Silent signals: identifying dung odour profiles that indicate age, sex and status in white rhino olfactory communication

by

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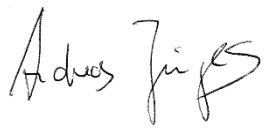

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Abstract

Olfaction is the primary mode of communication in most mammal species, with information transmitted via urine, faeces or specialised scent marks. Information can be accessed via volatile organic compounds (VOCs) emitted from these olfactory signals. White rhinoceroses (*Ceratotherium simum*) defecate communally in middens and it is possible that these middens act as signposts, where individuals can leave olfactory messages and obtain information from messages left by other individuals. If this is the case, then it is unclear what odours are transmitted via their dung and how much information white rhinos can ascertain from these odours. White rhinos have poor eyesight but an acute sense of smell and therefore rely heavily on olfactory communication. To explore whether white rhinos transmit information using chemical signals I collected scent samples (N= 83 individuals) from the faeces of white rhinos of different sex (male, female), age (calf, sub-adult, adult) and status (male: territorial vs. non-territorial, female: oestrus vs. non-oestrus). Scent samples were collected using headspace extraction methods, which extract only volatile compounds emitted from the faeces, with subsequent analysis via gas chromatography-mass spectrometry (GC-MS). I distinguished 326 volatile compounds and identified 285 of these from the dung of the 83 individuals. The relative proportions of nine compounds were important for predicting the sex of an individual, mainly alkanes and alkenes. Relative proportions of seven compounds (mainly alcohols and alkanes) were important for predicting age. Territorial males emitted significantly higher median proportions of acids and aldehydes than non-territorial males. Oestrous females emitted significantly lower median proportions of acids, alcohols and alkanes than non-oestrous females. These results support the idea that middens are areas of information exchange and that sex, age, territorial status and reproductive status are identifiable via odours at middens. Ultimately, these results provide insight into the information transmitted via white rhino dung and its use in olfactory communication. Moreover, it opens the door for understanding behavioural responses of white rhinos towards olfactory cues.

Declaration

I, Courtney Marneweck, declare that this thesis, which I hereby submit for the degree Master of Science at the University of KwaZulu-Natal, is my own work and has not previously been submitted for a degree at this or any other tertiary institution.

Signature: 

Date: 11 March 2014

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Acknowledgements

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Chapter 1: Literature Review and Introduction

Animals communicate primarily in three ways, by visual, vocal and/or olfactory signals. Many species use visual signals, for example, female chacma baboons (*Papio ursinus*) signal to males by displaying swollen rears when they are ready to mate (Bielert and Anderson, 1985). Male flycatchers (*Ficedula albicollis*) possess white forehead patches which function as a badge of territorial status (Pärt and Qvarnström, 1997). Passive visual signals such as colourful plumage are inexpensive to maintain, but acquiring specific pigments can be costly (Andersson, 2000). There are also habitat constraints involved, with low light and thick bush making contrast of colours difficult to see (Andersson, 2000). Finally, conspicuous displays and markings may make individuals vulnerable to predation (Endler, 1978).

Vocalisations are another way to communicate with other individuals. Both sexes of lion (*Panthera leo*) roar to broadcast territory ownership, stay in contact with other individuals and even attract mates (Schaller, 1972). However, with vocal communication there is a risk as competitors can listen to these calls leading to costly encounters (Grinnell and McComb, 2001). Although vocalisations can be inexpensive to produce (Horn et al., 1995) they have disadvantages. Habitat features such as mountains and rivers can cause acoustic distortion (Wiley and Richards, 1978) and like visual signals, vocalisations also attract attention from predators (Tuttle and Ryan, 1982). Further, both vocal and visual communication are only short-term as the message is gone as soon as the individual stops calling or leaves sight. Olfactory communication, on the other hand, allows signals to persist in the environment after the sender has gone.

1.1. Olfactory communication

Olfactory communication can be defined as the perception of molecules generated from one animal and transmitted through the air, which results in an alteration of behaviour in the receiver (Bossert and Wilson, 1963). There are many advantages to olfactory communication. Firstly, chemical signals provide an honest indicator of characteristics of the depositor (Zala et al., 2004). Female mice (*Mus musculus*) were able to detect the health status of males from urinary odours and showed preference for non-parasitized males (Kavaliers and Colwell, 1995). Further, the scent gland secretions of male lemurs (*Lemur catta*) show their genetic quality (Charpentier et al., 2008) allowing females to make optimal mate choices.

A second advantage is that olfactory communication ensures that information is available even after the sender has gone (i.e. long-term presence) (Mitro et al., 2012). Linklater et al. (2013) discovered that black rhinoceros (*Diceros bicornis*) faeces were effective in stimulating investigatory behaviour 32 days after being deposited, suggesting the faeces were still functional as an olfactory signal. Male golden hamsters (*Mesocricetus auratus*) stopped responding to flank marks only after they were 50 days old. However, they still responded to vaginal marks that were 100 days old (Johnston and Schmidt, 1979). These examples suggest that longevity differs between chemical signals.

Olfactory signals can also provide spatial information, i.e. territory ownership or information on the movements of a particular individual. For example, klipspringers (*Oreotragus oreotragus*) use pre-orbital gland scent marks to advertise their territory boundary (Roberts and Lowen, 1997) and male grey tree squirrels (*Sciurus griseus*) locate females in oestrus by following their scent trails (Thompson, 1977). Olfactory signals also deliver physiological information. Male horses (*Equus cabalus*) can detect oestrus specific odours from female urine (Ma and Klemm, 1997) and female mice can detect the health of males from urinary marks (Kavaliers and Colwell, 1995).

However, there are disadvantages of chemical signals. Firstly, they cannot be directed in specific directions or toward specific individuals (Eisenberg and Kleiman, 1972). Further, diffusion causes a concentration gradient termed the active space by Bossert and Wilson (1963) and individuals must rely on wind speed and direction to expand this active space. Moreover, chemical signals can be costly to produce. Spotted hyenas (*Crocuta crocuta*) can deposit up to 40% of their body weight annually in their lipid-rich scent marks (Gorman, 1990) and male mice suffer reduced body size and growth rate when they increase their scent mark rate (Gosling et al., 2000). Like visual and vocal signals, olfactory communication also carries a predation risk to both senders and receivers (Hughes et al., 2010). Predators can eavesdrop on signals which can cause inter-specific communication to become an unwanted predator attractant. Localised scent marking areas can then become risky to investigating receivers with decreased vigilance (Hughes et al., 2010).

1.2. Modes of information transfer

Chemical signals are transmitted and received via volatile organic compounds (VOCs). The volatility of a compound depends on its molecular weight, with larger and heavier compounds being less volatile (Stoddart, 1976). Additionally, Wheeler (1977) suggested that the ideal molecular weight for a compound to be effective as a chemical signal lays between 50 and 300. Alberts (1992) found that the mean molecular weight for mammalian range marks (i.e. marks identifying territory ownership or home range) was 208 compared to the mean molecular weight of mammalian sex attractants at 91. Highly volatile compounds may be released immediately as alarm signals, such as the alarm secretions emitted from rats (*Rattus norvegicus*; Abel and Bilitzke (1990)). The sudden burst and high volatility allows for a rapid fade-out meaning that the signal will not persist in the environment after the threat has disappeared (Bossert and Wilson, 1963). In contrast, volatile compounds may be emitted continuously at a constant rate through time, allowing for a relatively constant depletion (Bossert and Wilson, 1963). These signals tend to be used for marking territories and are usually made up of heavier compounds with less volatility so they can persist long enough to be received by others in the area (Alberts, 1992). Further, VOCs may also be emitted as chemical trails. Female rats in oestrus actively leave trails of urine and sebaceous secretions to induce male pursuit (Birke, 1978) and male grey tree squirrels locate females in oestrus by following their scent trails (Thompson, 1977).

It is one thing to be able to perceive VOCs in the environment, but animals must also be able to detect changes in VOCs associated with specific characteristics or changes in state or physiology in order to respond. VOCs may change in concentration in relation to individual characteristics, for example, the concentration of certain VOCs in the urine of lions differ in concentration according to sex (Andersen and Vulpius, 1999). On the other hand, VOCs may appear, or disappear, at the onset of characteristic. Oestrus specific urinary VOCs appear at the onset of oestrus in cows (*Bos taurus*; Kumar et al. (2000) and decrease in concentration at the onset of oestrus in horses (Mozūraitis et al., 2012). Moreover, VOCs can cause an immediate behavioural response called the releaser effect (Bossert and Wilson, 1963). For example, aardwolves (*Proteles cristata*) increased their scent marking rate, over marked and changed dens more often when they encountered scent marks from same sex individual in their territories (Sliwa and Richardson, 1998). Alternatively, they can cause a physiological influence called a primer effect (Bossert and Wilson, 1963), as in the urine of male mice that can induce oestrus in females (Jemiolo et al., 1986).

1.3. Information transferred using olfactory signals

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 may be effected by hormonal changes (Brown et al., 1996).

Behavioural studies suggest that sex is identifiable through odour. Both sexes of black rhinoceros investigated male faeces more than female faeces suggesting they were able to identify the sex of conspecifics via faecal odour (Linklater et al., 2013). Sex differences in odour may occur from different concentrations of the same odour compounds between males and females. For example, Andersen and Vulpius (1999) discovered that males and females had significantly different concentrations of volatile ketones emitted from their urine. The scent marks of male and female giant pandas (*Ailuropoda melanoleuca*) also differed significantly with regard to acids, with males having higher concentrations (Hagey and Macdonald, 2003).

The importance of compound concentration has even been shown in birds, with the alcohols emitted from preen secretions and preen down feathers of kittiwakes (*Rissa tridactyla*) differing in concentration between the sexes (Leclaire et al., 2011). As the sex of an individual is under genetic control and cannot be altered or varied, it is fixed (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. This difference contributes to sexually distinct odours in mice (Yamazaki et al., 1986). Therefore, in some species, an individual's sex can be identifiable via the odours it secretes and this may be through faecal, urinary or specialised scent marks. On the other hand, the concentration of VOCs may not account for sex differences, but rather sex specific VOCs are emitted from one or both genders. For example, human (*Homo sapiens*) scent possesses gender specific volatile compounds (mainly acids for males and mainly esters for females) (Penn et al., 2007). In contrast, a single volatile compound (a quinolone compound) was unique to the urine of male ferrets, *Mustela furo* (Zhang et al., 2005). However, it may be more complex than a single compound separating the sexes. Within the urinary volatiles of white-tailed deer (*Odocoileus virginianus*) 36 were female specific (phenols) and 28 male specific (including nitriles and sulphur compounds) (Miller et al., 1998).

Animals may also be able to identify the age of depositors through the scent of faeces, urine and/or scent marks. Linklater et al. (2013) suggested that black rhinos could identify the faeces of adults (>6 years old) from sub-adults (2-4 years old) via odour alone. Further, Kolar et al. (2002) suggested that female white rhinos (*Ceratotherium simum*) were able to discriminate the different ages of males from urine marks. However, both of these studies focused on behavioural responses to dung and urine and thus did not determine what VOCs signalled age. As reproductive hormone concentrations vary with age (Seraphin et al., 2008) it is likely that this alters the concentration of odours related to these hormones. Haze et al. (2001) discovered that the concentration of several skin volatiles (mainly aldehydes) of humans were positively correlated with aging. Additionally, differences in the ratio of urinary volatiles (mainly nitrogen compounds and acids) have been attributed to differences between adult (3-10 months) and aged (17+ months) mice (Osada et al., 2003). On the other hand, age specific VOCs may appear at the onset of sexual maturity along with the presence of higher concentrations of the reproductive hormones (i.e. testosterone and oestrogen for males and females, respectively). For example, four volatile compounds were identified exclusively from the faeces of wolf pups, *Canis lupus signatus* (esters) and 55 volatiles exclusive to adult faeces, including acids (Martín et al., 2010).

In many species, territorial males have higher testosterone concentrations than non-territorial males (e.g. white rhino (Rachlow et al., 1998); bird *sp*. (Wingfield et al., 1987, Hegner and Wingfield, 1987)). Testosterone itself is not a volatile compound and thus cannot be detected by the olfactory system due to its chemical structure (Luetjens and Weinbauer, 2012). Therefore its presence alone cannot be identified via faecal odours. Although mammals cannot degrade steroid hormones, once they are excreted they can be broken down by bacteria and, consequently, volatile compounds are produced (Chiang et al., 2010). Therefore, changes in testosterone concentration may cause changes in the concentration of VOCs. Gosling and Roberts (2001) suggested that odours of male mice could reflect these testosterone levels. Testosterone stimulates aggressive behaviour in males (e.g. mice, Tollman and King (1956); ungulates, Bouissou (1983)) and if non-territorial males are able to detect its presence via a compound(s) associated with testosterone, they will likely avoid areas containing these compounds. For example, European moles (*Talpa europaea*) actively avoid the scent marks of their territory neighbours (Stone and Gorman, 1990) and terrapins (*Mauremys leprosa*) can detect larger and stronger males from chemical cues they emit into the water and actively avoid pools containing such males (Ibáñez et al., 2012). This suggests

that non-territorial individuals can identify physiological characteristics from the odour of territorial marks and are avoiding potential risks of confrontation. The identification of territorial status may be due to the concentration of volatile compounds. For example, the anal gland secretions of territorial male European moles are characterised by high concentrations of acids (Khazanehdari et al., 1996) and it was suggested that these were involved in territory maintenance. Further, male horses emitted higher concentrations of alcohols in their urine during the breeding season and it was suggested that this was used to over-mark and disguise female oestrus signals (Mozūraitis et al., 2012). In contrast, there may be a territorial specific compound emitted as an olfactory signal as in male blackbuck (*Antelope cervicapra*), where three urinary volatile ketones were specific to dominant males during their dominance period (Rajagopal et al., 2010). This has also been identified in white-tailed deer, where nine VOCs (including alcohols, alkanes, alkenes and ketones) were specific to the urine of dominant males during the breeding season (Miller et al., 1998). Miller et al. (1998) suggested that these VOCs were a result of circulatory androgens being subsequently metabolized and excreted in the urine.

Another type of information that can be transmitted via olfactory cues is reproductive status. In some species, males are able to detect oestrus in females via odours emitted from faeces and urine. For example, male mohor gazelles (*Gazella dama mhorr*) are more attracted to females excreting higher concentrations of faecal oestrogen (Pickard et al., 2003), suggesting they are able to detect oestrogen from the scent of the faeces. Male Przewalski horses (*Equus ferus przewalskii*) over-mark the faeces and urine of females with their own urine and mostly during the breeding season (King and Gurnell, 2007). This suggests that the males are able to identify the reproductive status of females from faecal odours and mask any possible indicators of this to other males by placing their own scent on top to reduce intra-sexual competition. Also, male African elephants (*Loxodonta africana*) displayed more investigatory behaviour towards oestrous urine suggesting that they could detect the state using odour alone (Bagley et al., 2006). Female tigers (*Panthera tigris)* mark intensively before oestrus indicating an advertisement of reproductive state (Smith et al., 1989). Oestrus specific compounds can appear at the onset of oestrus. A benzenoid and an alcohol compound are only emitted from oestrous water buffalo faeces *Bubalus bubalis* (Karthikeyan et al., 2013). For cows, an ester and alkane compound were specific to oestrous urine (Kumar et al., 2000). In addition, an alkane, ester and two sesquiterpenes were oestrus specific in mouse urine (Achiraman and Archunan, 2006). Alternatively, oestrus specific compounds

can decrease in concentration at the onset of oestrus as certain alcohols do in female horse urine (Mozūraitis et al., 2012).

1.4. Sources of olfactory signals

Olfactory signals can originate from one of three main pathways: specialised scent glands, urine or faeces. Specialised scent glands exist in many species. Anal pouches are found in all four hyaenid species, with spotted hyenas using their specialised anal scent glands to communicate social status and individuality (Burgener et al., 2009). Specialised scent glands can also occur within the skin. The subcaudal scent glands found in European badgers (*Meles meles*) convey multiple information including sex, age, body condition and reproductive status (Buesching et al., 2002).

Urine marking occurs in rodents and also conveys multiple signals, for example, the urinary marks of mice display information on male territory ownership (Humphries et al., 1999), female reproductive state (Achiraman et al., 2010), age (Osada et al., 2008), health condition (Kavaliers and Colwell, 1995) and even individuality (Brennan, 2004). Ritualised spray urination is common in felids. Male tigers 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). Contrastingly, in leopards (*Panthera pardus*), only males spray urinate (Bothma and le Riche, 1995) 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.

