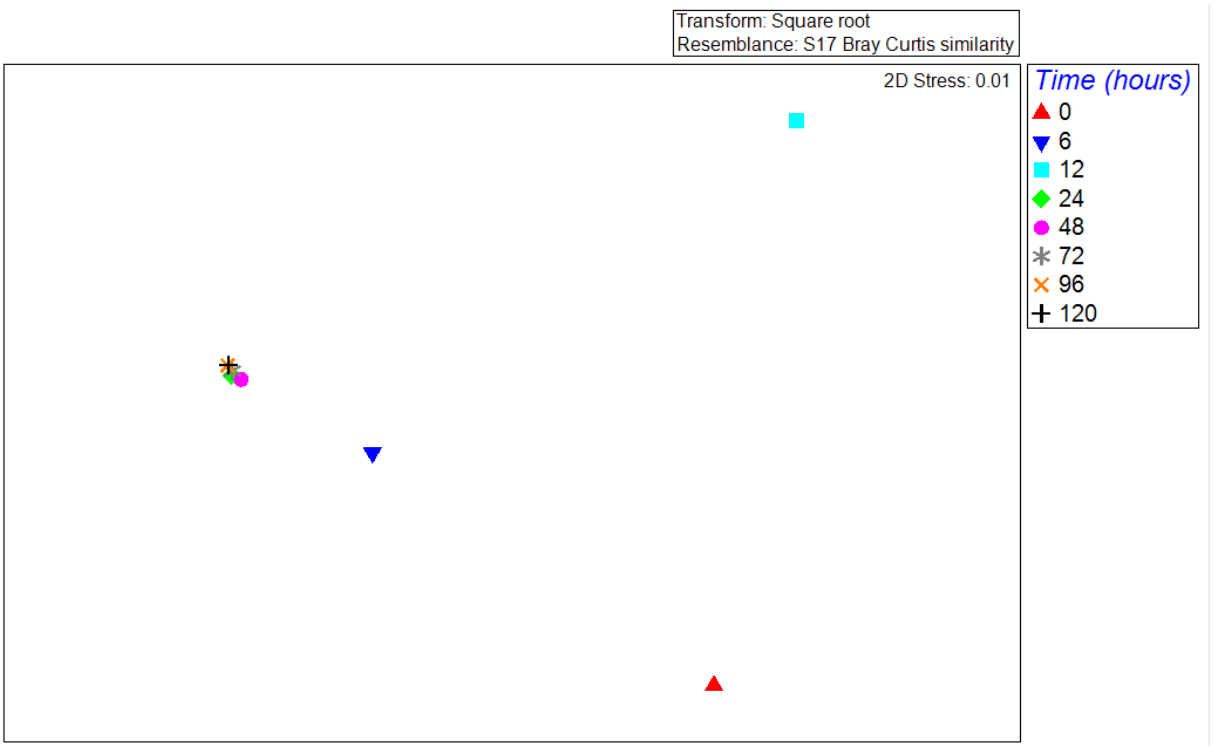

Figure A1. Multidimensional scaling (MDS) plot based on Bray-Curtis similarities of the variation of VOCs emitted from white rhino dung over 120 hours. After 48 hours the samples are clumped together meaning that the odours have lost any diverse information they were carrying, i.e. no longer effective as olfactory signals.