Another source of olfactory signals are faeces, which are commonly use to advertise territory boundaries and ownership. 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 faeces in localised defecation sites at territory boundaries. Marking with faeces is commonly associated with other ritualised behaviour such as kneeling, kicking and rubbing (Gosling, 1985). Wildebeest (*Connochaetes taurinus*) lay and roll in faecal marking sites (Estes, 1969), whereas blackbuck perform a paw/urinate/defecate sequence (Dubost and Feer, 1981).

1.5. Communal defecation

The transfer of information via olfactory signals is stable 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 these messages. Latrines (e.g. European badgers, Roper et al. (1993)) and middens (e.g. mountain gazelles, Attum et al. (2006)) are sites where individuals defecate communally. Therefore, communal defecation sites can act as a centre for information exchange (Eisenberg and Kleiman, 1972). In addition to transferring important biological information, the deposition of urine and faeces at latrines/middens does not demand additional energetic costs as extra scent marking does. For example, spotted hyenas can deposit up to 40% of their body weight annually in their lipid-rich scent marks (Gorman, 1990), whereas deposition of faeces at communal sites ensures little additional energy expenditure. Further, faeces are large, readily produced and persist in the environment making them ideal vectors for olfactory signals (Gosling, 1985). Therefore, the energetic and social economics of communal defecation areas are highly profitable to both (1) an individual leaving information and (2) individuals accessing that information (Brown and Macdonald, 1985).

Communal defecation sites are typically found in species with territorial organisation (Gosling and Roberts, 2001). European badgers (Hutchings et al., 2002), klipspringers (Dunbar and Dunbar, 1974), Indian rhinoceros (*Rhinoceros unicornis*; Laurie (1978)) and impala (*Aepyceros melampus*; Jarman (1979)) all utilise defecation sites to advertise territorial ownership. These dung piles are traditionally located either in the approximate centre of a home range, as in brown hyenas (*Hyaena brunea*, Mills et al. (1980)) or along home range boundaries, as in oribi (Brashares and Arcese, 1999). In this way, communal defecation serves to warn potential intruders of territory ownership. Perhaps the most obvious use of a communal defecation site is that used by the white rhinoceros where this species utilises middens extensively in both space and time.

1.6. White rhinoceros

The white rhinoceros is the world's largest purely graminivorous animal and is therefore limited to grassland savannahs (Skinner and Chimimba, 2005). Males are generally larger than females, with females weighing ~1600 kg and males from 2000-2400 kg (Owen-Smith, 1973). Mixed age and sex social groups are common in white rhinos, but territorial males remain solitary. Territorial males hold defined territories ranging from 0.74-2.60 km^2 (Owen-Smith, 1973). Territorial males mark their territories by spray urinating and defecating in middens both along territory boundaries and throughout their area. When a territorial male defecates, the faeces are scattered by a kicking action with the back feet (Owen-Smith, 1973).

Adult females have larger home ranges of 6.1-20.5 km^2 that overlap extensively with those of other adult females and can include the territories of up to seven territorial males (Owen-Smith, 1973).

White rhinos have poor eyesight but an acute sense of smell, therefore their communication relies heavily on olfactory signals such as those deposited in middens (Owen-Smith, 1973). White rhinos of all ages and sex defecate communally at middens (Owen-Smith, 1973). Thus, middens may act as message boards holding records of territory ownership, reproductive state of females and even individual identities of rhinos in the area (Owen-Smith, 1973). If this is true, then middens would be important centres of information exchange (Eisenberg and Kleiman, 1972) for white rhinos. The combination of these factors makes white rhinos a perfect model species to study olfactory communication.

Despite this, studies of white rhino olfactory communication have been mostly descriptions of behaviour with little investigation regarding volatile odorous compounds emitted as faecal signals. For example, Cinková and Richard (2013) suggested that white rhinos were able to distinguish sex from faecal odours, however, they were unable to 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 faeces 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 what odour compounds were eliciting such a behavioural response. Rachlow et al. (1998) found that territorial male white rhinos had higher faecal testosterone than non-territorial males, and although testosterone itself is not identifiable via odour due its structure (Luetjens and Weinbauer, 2012), it may be possible that individuals are able to identify the territorial status of a male based on odours from their deposited faeces.

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 (Owen-Smith, 1973). Grün (2006) findings support this, where males spent a significant amount of time investigating the faeces from breeding females, suggesting the identification of oestrus odours. As an individual’s age is potentially identifiable through faecal scent in the black rhino (Linklater et al., 2013), it is possible that age is also attainable through faecal scent in the white rhino. However, to date this has not been quantified.

Despite all of the behavioural evidence in many studies conducted on white rhinos (Owen-Smith, 1973, Rachlow et al., 1998, Kretzschmar et al., 2001, Grün, 2006), there is a gap in our knowledge regarding what volatile compounds are behind these olfactory signals of sex, age, territorial state and reproductive state. Middens are located throughout territories, concentrated particularly at territorial boundaries, alongside drinking areas and regularly used trails (Owen-Smith, 1975) which makes it very important to understand their function. However, in order to assess the function that middens play within white rhino ecology, we need to start by assessing what information is held within the odours at middens and how much information white rhinos can ascertain from these odours.

1.7. Broad Aim

The overall aim of my study is to identify the volatile organic compounds (VOCs) emitted from white rhino faeces associated with individual characteristics (i.e. sex, age and status).

1.8. Objectives

1. Identify VOCs from undigested plant material
2. Assess the role of season in VOC changes
3. Determine the VOCs associated with of sex
4. Determine the VOCs associated with of age
5. Determine the VOCs associated with adult male territorial state
6. Determine the VOCs associated with adult female reproductive state

1.9. Hypotheses

Using the information gathered from the literature, I hypothesise the following with regard to the VOCs emitted from white rhino faeces:

1. As some VOCs will represent undigested plant material I predict that;
    a. There will be no difference in the concentration of these plant based compounds with regard to sex, age, territorial state or reproductive state or
    b. In contrast, any differences found within these compound concentrations will be due to dietary changes associated with age (i.e. the milk diet of calves).
2. As food availability differs seasonally I predict that;
    a. The odour profiles of individual characteristics will differ seasonally.

    b. However, the odour profiles of individual characteristics may be the same throughout the year if the rhinos' diets remain relatively stable
3. As the sex of an individual is a fixed characteristic, I predict that male and female white rhinos will emit different odours from their faeces. With regard to the chemical marker(s) for sex there will be either;
    a. Sex specific compound(s) emitted from the faeces of each sex or
    b. The concentration of specific volatile compound(s) emitted from the faeces will vary between the sexes.
4. As hormones vary with age (Seraphin et al., 2008), I predict that different aged white rhinos will emit different odours from their faeces. With regard to the chemical marker(s) for age there will be either;
    a. Age specific compound(s) emitted from faeces or
    b. The concentration of compound(s) emitted from faeces will vary between ages.
5. As testosterone levels are higher in territorial males than non-territorial males (Rachlow et al., 1998), I predict that territorial males will emit different odours from faeces than non-territorial males as a result of compounds associated with testosterone. With regard to the chemical marker(s) for territorial state there will be either;
    a. Territorial specific compound(s) emitted from faeces or
    b. The concentration of compound(s) emitted from faeces will vary between territorial and non-territorial states.
6. As progesterone levels differ between oestrous and non-oestrous females (Schwarzenberger et al., 1998), I predict that oestrus females will emit different odours from faeces than non-oestrus females as a result of compounds associated with progesterone. With regard to the chemical marker(s) for reproductive state there will be either;
    a. Oestrus specific compound(s) emitted from faeces or
    b. The concentration of compound(s) emitted will vary between oestrous and non-oestrous females.

Chapter 2: Materials and Methods

2.1. Study site

I conducted this study in the iMfolozi section of the 960 km^2 Hluhluwe-iMfolozi Park (HiP) KwaZulu-Natal Province, South Africa (Figure 1). The area of study was within the northwestern section of iMfolozi known as Mbhuzane, S 28˚20', E 31˚51'. iMfolozi has approximately 600 mm of rainfall per year and a range of vegetation from grasslands to *Acacia* woodlands (detailed vegetation description by Whateley and Porter (1983)). I selected HiP for this study due to the large white rhino population, ~2050 individuals in 2008, of which ~1800 are located within the iMfolozi section (Ezemvelo KZN Wildlife, unpublished data).

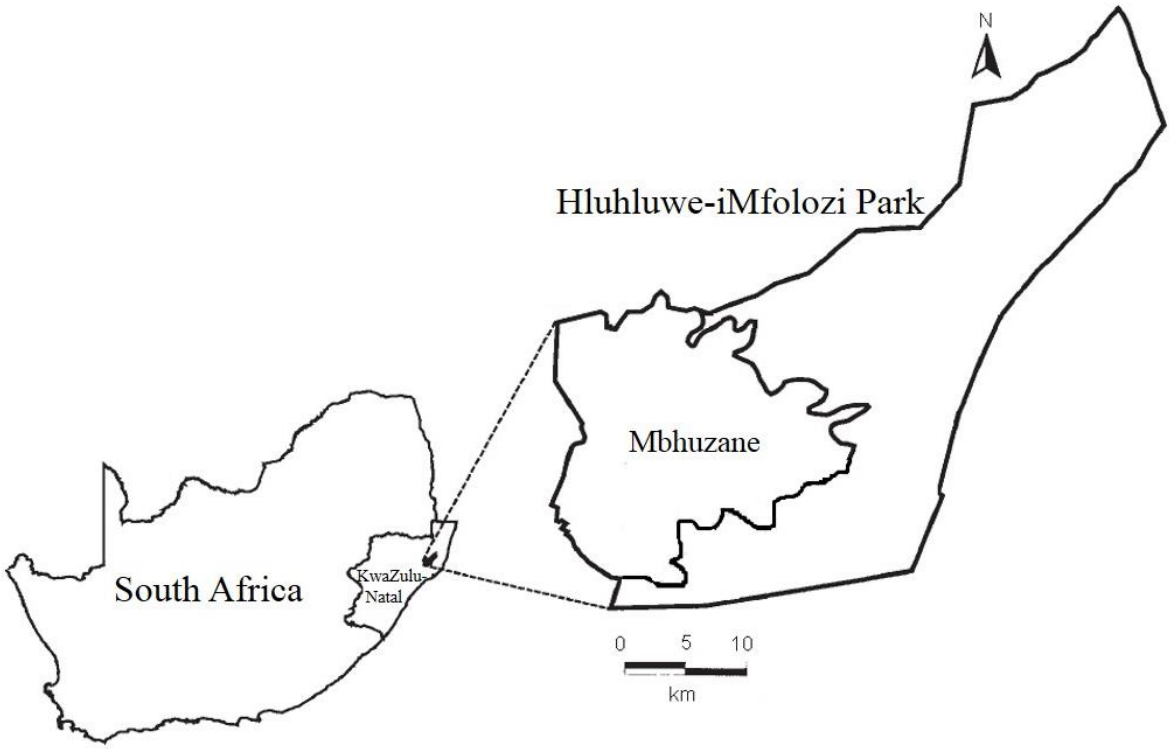

Figure 1. Location of the Hluhluwe-iMfolozi Park (HiP) within South Africa.

2.2. Location, identification and classification of white rhinos

I did not tag or mark individual rhinos, thus I identified individuals from variations in horn shape, body size, skin folds and other distinguishing characteristics (Adcock and Emslie, 2003, Patton and Campbell, 2011). Individuals were located on foot either opportunistically or by tracking footprints and signs. Defecation occurs throughout the day, with Owen-Smith

(1973) recording a territorial male white rhino defecating each day at approximately 0800, 1200 and 1700, and up to 6 times in 24 hours. This regularity of defecation made collection relatively easy.

From June through to November 2012, I collected 79 individual dung scent samples (Table 1). Each sample represents the dung scent of a different individual white rhino. I classified each individual by sex, age, territorial state for adult males and reproductive state for adult females. Individuals were grouped into three age categories: calves (0-2 years), sub-adults (2-7 years) and adults (>7 years) based on the age classification of Hillman-Smith et al. (1986). The age of the individual was determined based on body size and horn development. I divided the sampling period into wet and dry seasons, with the wet season October to March (White et al., 2007). There was a break in data collection over August-September, therefore, I classified all samples collected in the first field session as dry season (N= 54) and all samples collected in the second field session as wet season (N= 25).

Table 1. Age classification of white rhinoceros and number of samples collected.

		Number of samples collected			
Group	**Age**	**Male**	**Female**		**Sub-total**
Calf	0 - 2 years	10	14		24
Sub-adult	2 - 7 years	11	10		21
Adult	>7 years (non-territorial[a]/non-oestrous[b])	8	11		19
Adult	>7 years (territorial[a]/oestrous[b])	10	5		15
	Sub-total	39	40	**Total**	**79**

[a] males; [b] females

Territorial male rhinos are the only individuals who kick their dung, leaving distinctive foot scrape marks in the centre of the midden (Owen-Smith, 1975). In addition, they also spray urinate to mark their territory border (Owen-Smith, 1975). I used these distinctive features to differentiate between territorial and non-territorial males. There are also behavioural interactions between adult females and males that indicate female oestrus, for example, a territorial male will follow and physically block the progression of the female if she moves towards his territory boundary and he squeals loudly (Owen-Smith, 1973). I defined the onset of oestrus by this behaviour plus repeated courtship advances by the male, both successful and unsuccessful, as in Owen-Smith (1975).

2.3. Faecal scent collection

To ensure that I knew the sex, age and territorial/reproductive status of the rhinos from which I collected scent samples, I only collected data from rhinos that I observed defecate. Scent samples were collected using headspace extraction methods to entrap volatile organic compounds (VOCs) emitted from the faeces (as described in Johnson and Jürgens (2010)). Headspace refers to the air above the dung, so I only extracted the VOCs that an individual sniffing at a midden would inhale. I collected headspace samples by enclosing ~800 g of faeces (i.e. one bulbous) in a polyacetate bag and sucking air for 25 minutes through a small filter filled with 1 mg of Tenax® and 1 mg of Carbotrap® using a micro pump with a realized flow rate of 150 ml/min (Figure 2). At each sampling session, I took control samples at the same time from an empty polyacetate bag in the surrounding area of the midden for the same duration. To ensure freshness of the sample, I collected all faecal samples within 10 minutes of defecation.

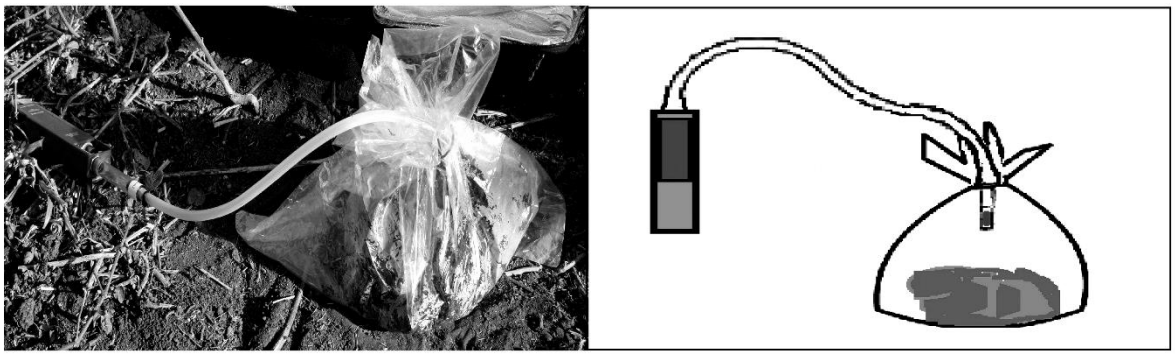

Figure 2. Photograph and diagram of micro pump extracting VOCs from faecal headspace.

2.4. Gas chromatography-mass spectrometry analysis of faecal scent

GC-MS analyses of the samples were carried out using a Bruker 450 GC with a 30 m x 0.25 mm internal diameter (film thickness 0.25 µm) Varian VF-5ms column, connected via Quick-Switch (Bruker), to a Varian VF-1ms column (10m; film thickness 0.25 µm, 0.25 mm internal diameter) which was coupled to a Bruker 300 quadrupole mass spectrometer in electron-impact ionization mode at 70 eV (detector set to Extended Dynamic Range, EDR). Thermodesorption cartridges were placed in a Bruker 1079 injector equipped with a ChromatoProbe thermal desorbtion device (Amirav and Dagan, 1997, Dötterl et al., 2005). The flow rate of helium carrier gas was 1.8 ml min-1. The injector was held at 40 °C for 2 minutes with a 20:1 split. The temperature was then increased to 200 °C at 200 °C min-1,

held for 10 minutes, increased to 250 °C at 200 °C min-1 and held for a further 10 minutes. After the initial 2 minutes, the split was removed for thermal desorption and then a 100:1 split was introduced after 4.2 minutes to flush the injector. Meanwhile, the GC oven was held at 40 °C for 3 minutes and then turned up to 240 °C at 10 °C min-1 and held there for 12 minutes. Compounds were identified using the Varian Workstation software with the NIST 2011 mass spectral library mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011; MS search software version 2.0 d). The identification of compounds was verified with retention times of authentic standards and published Kovats indices wherever possible (see Appendix 1). Compounds found at similar relative amounts in control samples were considered contaminants (e.g. Limonene) and excluded from further statistical analyses.

2.5. Statistical analyses

Aitchison and Egozcue (2005) state that the absolute values of components in a composition are meaningless unless they are compared using proportions. To control for this, and variability between samples, I used the proportion of each compound (on a scale of 0-1) to assess its relative abundance within a scent sample rather than its absolute concentration. Due to the nature of this data, failing tests of normality and containing a high frequency of zeros, I used non-parametric analyses.

Van Dam and Poppy (2008) specified the need to adopt methods from bioinformatics in the field of plant volatile analysis as both deal with large amounts of data. I adopted this for the analysis of faecal volatiles in my study. I used random forests (RF), a machine learning algorithm developed by Breiman (2001), to assess the variable importance for predicting sex and age from the faecal volatiles of white rhinos. VOC datasets are comparable to gene expression datasets as they both have more variables than they do samples (Ranganathan and Borges, 2010). In this case, each VOC is a single variable. Ranganathan and Borges (2010) listed several features of RF that make it suitable for volatile analysis. For example, it allows for more variables than samples, it has high classification efficiency (even with background noise) and it can create a minimal set of variables, which can be used as group predictors. A key advantage of RF is that it does not over fit the data (Breiman, 2001). For example, even if minor variations in variables (i.e. VOC proportions) are involved in the building of thousands of decision trees, they are not given unjustified

importance in the final model and therefore a minimum set of importance predictor variables is created (Ranganathan and Borges, 2010).

The RF builds decision trees using bootstrapping from samples, and selects a set of variables (in this case, VOCs) at the terminal node of the decision trees created (Ranganathan and Borges, 2010). The RF then creates a ranked variable importance list by running permutations of decision trees (Strobl et al., 2009a). One decision tree determines the most reliable variables (VOCs) for predicting a specific characteristic (i.e. sex or age) and the repetitions create a robust and accurate ranked list (Strobl et al., 2009b). It is necessary for data with large variable lists to have a higher number of permutations (Hothorn et al., 2013), the default of which is 500. For my RF analyses, I set the number of decision tree permutations at 10,000 to increase robustness as I had a very large variable list (Strobl et al., 2009b). The number of randomly selected predictor variables for each split in the decision tree (i.e. *mtry*), has a default of 5. However, it is suggested to use the square root of the number of variables (Strobl et al., 2009b), thus I set the *mtry* to 12 (i.e. $\sqrt{}$ (N= 157).

In the first analysis, I used sex as the predictor and each individual compound as a variable. In the second analysis, I used age as the predictor and each individual compound as a variable. I set the min_criterion to 0.95, which represents the significance level (i.e. 1 – p value) (Hothorn et al., 2013). This analysis is repeated several times to ensure a stable variable importance list (Strobl et al., 2009a). The absolute values of the importance scores are not interpreted (Strobl et al., 2009b), thus I reported the output variable importance as a relative ranked list of the significant predictors. I considered variables important if their variable importance score was above the absolute value of the lowest ranking negative-scoring variable. This is because the importance of irrelevant values varies randomly around zero (Strobl et al., 2009b). The absolute value of the lowest ranking variable is represented by a threshold value on figures; all variables above the threshold value are considered important. As they are insignificant to the outcome prediction, I excluded all non-important variables (i.e. those scoring ≤ 0) from the figures.

With regard to territorial/reproductive state of adults, sample size was too low to compute using RF. As a result, I ran non-parametric Mann-Whitney U tests to determine differences between the median proportion of VOCs grouped by their biosynthetic pathways (Knudsen et al., 2006) (see Table 2) between (1) territorial and non-territorial adult males and (2) oestrous and non-oestrous adult females. I also used Mann-Whitney U tests to investigate differences in median proportion of the compound groups between the wet and dry seasons.

Using Mann-Whitney U tests, volatiles occurring from undigested plant material will be defined as VOCs that have no statistical difference between any test group (season, sex, age, territorial and reproductive state). I set the significance level at 0.05. For figures and descriptive statistics, I calculated the median and interquartile range (IQR) and excluded extreme outliers (values greater than three times the IQR) from figures only. I performed all statistical analyses using the software R version 3.0.1 for windows (http://www.r-project.org), with RF functions in the user contributed package 'party' (Hothorn et al., 2013). I created all figures using the statistical package IBM SPSS 21 ©.

Chapter 3: Results

Gas chromatography-mass spectrometry (GC-MS) analyses distinguished three hundred and twenty six (326) volatile organic compounds (VOCs) from the headspace of white rhino faeces (see Appendix for full list). I grouped these compounds according to their biosynthetic pathways (see Knudsen et al. (2006)) into 14 classes (Table 2). Four of these classes represent emissions from undigested and waste plant material (i.e. benzenoids, monoterpenes, sesquiterpenes and nitrogen compounds). These classes, along with unidentified compounds (N= 169), were excluded from the random forest analyses. Moreover, I excluded unidentified compounds from all analyses.

Table 2. Frequency of compounds within each of the 14 distinguished classes.

Compound class	**Number identified**
Aliphatic Acid	14
Aliphatic Alcohols	16
Aliphatic Aldehydes	11
Aliphatic Alkanes	31
Aliphatic Alkenes	12
Aliphatic Esters	3
Aliphatic Ketones	7
Benzenoids	19
Miscellaneous compounds	57
Monoterpenes	19
Nitrogen compounds	3
Sesquiterpenes	86
Sulphur compounds	6
Unidentified compounds	42
Total	**326**

3.1. Identification of dietary plant material from VOCs emitted from white rhino faeces

With regard to age, faeces from calves emitted significantly lower proportions of sesquiterpenes than both adults ($U=1616174$, $p<0.05$; Figure 3 & 4) and sub-adults

(U=1687497, p<0.05 Figure 3 & 4). Calves' faeces also emitted significantly lower relative amounts of monoterpenes than sub-adults (U=77734, p<0.01; Figure 5). However, there was no significant difference in the median proportion of monoterpenes when compared to adults (U=77675, p=0.167).

There were no significant differences between the median proportions of plant material VOC classes emitted from white rhino faeces with regard to sex (i.e. benzenoids U=15287, p=0.542, monoterpenes U=15773, p=0.910, sesquiterpenes U=324316, p=0.906 or nitrogen compounds U=7862, p=0.212). Nor were there any significant differences between median proportions of plant material for territorial state (i.e. benzenoids U=14100, p=0.705; monoterpenes U=13293, p=0.207; sesquiterpenes U=293278, p=0.770 or nitrogen compounds U=397, p=0.367), or reproductive state (i.e. benzenoids U=11379, p=0.506; monoterpenes U=11714, p=0.804; sesquiterpenes U=235557, p=0.270 or nitrogen compounds U=296, p=0.990).

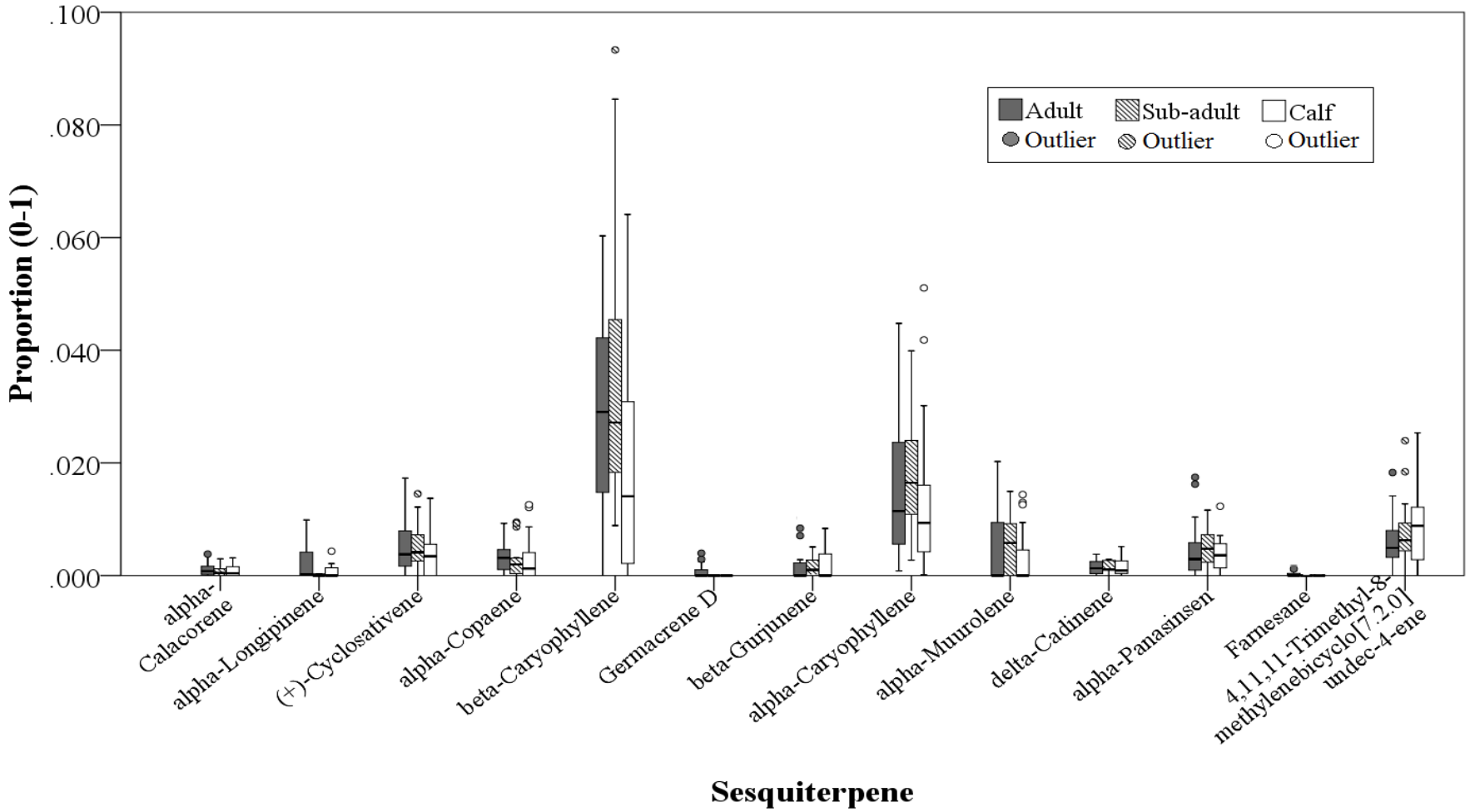

Figure 3. Median proportion (+/- 95% confidence intervals) of known sesquiterpenes (N= 13) emitted from the faeces of all individuals. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

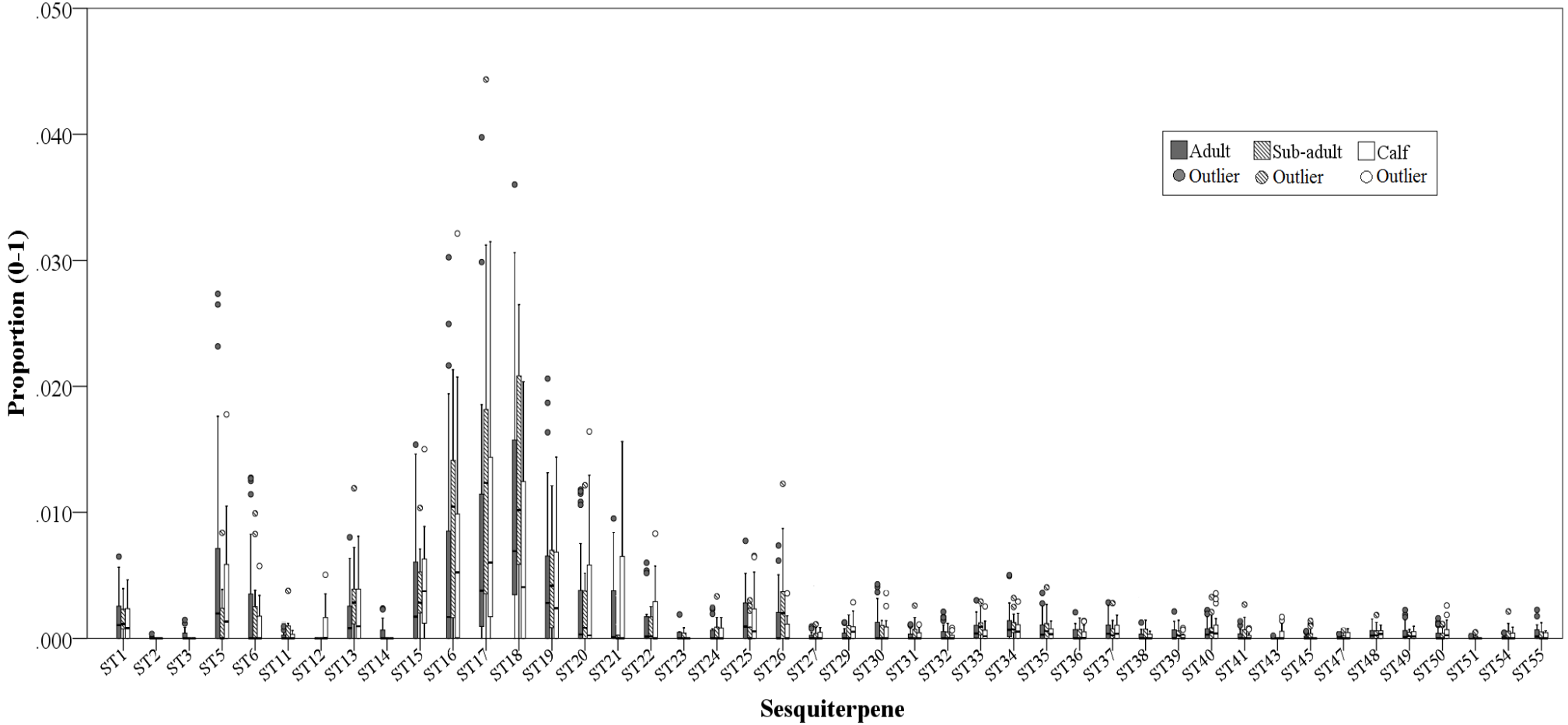

Figure 4. Median proportion (+/- 95% confidence intervals) of unknown sesquiterpenes (N= 44) emitted from the faeces of all ages. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median.

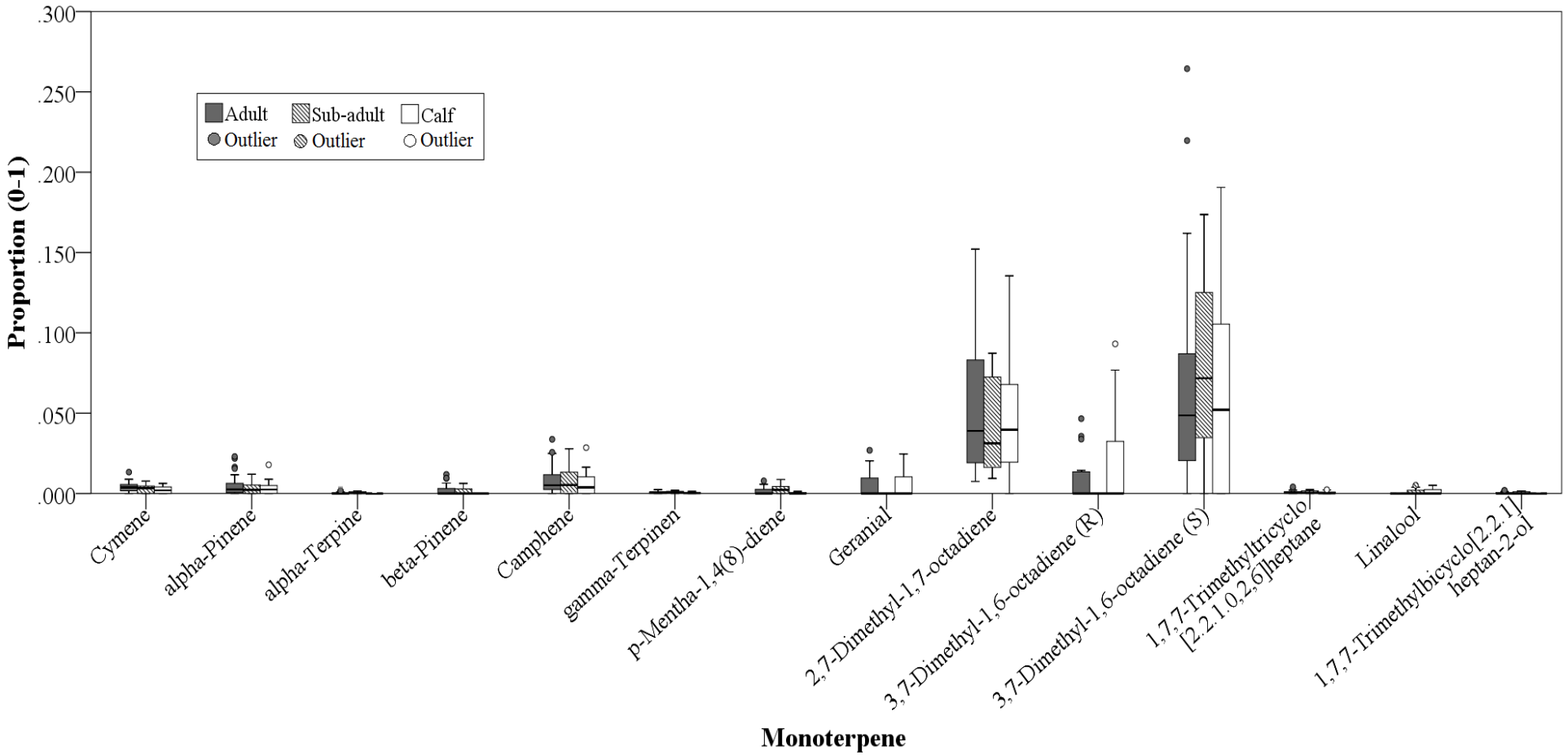

Figure 5. Median proportion (+/- 95% confidence intervals) of monoterpenes (N= 14) emitted from the faeces of all ages. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

3.2. The effect of season on VOCs emitted from white rhino faeces

Seasonally, all the rhinos emitted significantly higher median proportions of alcohols (U=149066, p<0.001; Figure 6) and alkanes (U=810543, p< 0.001; Figure 7) in the dry season compared to faeces from the wet season. They also emitted significantly higher median proportions of sesquiterpenes (U=554313, p<0.001; Figure 8 & 9) and nitrogen containing compounds (U=4443, p<0.001; Figure 10) from faeces in the wet season compared to the dry season.

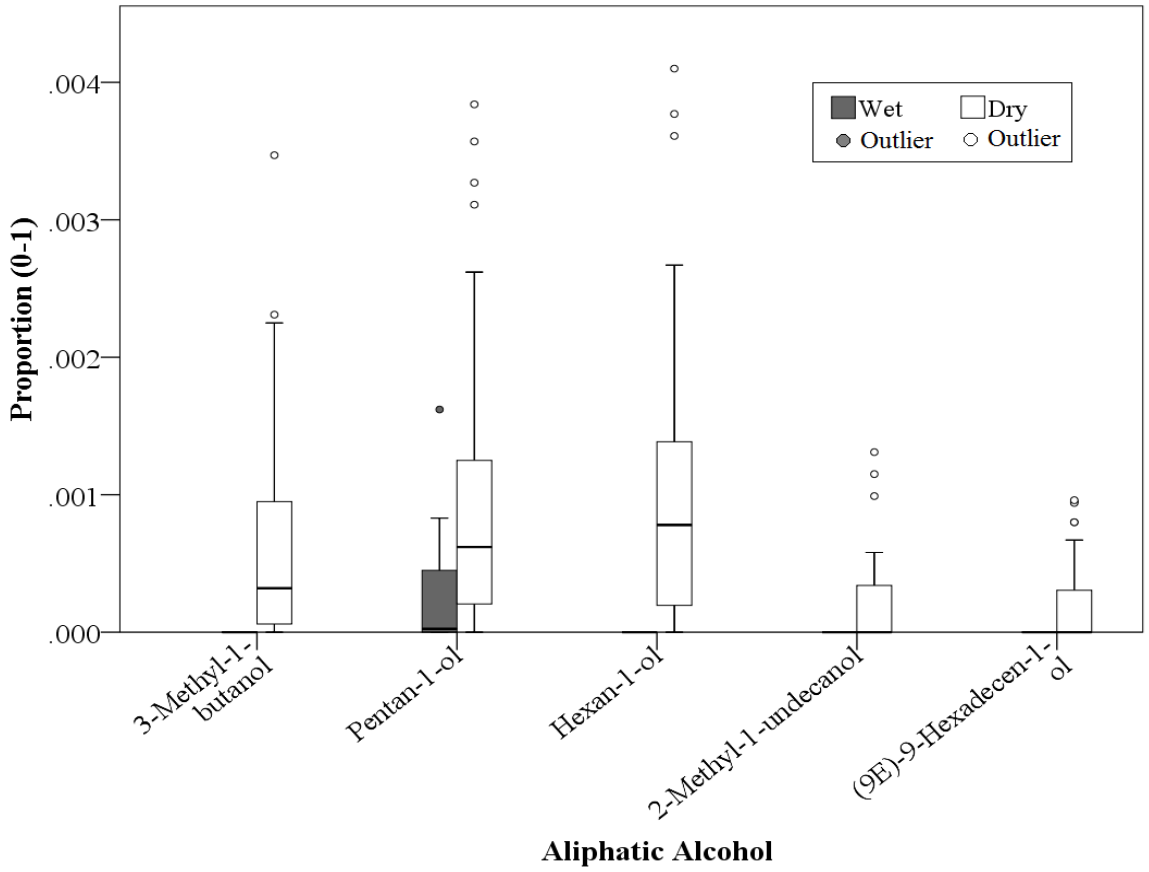

Figure 6. Median proportion (+/- 95% confidence intervals) of aliphatic alcohols (N= 5) emitted from the faeces of all individuals during the wet and dry seasons. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

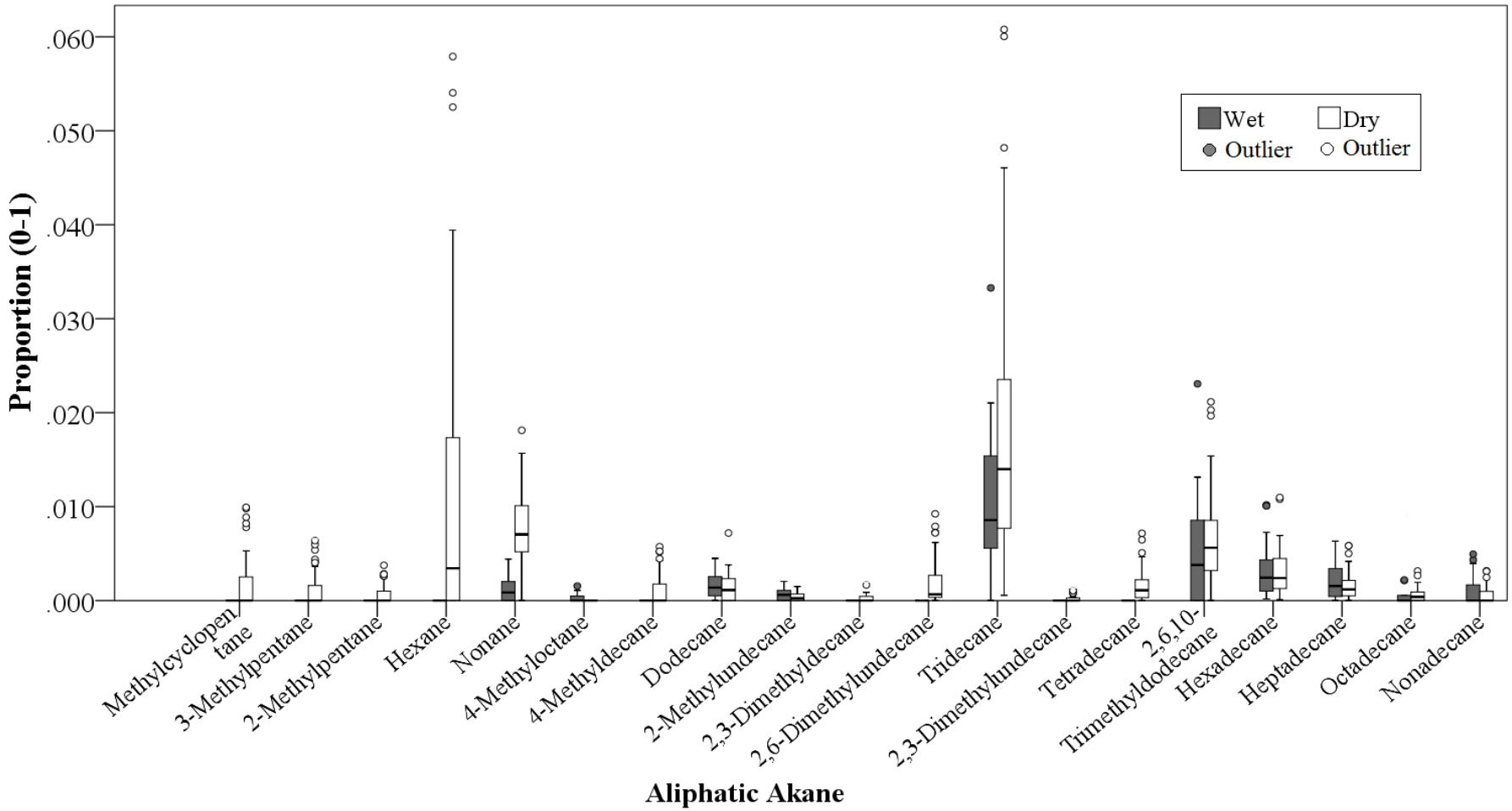

Figure 7. Median proportion (+/- 95% confidence intervals) of aliphatic alkanes (N= 19) emitted from the faeces of all individuals in the wet and dry seasons. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

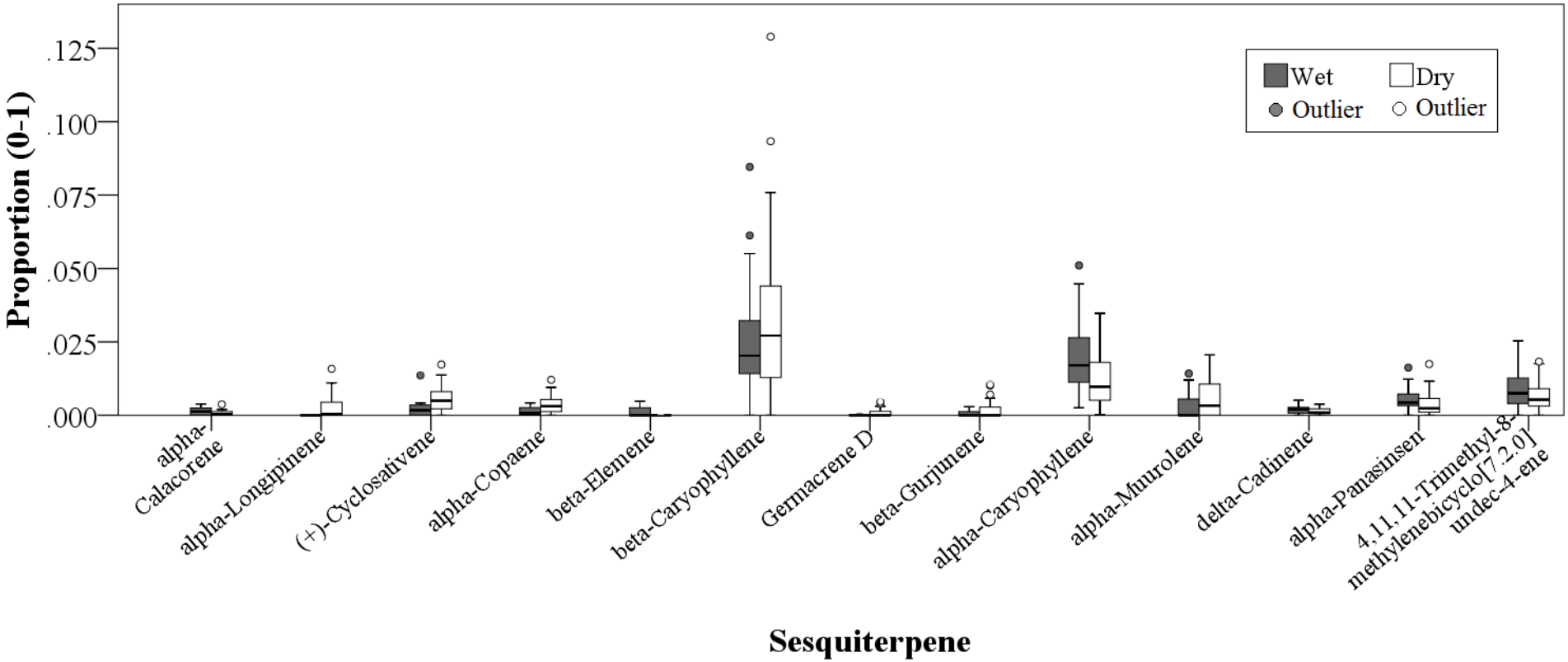

Figure 8. Median proportion (+/- 95% confidence intervals) of known sesquiterpenes (N= 13) emitted from the faeces of all individuals during the wet and dry seasons. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

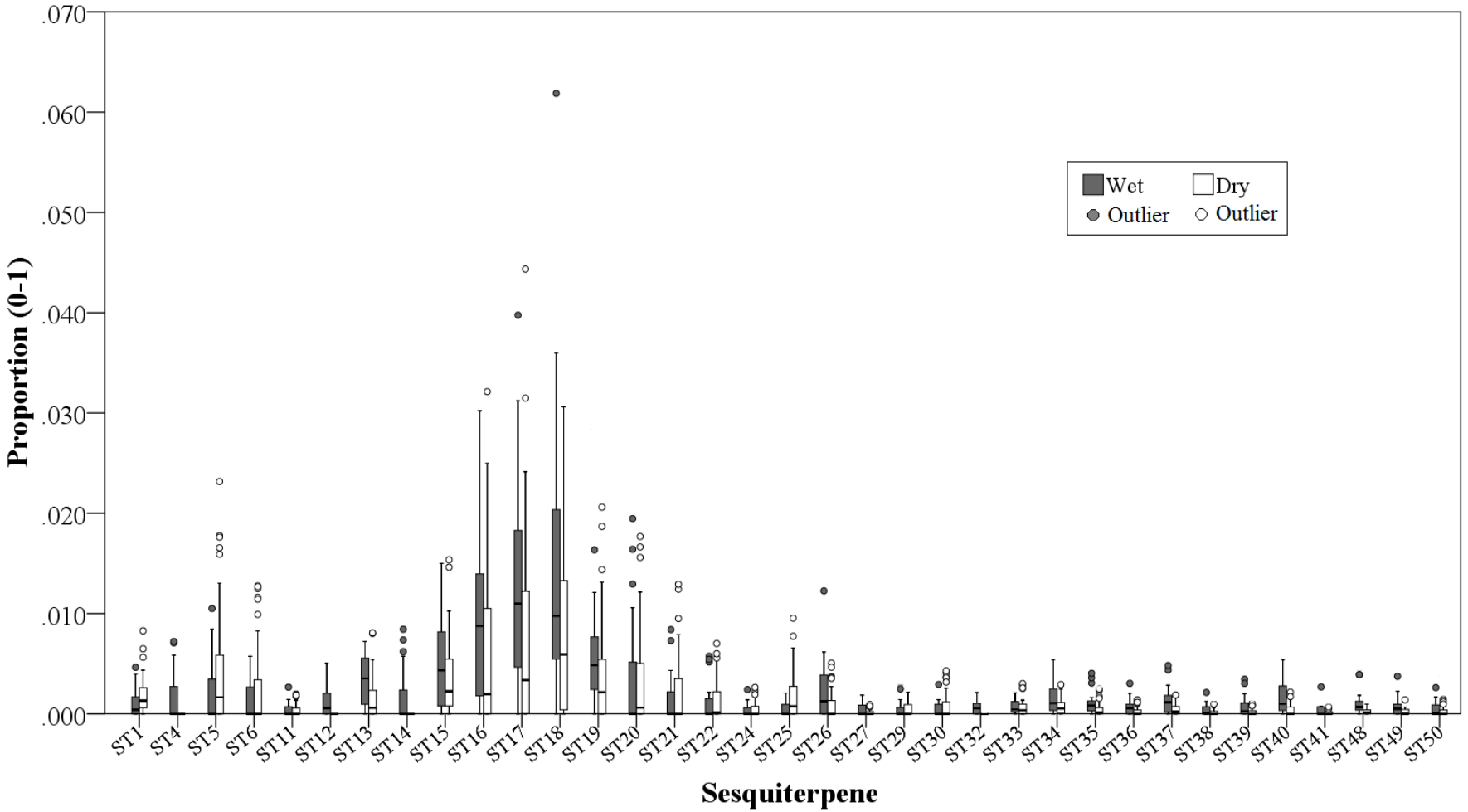

Figure 9. Median proportion (+/- 95% confidence intervals) of unknown sesquiterpenes (N= 35) emitted from the faeces of all individuals during the wet and dry seasons. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median.

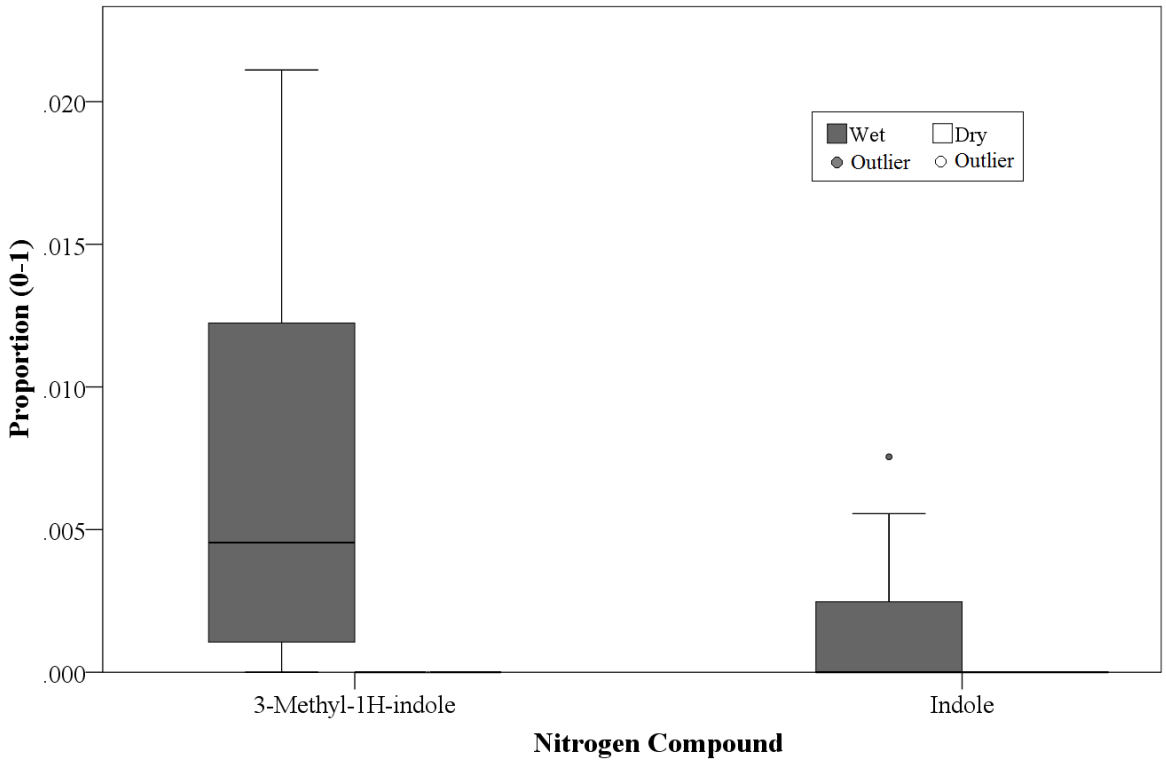

Figure 10. Median proportion (+/- 95% confidence intervals) of nitrogen compounds (N= 2) emitted from the faeces of all individuals during the wet and dry seasons. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median.

I did not find any significant differences between the median proportions of any other VOC class with regard to season (i.e. aliphatic acids U=180915, p=0.662; aliphatic aldehydes U=88120, p=0.564; aliphatic alkenes U=102002, p=0.772; aliphatic ketones U=33328, p=0.118; aliphatic esters U=6591, p=0.168, benzenoids U=245342, p=0.117; monoterpenes U=237912, p=0.461; sulphur compounds U=25566, p=0.440; miscellaneous compounds U=2374738, p=0.353). The differences reported are due to plant based VOCs, therefore, I can report that the seasonal results are due to changes in food quality and/or forage species composition. Further, as there is no other effect of season on any other VOC class I can report that all following results are therefore due to physiological differences and not dietary or seasonal changes in food quality.

3.3. Identification of sex using VOCs emitted from white rhino faeces

The ranked variable importance list from RF identified nine compounds as being important for identifying the sex of an individual rhino from the headspace of its faeces. The most important compound for determining sex was (3E)-3-Decene, where males have a higher median proportion than females (Figure 11). 6-Methyloctadecane and (E)-Oct-2-ene were respectively the second and third most important compounds for determining sex, with females having higher median proportions than males in both regards (Figure 11).

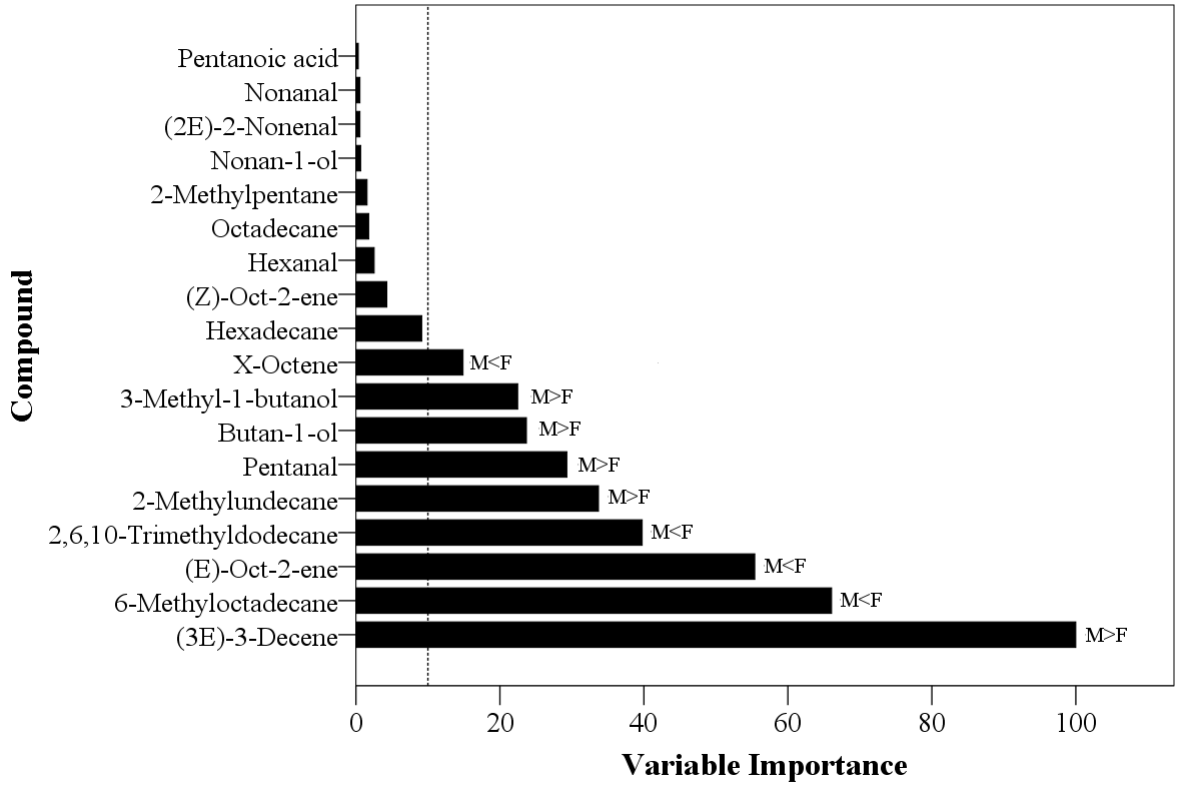

Figure 11. Ranked variable importance list for sex. This shows the relative importance of specific VOCs for determining sex from headspace of white rhino faeces. Longer bars indicate greater importance (i.e. variable importance of 100 indicates the most important compound). The dotted line indicates the threshold value. All VOCs above (i.e. to the right) of this threshold are considered important. Text noted next to each bar indicates which sex has the higher median proportion emitted from faeces (M= male, F= female). Since (3E)-3-Decene is the most important variable for predicting sex, I scaled all other VOCs according to (3E)-3-Decene.

As described in Chapter 2, I achieved a variable importance list from a 'forest' of decision trees. Figure 12 shows one decision tree for determining variable predictor accuracy for sex.

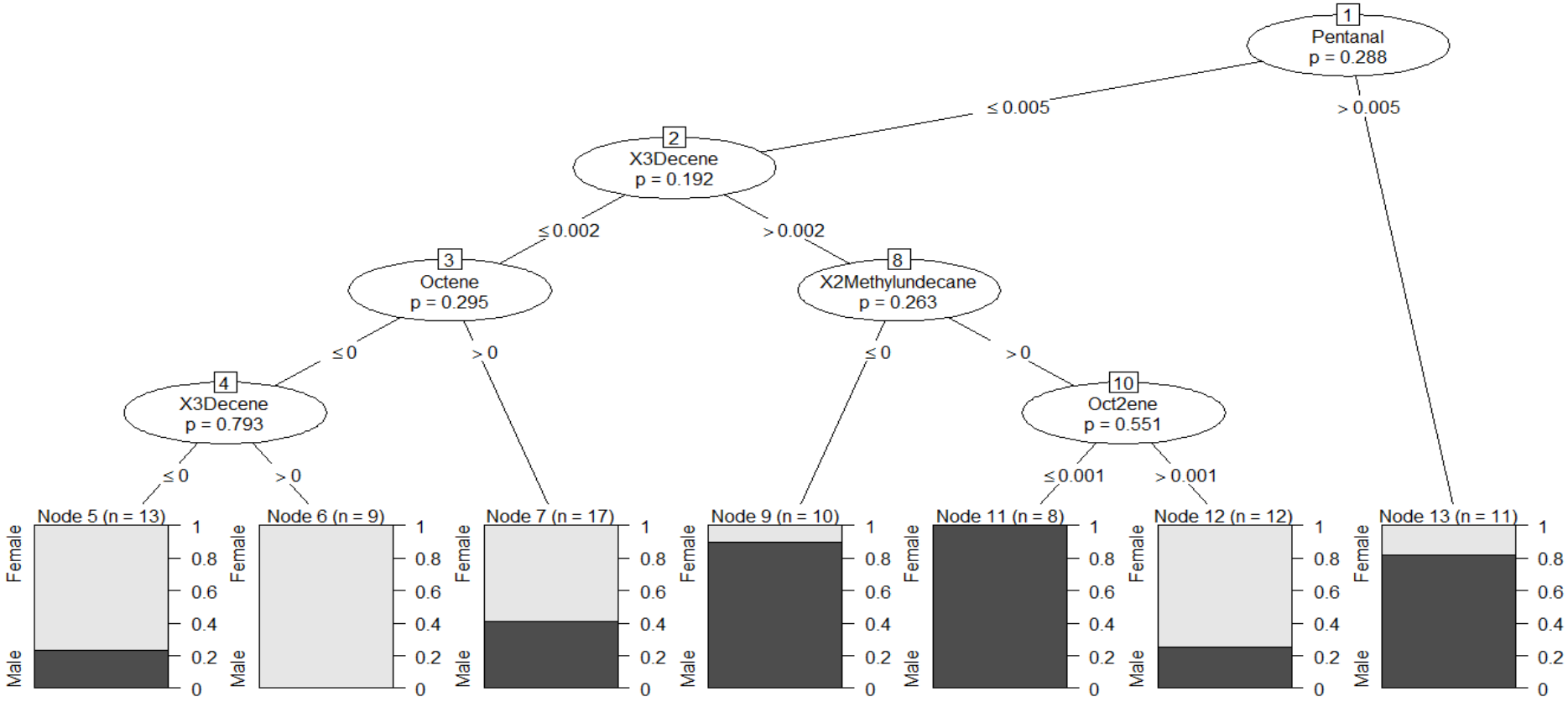

Figure 12. Diagram of a single decision tree representing the variables (i.e. VOCs) that are important for predicting sex from faecal odour of white rhinos. The seven terminal nodes show the predictor accuracy for determining sex (black= male, white= female). Values of one indicate 100% predictor accuracy. For example, at node 6 the predictor variables are 100% accurate in predicating a female, this means that if (1) Pentanal is ≤0.005, (2) 3-Decene is ≤0.002 (3) Octene is ≤0 and 3-Decene is >0 we can say with 100% accuracy that this scent is from a female.

3.4. Identification of age using VOCs emitted from white rhino faeces

The ranked variable importance list identified seven compounds as being important for determining the age of an individual white rhino from the headspace of its faeces (Figure 13). Isobutyric acid was the most important compound for determining age. Adults have the highest proportion, followed by sub-adults and calves, respectively. (9E)-9-Hexadecen-1-ol is the second most important compound for determining age, with adults having the highest proportion, followed by sub-adults and then calves.

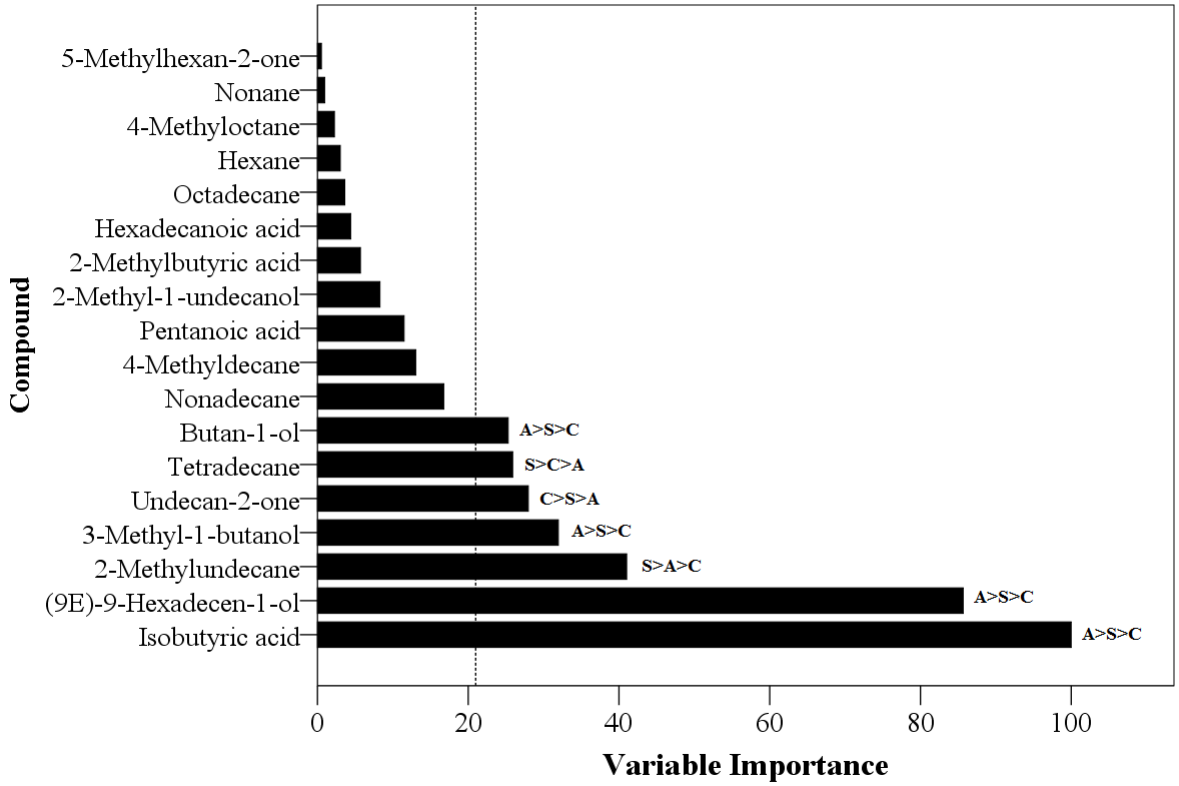

Figure 13. Ranked variable importance list for age. This shows the relative importance of specific VOCs for determining age from headspace of white rhino faeces. The dotted line indicates the threshold value; all VOCs above (i.e. to the right) of this threshold are important. Text noted next to each bar indicates which age has the higher median proportion emitted from faeces (A= adult, S= sub-adult, C= calf). Since Isobutyric acid is the most important variable for predicting age, I scaled all other VOCs according to Isobutyric acid.

3.5. Identification of male territorial state using VOCs emitted from white rhino faeces

When I focussed my analysis on adult males, I found that territorial males emitted significantly higher median proportions of aliphatic acids ($U=7748$, $p<0.001$; Figure 14) and aliphatic aldehydes ($U=4208$, $p<0.05$; Figure 15) than non-territorial males. The only

exceptions were the aliphatic aldehydes Pentanal and Decanal, which were lower in territorial males (Figure 15).

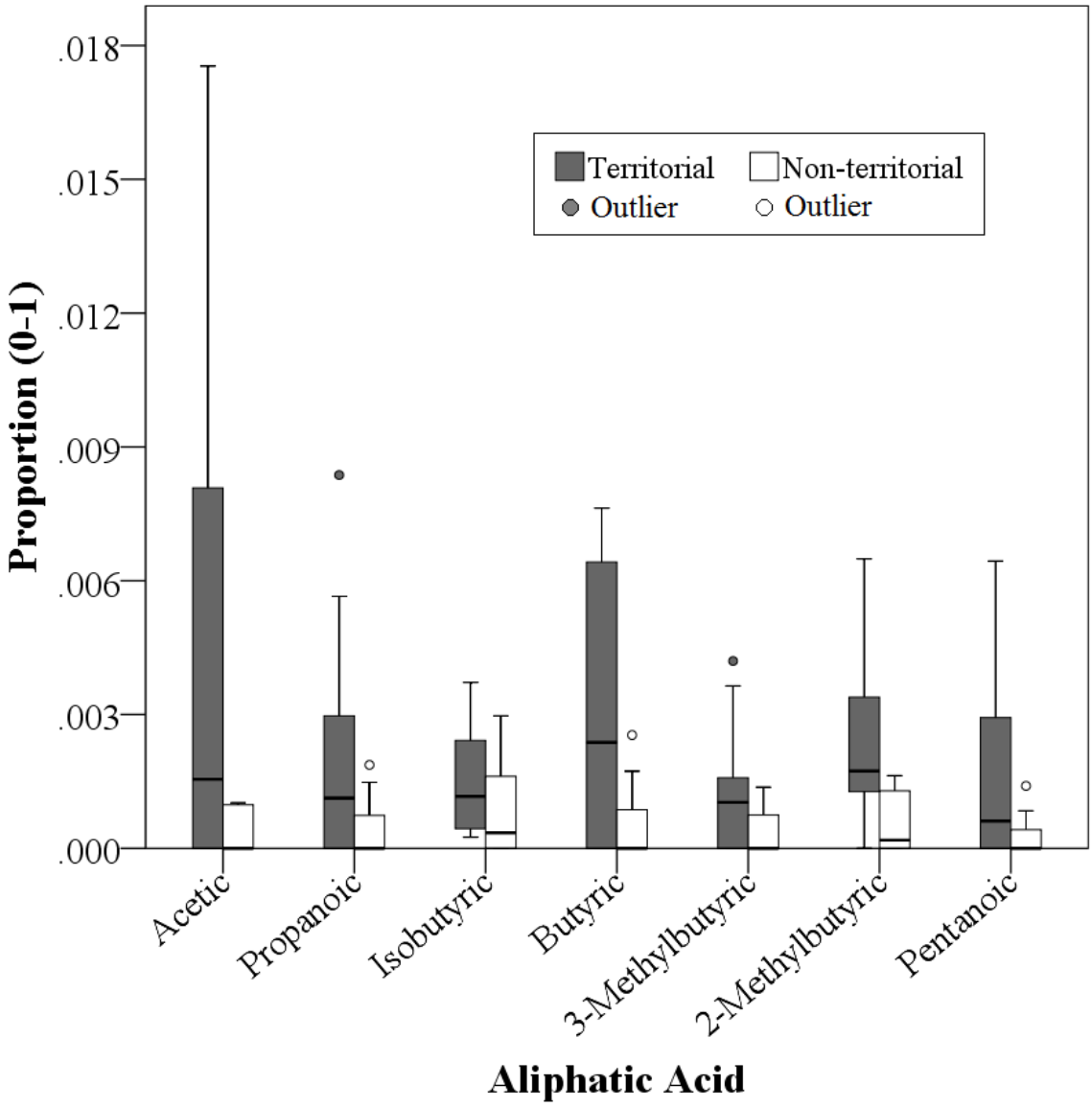

Figure 14. Median proportion (+/- 95% confidence intervals) of aliphatic acids (N= 7) emitted from the faeces of adult males. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

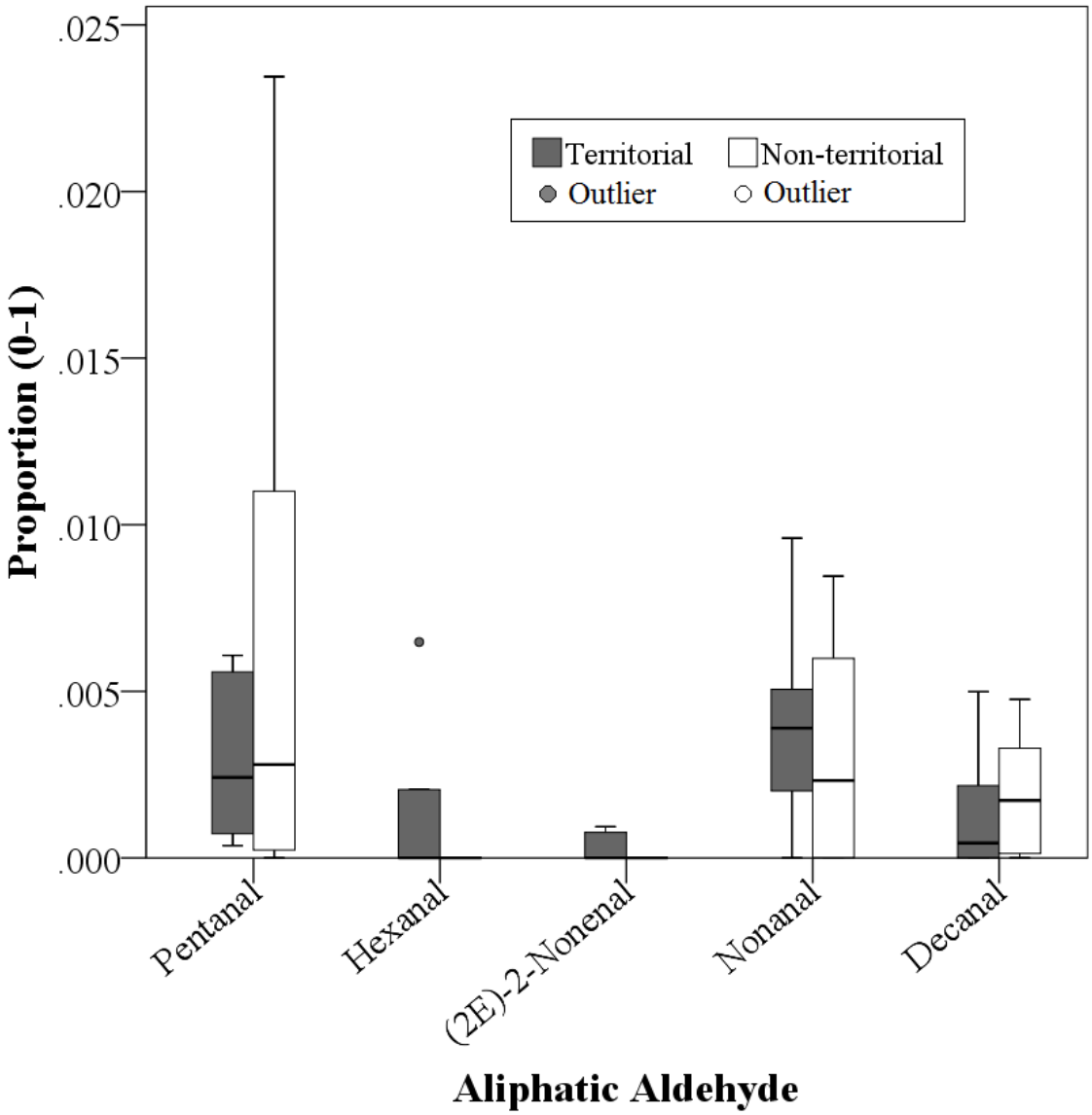

Figure 15. Median proportion (+/- 95% confidence intervals) of aliphatic aldehydes (N= 5) emitted from the faeces of adult males. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

In contrast to the aliphatic acids and aliphatic aldehydes, I did not find significant differences in the median proportion of aliphatic alcohols (U=9896, p=0.624), aliphatic alkanes (U=37687, p=0.691), aliphatic alkenes (U=5441, p=0.485), aliphatic ketones (U=1951, p=0.965) or miscellaneous compounds (U=132924, p=0.638) emitted between territorial males and non-territorial males. Moreover, no aliphatic esters or sulphur compounds were present in the VOCs of faeces from adult males.

3.6. Identification of female reproductive state using VOCs emitted from white rhino faeces

When focusing on adult females, I found that faeces from oestrous females emitted significantly lower median proportions of aliphatic acids ($U=5763$, $p<0.05$; Figure 16), aliphatic alcohols ($U=5103$, $p<0.001$; Figure 17) and aliphatic alkanes ($U=26983$, $p<0.05$; Figure 18) than faeces from non-oestrous females. Overall, when an adult female comes into oestrus, aliphatic acid levels are greatly reduced (Figure 16), aliphatic alcohols disappear (Figure 17) and some of the aliphatic alkanes (e.g. Hexane and Tridecane) decrease (Figure 18).

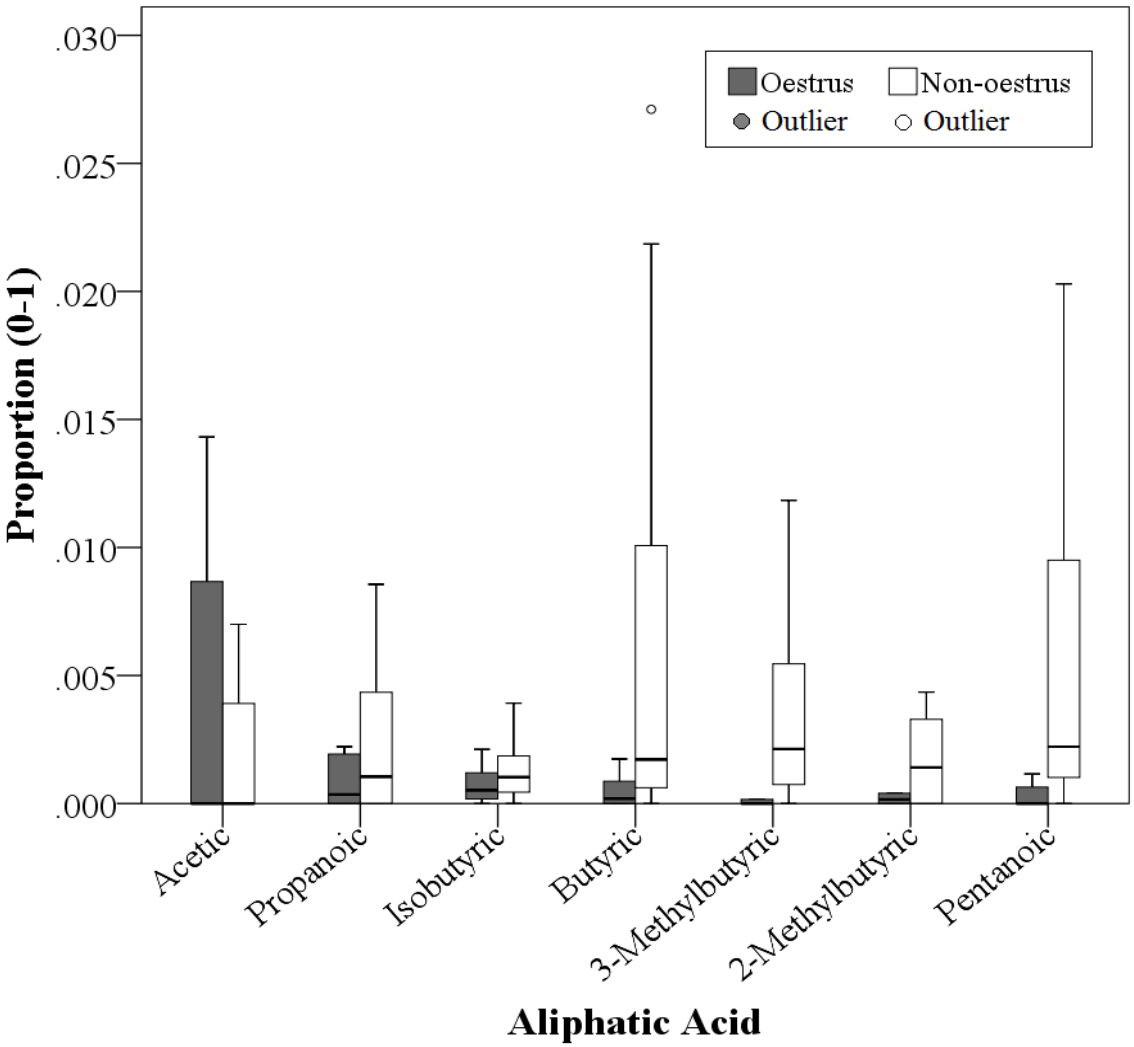

Figure 16. Median proportion (+/- 95% confidence intervals) of aliphatic acids (N= 7) emitted from the faeces of adult females. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

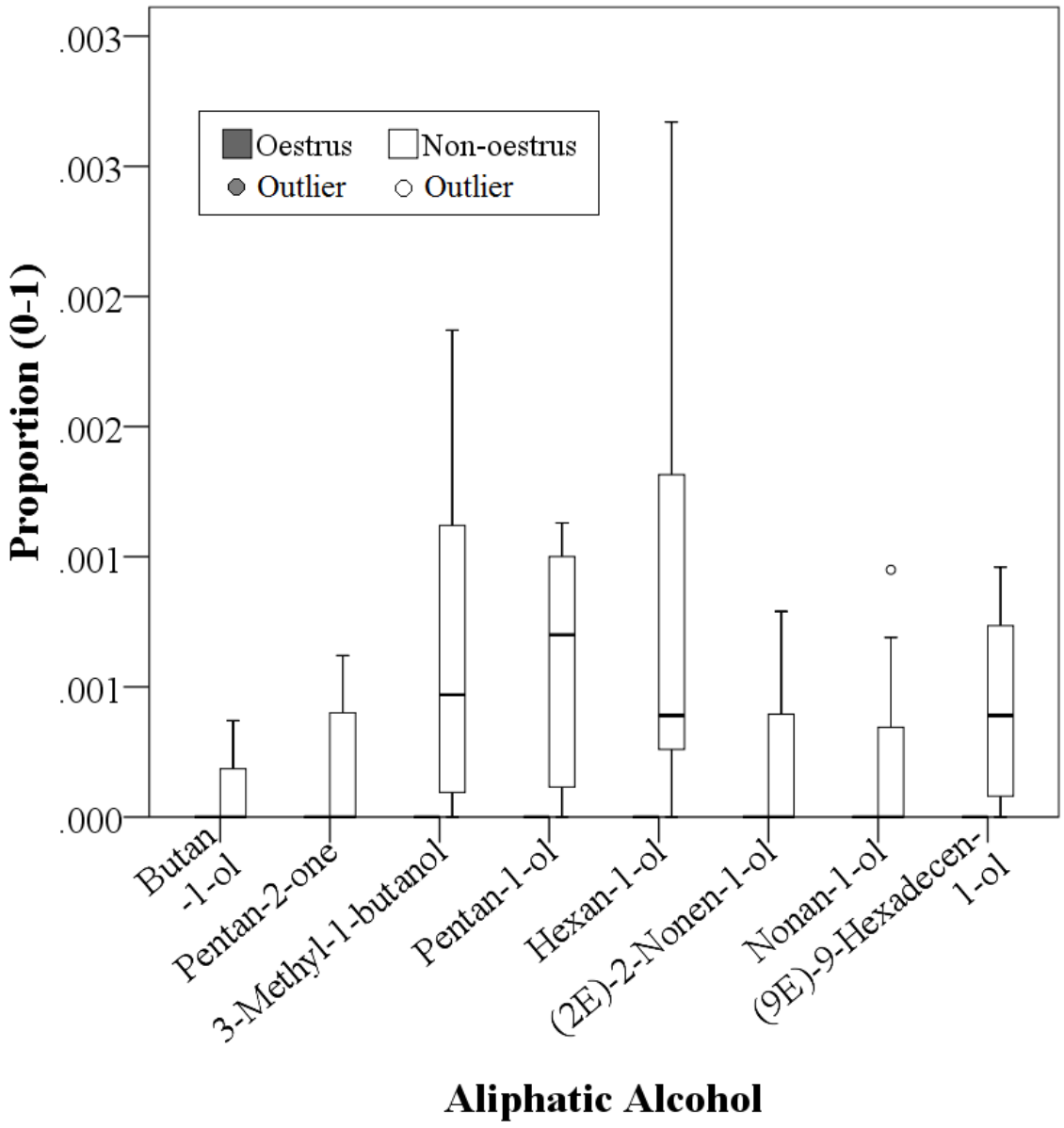

Figure 17. Median proportion (+/- 95% confidence intervals) of aliphatic alcohols (N= 8) emitted from the faeces of adult females. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median. Compounds are plotted by increasing molecular weight.

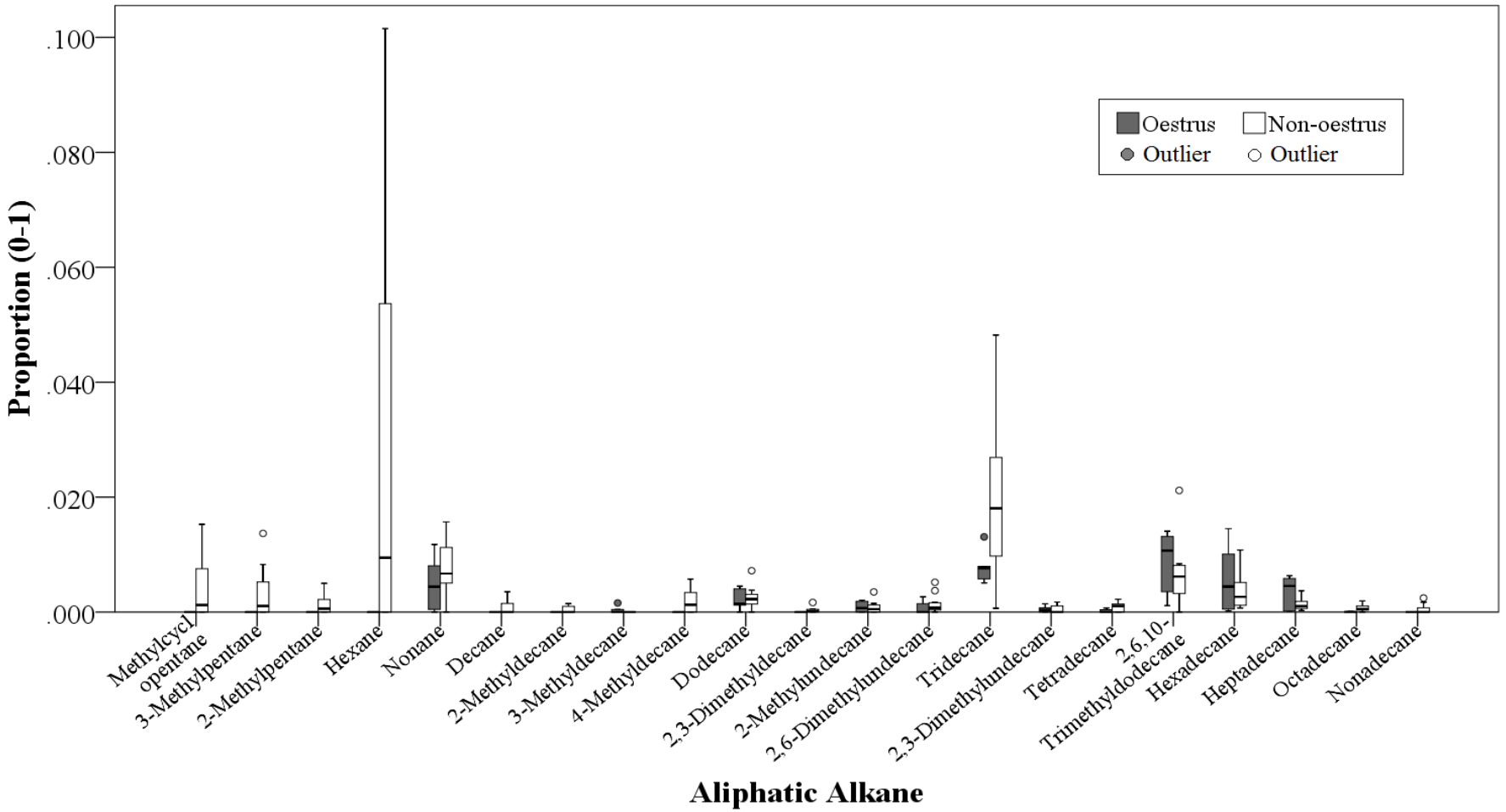

Figure 18. Median proportion (+/- 95% confidence intervals) of aliphatic alkanes (N= 21) emitted from the faeces of adult females. The upper and lower limits of the box represent the upper and lower quartiles and the thick middle band represents the median.

Between oestrous and non-oestrous females, I did not find any significant differences in the median proportion of aliphatic aldehydes (U=3660, p=0.346), aliphatic alkenes (U=4673, p=0.844), aliphatic ketones (U=1585, p=0.764) or miscellaneous compounds (U=111132, p=0.971) emitted from faeces. As with the adult males, there were no aliphatic esters or sulphur compounds within the VOCs emitted from the faeces of adult females.

Chapter 4: Discussion

Many behavioural studies have indicated the importance of odour in rhinoceros communication. Linklater et al. (2013) suggested that black rhinos could detect age, sex and even individuals from faecal odour. Other studies have shown that foreign faeces increased the vigilance of territorial white rhino bulls (Grün, 2006) and stimulated an increased marking rate (Aberham, 2001). Research into rhino olfactory communication has been generally behavioural and previous attempts to identify specific volatile organic compounds (VOCs) have had little success. For example, Grün (2006) was only able to identify 14 VOCs from white rhino faeces. I distinguished 326 VOCs and identified 285 that I categorised according to their biochemical pathways into 14 classes (see Table 2). In addition, I was able to determine differences in VOCs emitted from the faeces of white rhinos of different sex, age, territorial state of males and reproductive state of females. The differences between males and females were mainly due to alkanes and alkenes while for age, the differences were mainly due to alkanes and alcohols. Acids and aldehydes caused differences between territorial and non-territorial adult males and the differences between oestrous and non-oestrous adult females were due to acids, aldehydes and alkanes. Notably, Grün (2006) discarded the benzenoid Toluene from his samples as an environmental contaminant that rhinos breathed in from vehicle exhaust fumes. However, toluene was present in a high proportion in all but one of my samples. Since these were wild white rhinos in a large conservation area, it is not likely that this is an industrial environmental contaminant.

4.1. Identification of dietary plant material from VOCs emitted from white rhino faeces

Calves emitted significantly lower median proportion of sesquiterpenes than adults and sub-adults. Calves also emitted a significantly lower median proportion of monoterpenes compared to sub-adults. The likely explanation for this is the difference in dietary patterns from calves to sub-adults to adults. Calves aged 0-2 years have primarily a milk diet from their lactating mother (Owen-Smith, 1973). Monoterpenes and sesquiterpenes are plant based compounds (Gershenzon and Croteau, 1991), therefore, these VOCs will not be emitted in calf faeces but will be more prevalent in sub-adult and adult faeces.

I found that the difference between the median proportions of plant originated volatile compounds (i.e. benzenoids, monoterpenes, sesquiterpenes and nitrogen compounds) did not differ with regard to sex, territorial state of males or reproductive state of females.

Ultimately, this provides support of the conclusions that I have drawn with regard to VOC differences between these different classes. As the plant originated VOCs are not affected by sex or state, it verifies that the VOCs that are affected by sex or state are in fact a result of physiological differences and not differences in feeding ecology. Hence, my classification of the plant originated compounds (i.e. benzenoids, monoterpenes, sesquiterpenes and nitrogen compounds) as having no olfactory significance is validated.

4.2. The effect of season on VOCs emitted from white rhino faeces

There was a significantly higher median proportion of sesquiterpenes in the wet season compared to the dry season for all individuals. Sesquiterpenes originate from plant material (Gershenzon and Croteau, 1991) and there is generally a higher quality of forage during the wet season than during the dry season due to increased water availability facilitating plant growth (Poorter and Nagel, 2000). Although the overall food intake rate of white rhinos does not differ with season (Shrader et al., 2006) the reduction in food quality, causing a lower intake of protein, sodium and phosphorous (Georgiadis and McNaughton, 1990) would explain the reduction in sesquiterpenes during the dry season. Further, the higher proportion of sesquiterpenes in the wet season may be due seasonal effects on sesquiterpenes emission. The emission of sesquiterpenes from Scots pine (*Pinus sylvestris* L.) leaves occurred mainly during the summer months (Hakola et al., 2006). Or more simply, due to the effect of temperature on emission rates as in the higher emission of sesquiterpenes from orange leaves (*Citrus sinensis* and *Citrus clementi*) under higher temperatures (Ciccioli et al., 1999).

There was also significantly higher proportion of aliphatic alcohols and alkanes in the dry season compared to the wet season. Both alcohols and alkanes from plant cuticle wax can be used to assess diet composition in herbivores (Bugalho et al., 2004) and the higher proportion of both types of compounds would suggest a dietary difference between the seasons. Shrader et al. (2006) found that the use of Themeda and other grasslands by white rhinos increased during the dry season as a way of coping with decreasing food quality. The higher proportions of alcohols and alkanes emitted from white rhino faeces in the dry season are likely due to the shift in diet composition to higher quality grasses to cope with the lower quality forage in the dry season. Therefore, the dietary and seasonal results support that all other results must be due to physiological differences and not due to dietary or seasonal differences.

4.3. Identification of sex using VOCs emitted from white rhino faeces

I identified distinct sex odour signatures for white rhinos, with nine specific VOCs being important. Males had significantly lower median proportions of two alkanes (6-Methyloctadecane and 2,6,10-Trimethyldodecane) and two alkenes [(E)-Oct-2-ene and X-Octene] than females. Males had significantly higher proportions of one alkane (2-Methlyundecane), one alkene [(3E)-3-Decene], one aldehyde (Pentanal) and two alcohols (Butan-1-ol and 3-Methyl-1-butanol) than females. The differences observed were due to changes in concentration and not sex specific VOCs. Gut morphology and feeding ecology are unlikely to be a cause for odour difference as all individuals eat the same food types (Owen-Smith, 1988, Shrader, 2003, Shrader et al., 2006). Further, Kwak et al. (2008) suggested that there are a stable set of volatile compounds that are not influenced by dietary changes. This would then mean that individual characteristics, such as sex and age, would remain distinguishable regardless of environmental variation (i.e. diet). Sex is genetically determined, the presence of X or Y chromosomes causes different genes to be expressed which ultimately causes distinguishable odours between the sexes (Yamazaki et al., 1986). The genetic differences between males and females will likely explain the sex differences I observed.

Further, the bacterial role in mammals that use specialised scent glands has been widely documented (see review by Archie and Theis (2011)). Microbes flourish because the specialised glands provide a warm, moist and nutrient rich habitat for them to grow. However, less importance goes to the microbial contribution for mammals that use faeces or urine to scent mark. Bacteria break down organic material and produce VOCs that contribute to host odour, which is explained by the fermentation hypothesis (Albone and Eglinton, 1974). The fermentation hypothesis proposes that the symbiotic bacteria living within scent glands produce volatile compounds that contribute to mammalian recognition cues. The variation in composition and abundance of these bacterial communities then creates a unique 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).

The fermentation hypothesis explains odours as a result of resident bacteria within a scent gland, however there are also resident bacteria within the gut that are regularly expelled with faecal matter. The microbial community of white rhino faeces has been recorded in

detail, with 50 genera of bacteria identified (Bian et al., 2013) compared to the 31 genera identified from tarsal tufts of white-tailed deer (Alexy et al., 2003). If the fermentation hypothesis is correct and odours are the result of bacteria, gut bacteria should therefore also contribute significantly to host odour. Therefore, it could likely explain the differences between the faecal odours of white rhinos of different sexes and further, not only would this then encode individual recognition cues, but also more specific cues such as age and territorial/reproductive state.

4.4. Identification of age using VOCs emitted from white rhino faeces

I identified distinct age scent signatures, with seven volatile compounds being important. Adults had the highest proportions of the alcohols [(9E)-9-Hexadecen-1-ol, 3-Methyl-1-butanol and Butan-1-ol] and Isobutyric acid. Sub-adults had the highest proportion of the alkanes (2-Methylundecane and Tetradecane) and calves had the highest proportion of the ketone (Undecan-2-one). The differences observed were due to changes in concentration and not age specific VOCs. Gut digestive development and diet are likely causes of these age differences in VOCs of white rhino faeces.

White rhino calves aged 0-2 years have a primarily milk diet (Owen-Smith, 1973). The sub-adult age group begins with the weaning years at approximately 18 months and an adult diet is comprised of grass only (Owen-Smith, 1973). Calves on a milk diet will consequently have a different gut microbial community than adults on a solid diet and therefore a different faecal odour. Adult owl monkeys (*Aotus nancymaae*) were discriminated from young (0-4 years) via six volatile compounds of plant origin, suggesting that the higher concentrations present in adults were the result of dietary differences (Macdonald et al., 2008). Individuals must adapt from a sterile environment at birth to the introduction of microbes in the mother's milk and then to the new microbes present in a solid diet causing drastic changes in gut microbial flora (Lee and Gemmell, 1972). The gut bacteria have a large impact on the production of VOCs and therefore their presence-absence will affect faecal odour.

Reproductive hormones (i.e. testosterone and oestrogen) are non-volatile compounds, meaning that they are not detectable via odour. However, the circulating levels of testosterone in males and oestrogen in females will be subsequently broken down when expelled in faecal matter (D'Ascenzo et al., 2003, Chiang et al., 2010) and this will intuitively create different odours. Endocrine development will therefore likely cause a change in faecal

scent, especially within the sub-adult stage as individuals come into sexual maturity. At the onset of sexual maturity testosterone levels can increase dramatically in males (Ariyaratne and Mendis-Handagama, 2000) and oestrogen can increase rapidly at the onset of ovulation in females (Gesquiere et al., 2007). Female white rhinos have their first oestrus period at approximately five years of age (sub-adult) although the birth of first offspring generally only occurs at 6.5-7 years (Owen-Smith, 1975). With regard to males, sexual development occurs at a different rate to females. Sub-adult males can become solitary at approximately eight years of age (adult), but tend to only establish a territory when they are 9-12 years old (Owen-Smith, 1975). The establishment of a territory is also linked to an increase in testosterone levels (Kretzschmar, 2002). It is possible that the production of reproductive steroid hormones (i.e. testosterone in males and oestrogen in females) appearing in higher levels in the sub-adult females and adult males will likely alter the faecal scent. Therefore, I suggest that the observed differences between white rhinos of different ages with regard to VOCs are due to a combination of gut microbial development and endocrine regulation.

4.5. Identification of male territorial state using VOCs emitted from white rhino faeces

I found that territorial adult male white rhinos had significantly higher median proportions of all volatile acids and 60% of aldehydes than non-territorial males. The differences observed were due to changes in concentration and not territorial specific VOCs. This is likely linked to the fact that adult male white rhinos have significantly higher testosterone concentrations than either male sub-adults or calves (Kretzschmar, 2002). Moreover, territorial male white rhinos have significantly higher concentrations of faecal testosterone than non-territorial adult males (Rachlow et al., 1998). As a result, there is more testosterone present for bacterial break down in territorial male faeces and this subsequently creates more testosterone degradation volatiles (e.g. the ketone, androsta-1,4-diene-3,17-dione (Chiang et al., 2010)) than would be present in non-territorial male faeces.

It may be that these volatiles are simply associated with testosterone. Two empirical studies have identified testosterone dependant volatile compounds. Firstly, Achiraman and Archunan (2005) discovered a testosterone dependant urinary volatile in male mice. The alkane, 3-Ethyl-2,7-dimethyl octane, was involved in attracting females and its production was under the control of androgen. Secondly, in wolves, two sulphur compounds are testosterone dependant volatiles (Raymer et al., 1986). Raymer et al. (1986) determined this when these compounds disappeared when adult males were castrated and reappeared upon

subsequent testosterone treatment. In contrast, volatiles present may be a direct result of testosterone degradation. Mammals cannot degrade steroid hormones but after excretion they can be broken down by bacteria and consequently, several volatile ketones are produced (Chiang et al., 2010). Although there was no significant difference between the proportion of faecal ketones of territorial and non-territorial males, it is possible that the elevation of acids and aldehydes emitted from territorial males is associated with the bacterial degradation of higher concentrations of testosterone.

It has been suggested that the high concentration of aldehydes present in the inter-digital secretions of red hartebeest could only be used as a short-term territorial marking signal (Reiter et al., 2003). This is because aldehydes are highly susceptible to autoxidation (Nonhebel et al., 1979) (i.e. spontaneous oxidation of a compound in air). Through this reaction, aldehydes convert to acids. Therefore this conversion, which is linked to testosterone degradation and/or volatiles associated with testosterone, could function as a signal of territorial ownership and possibly a signal of deposition time. My observed differences of higher proportions of acids and aldehydes emitted from territorial male faeces compared to non-territorial males support this. Territorial male white rhinos mark their territory regularly, visiting boundary middens approximately every two days (Owen-Smith, 1975). It is possible that the higher median proportion of volatile acids and aldehydes emitted from faeces act as a signal of territory ownership.

4.6. Identification of female reproductive state using VOCs emitted from white rhino faeces

Adult female white rhinos in oestrus emitted significantly lower proportions of acids, alcohols and alkanes compared to non-oestrous females. The differences observed were due to changes in concentration and not oestrus specific VOCs. In many other species, these observed compound classes have been reported with regard to oestrus. Three oestrus specific compounds have been identified in cow faeces (Acetic acid, Propionic acid and the alkane 1-Iodoundecane) (Sankar and Archunan, 2008). The compound 1-Iodoundecane was also identified as an oestrus specific compound in cow urine, along with the ester Di-n-propyl phthalate (Kumar et al., 2000). Moreover, the alkane 1-Iodo-2-methylundecane is also oestrogen dependant in female mice (Achiraman et al., 2010). However, in all of these examples, females showed a higher concentration or sudden appearance of these volatiles during oestrus. In contrast, I found the opposite pattern in my study, with oestrous females having lower proportions of acids, alcohols and alkanes. However, the reduction or absence

of a volatile compound does not mean an oestrous female is more difficult to detect. Rather, the scent, as a whole, alters and that thus males should likely be able to differentiate between oestrous and non-oestrous female faecal scent.

A potential explanation for the lower proportions of volatile compounds emitted from oestrous female white rhino faeces compared to species' above could be due to the fermentation-absorption process in hindgut fermenters (Titus and Ahearn, 1992). Volatile compounds can be absorbed in the hindgut before they are released with faecal matter and this can contribute significantly to host energy requirements (Marty and Vernay, 1984). Although hindgut fermenters differ in absorption rate compared to ruminants, Steuer et al. (2011) discovered that white rhinos are an exception. The mean particle retention time of 47 hours lies within the range of ruminants meaning that, for a hindgut fermenter, the white rhino is effective in absorbing volatile compounds in the hindgut before they are expelled with faecal matter.

Ovulation, gestation and lactation are energetically costly to females (Gittleman and Thompson, 1988) and as the absorption of volatile acids may be under hormonal control (Marty and Vernay, 1984) oestrous females may be absorbing higher levels of volatiles to use for energy and to counter-act the strain on body condition. The significantly lower levels of acids, alcohols and alkanes emitted from oestrous female white rhino faeces may be because they were absorbing them to prepare for the subsequent strain on body condition caused by gestation and lactation.

4.7. Future directions

The behavioural evidence for olfactory communication in white rhinoceros has been documented (Owen-Smith, 1975, Kretzschmar et al., 2001, Grün, 2006). However, prior to my study, the extent of the information transmitted was unknown. Here, I present information conveyed via faeces using VOCs associated with specific characteristics (i.e. sex, age, male territorial state and female reproductive state). To date, the recorded behavioural responses of individual white rhinos have not indicated the true importance of faecal signals to white rhino communication. The results of my study have unlocked an important aspect of white rhino olfactory communication. However, this study is only the first step in a process of truly understanding white rhino olfactory communication. Ultimately, my results allow additional

experiments to be conducted that would further expand our understanding of white rhino olfactory communication.

4.7.1. Persistence of faecal odours

As faecal signals persist in the environment, abiotic factors such as rain, pH, desiccation and physical destruction will have an effect on the volatiles and their subsequent emission rates. The pre-orbital scent gland secretions of klipspringers were only efficient as territorial signals for up to seven days (Roberts, 1998). In contrast, black rhinos investigated dung similarly irrespective of whether the dung one day old or 32 days old (Linklater et al., 2013). However, it is unclear as to when faeces stops acting as an olfactory signal in the white rhino. Microbes play an important role in the time release of urinary volatiles during musth of both male African and Asian (*Elephas maximus*) elephants (Goodwin et al., 2012) and this can carry a signal of deposition time for receivers. It is therefore important to look at how odour profiles change over time. As I have identified a baseline of VOCs in this study, I can now examine how they change over time and under different seasonal and environmental conditions. This will allow me to answer if (1) the same chemical signal remains but weakens over time, as suggested with klipspringer scent secretions (Roberts, 1998) or (2) the chemistry of the odour changes allowing for a deposition signal, as with urinary musth signals in elephants (Goodwin et al., 2012).

4.7.2. VOCs within urine

Territorial male white rhinos use urine to scent mark as well as faeces (Owen-Smith, 1971). As a result, it would be interesting to investigate how the urine odour profile differs with age and sex and how it differs to the odour profile of faeces. A study on black rhinos found that the importance of faeces and urine differed with sex. Male black rhino faeces were investigated more than female faeces, and female black rhino urine was investigated more than male urine (Linklater et al., 2013). For white rhinos, it is likely that urine also plays a significant role in rhino olfactory communication (Owen-Smith, 1988). However, the question that arises is what information is transmitted via urine?

Two hypotheses have been put forward by Ralls (1971) that marking with multiple sources of scent either (1) sends multiple messages or (2) the same message in multiple ways. Owen-Smith (1973) observed that male white rhinos displayed flehmen behaviour (i.e. the characteristic behaviour common to male ungulates whereby the upper lip is curled up and

the head is tilted back to facilitate the detection of olfactory signals associated with oestrous females) after sniffing the urine of oestrous females but not after sniffing their faeces. This would suggest that the oestrus signal is only present in urine. Zhang et al. (2005) found that the major volatile compounds in the anal gland secretions of ferrets were not present in urine. Thus, they suggested that the ferrets transmitted different information in these two secretions. In contrast, the same compound, 1-Iodoundecane, was identified as oestrus specific in both the faeces (Sankar and Archunan, 2008) and urine of cows (Kumar et al., 2000), suggesting that the same message could be recognisable from both means of olfactory signals.

It is clear that male white rhinos are able to detect an oestrous female, as they follow them for the entire period, do not allow them to leave their territory and make repeated advances (Owen-Smith, 1973). However, it is unclear whether this signal is apparent in both the faeces and urine of oestrous females. By collecting the volatiles of oestrous female urine this would allow me to answer if (1) the same volatiles are emitted from oestrous urine as in oestrous faeces suggesting the same signal in two ways as in cows (Kumar et al., 2000, Sankar and Archunan, 2008), or (2) the volatiles emitted from oestrous urine differ from that of oestrous faeces suggesting a different message as in ferrets (Zhang et al., 2005).

4.7.3. Validity of the identified VOCs for white rhino communication

Although I have identified scent profiles in my study, I cannot be certain as to which compound, or set of compounds, acts as the specific signal(s) for a certain characteristic. Sankar and Archunan (2008) found that the combination of the three faecal oestrus specific volatiles from female cows that elicited the greatest response from males, compared to any other combination or singular volatile. It is therefore essential to conduct field experiments with several different combinations of VOCs for each characteristic identified in my study (i.e. sex, age and territorial/reproductive state) in order to fully distinguish a genuine semio-chemical from 'background noise' via assessing behavioural responses to such cues. By using synthetically created volatiles injected into turf, I can examine behavioural responses to assess which compound, or set of compounds, are responsible for transferring information. For example, I would expect the scent signal of a rival territorial male to elicit increased scent marking and vigilance in a resident territorial male.

4.7.4. Frequency of midden visitation

Understanding the temporal persistence of key VOCs emitted from faeces allows for running of experiments to explore what determines the frequency with which individuals re-visit middens to assess information left by conspecifics. For example, is the visitation frequency different for a territorial male compared to an adult female? It is likely that different age and sex classes would have different midden visitation frequencies in order to increase their fitness. A second question that I could explore would be, is this visitation frequency dependent on other individuals in the area? For example, if there is a potential threat to a territorial male, does his midden visitation frequency increase? By manipulating scent at middens (as described in 4.7.3.) and using camera traps at all middens within a territory/home range, I would be able to assess, for example, how the visitation frequency of a territorial male changes after depositing a synthetic rival male scent.

4.7.5. Dung kicking by territorial males

The reason why territorial males kick their dung within middens could be because (1) it spreads the territorial male's scent across the midden, and therefore his scent dominates the pile, or (2) that the male is acquiring faecal odours on his feet and subsequently carrying the scent to greater distances around his territory (Owen-Smith, 1973). Using the scent collection techniques described in my study, a simple experiment could test these alternative hypotheses for dung kicking by comparing (1) scent samples taken from across areas of the midden differing in proportion coverage of fresh territorial dung and (2) scent samples of tracks imprinted with dung as a territorial males walks away from the midden.

4.8. Conclusions

The results of my study contribute to the greater knowledge of white rhino olfactory communication, and further our understanding of olfactory communication for mammals in general. By understanding the chemistry of mammalian odours, we can better understand how they use and perceive their environment and therefore provide better management. For example, black rhino reintroductions can have little success due to aggression causing high mortality rates (Brett, 1998). Linklater et al. (2006) investigated the use of scent broadcasting in the relocation of black rhinos. Contrary to their hypotheses, when they deposited the faeces of individuals 2 kms around their release site, the black rhinos actually moved further

distances than individuals with no scent deposited in their release site. With similar experiments to those I stated in the above sections, and using the methodology described in my study, scent broadcasting can be investigated further in order to fully understand the mechanisms behind it so it may be used it to its full potential in aiding the safe relocation of rhinos.

Moreover, the results of my study are applicable to rhinos in captivity. White rhinos have very poor reproductive success in captivity, which is still not fully understood. Lindemann (1982) discovered that the main requirement for successful breeding was the presence of more than one male, as sexual competition appeared necessary for the stimulation of male sexual behaviour. Within zoos, the presence of another white rhino male is not always achievable and so this study may be a platform for the exploration of a potential olfactory stimulus to substitute for male presence. For example, the acid and aldehyde VOCs I identified as being higher in territorial males can easily be synthetically produced, injected into turf and strategically placed within a male's enclosure to create the illusion of faeces from an intruder male. This may then stimulate male sexual behaviour. Further, females require the selection of a mate in order for oestrus cycling to be initiated (Owen-Smith, 1988), but in captivity it is not always possible to provide females with multiple males. However, it may not only the presence of multiple males, but rather the olfactory stimulus given by multiple males that could initiate oestrus cycling. For example, the oestrus cycling of female mice can be induced by male urine (Jemiolo et al., 1986). Further, Jemiolo et al. (1986) found that synthetic compounds had the same effect as original male urine. The same principle could be applied here, to create the illusion of multiple male's faecal marks by injecting turf with varying levels of territorial male associated VOCs, which could stimulate oestrus cycling via olfactory cues, and ultimately aid successful breeding.

In conclusion, my results present the chemical features associated with physical characteristics of sex, age, territorial state of adult males and reproductive state of adult females. Ultimately, providing chemical support for the behavioural evidence of the importance of middens in white rhino olfactory communication. We knew that middens were used in white rhino olfactory communication, but my study provides support that information about territory ownerships, reproductive status of females, sex and even the age of individuals can be distinguished via faecal odours at middens. My study also serves as a platform for further investigation that could link the VOCs identified in my study to specific behaviours (see above sections), further expanding our knowledge of both white rhino

behavioural ecology and olfactory communication. My study, along with the further studies outlined in the sections above, will achieve a well-rounded understanding with regard to the olfactory world of white rhinos which can be applied to both captive and free-living populations.

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Appendix

Appendix 1. List of all compounds distinguished from all samples of white rhino faeces. 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	Retention time	CAS #	KOVATS index
Aliphatic acids			
Acetic acid[c]	1.681	64-19-7	645
Propanoic acid[b]	2.514	79-09-4	710
Isobutyric acid[b]	3.396	79-31-2	765
Butyric acid[b]	4.019	107-92-6	789
3-Methylbutyric acid[b]	4.976	503-74-2	848
2-Methylbutyric acid[b]	5.397	116-53-0	863
Pentanoic acid[b]	5.800	109-52-4	841
Butylacetic acid[b]	8.038	1070-83-3	952
Butylethylacetic acid[b]	11.129	149-57-5	1123
Nonanoic acid[b]	14.126	112-05-0	1273
Undecanoic acid[b]	19.561	112-37-8	1561
Hexadecanoic acid[b]	25.602	112-39-0	1870
Octadecanoic acid[b]	28.380	57-11-4	2171
Octanoic acid[a]	29.666	124-07-2	
Aliphatic alcohols			
Butan-1-ol[b]	2.272	71-36-3	653
3-Methyl-2-butanol[a]	2.764	598-75-4	
3-Methyl-1-butanol[b]	3.159	123-51-3	737
Pentan-1-ol[b]	3.665	71-41-0	766
(3E)-3-Hexen-1-ol[b]	5.238	544-12-7	845
Hexan-1-ol[b]	5.637	111-27-3	860
6-Methyl-3-heptanol[b]	8.352	589-98-0	991
(2E)-2-Nonen-1-ol[a]	9.695	31502-14-4	
3-Nonanol[a]	9.909	624-51-1	
Nonan-2-ol[b]	10.939	628-99-9	1100
Undecan-1-ol[a]	11.005	112-42-5	
Nonan-1-ol[b]	12.432	112-42-5	1169
2-Methyl-1-undecanol[a]	15.881	10522-26-6	
2-Butyl-1-octanol[a]	16.220	3913-02-8	
(9E)-9-Hexadecen-1-ol[a]	27.377	64437-47-4	
Aliphatic aldehydes			
Pentanal[b]	2.645	110-62-3	693
Hexanal[c]	4.234	66-25-1	776
(2E)-2-Hexenal[b]	5.253	6728-26-3	850
Heptanal[c]	6.256	111-71-7	901

(2Z)-2-Heptenal[b]	7.445	57266-86-1	952
(2E)-2-Nonenal[a]	9.912	18829-56-6	
Nonanal[c]	10.976	124-19-6	1103
Decanal[c]	13.166	112-31-2	1205
Tetradecanal[b]	20.409	124-25-4	1574
(9Z)-9-Octadecenal[b]	27.830	2423-10-1	
(9Z)-9-Hexadecenal[b]	30.430	56219-04-6	
Aliphatic alkanes			
2-Methylpentane[b]	1.882	107-83-5	560
3-Methylpentane[b]	1.962	96-14-0	570
Hexane[c]	1.927	110-54-3	600
Methylcyclopentane[b]	2.265	96-37-7	635
2,4-Dimethylheptane[b]	4.649	2213-23-2	820
4-Methyloctane[b]	5.475	2216-34-4	862.85
Nonane[c]	6.411	111-84-2	900
Decane[c]	8.658	124-18-5	1000
4-Methyldecane[b]	9.004	2847-72-5	1059
6-Methyloctadecane[a]	11.506	10544-96-4	
3-Methyldecane[a]	12.340	13151-34-3	
2-Methylundecane[b]	12.194	7045-71-8	1163
2,3-Dimethyldecane[a]	12.497	17312-44-6	
Undecane[c]	13.099	629-59-4	1100
Dodecane[c]	13.222	112-40-3	1200
2,6-Dimethylundecane[b]	13.376	17301-23-4	1213
Pentadecane[a]	14.120	629-62-9	
2,3-Dimethylundecane[a]	14.432	17312-77-5	
2-Methyldecane[a]	14.347	6975-98-0	
Nonadecane[a]	14.517	629-92-5	
Tridecane[c]	15.217	629-50-5	1300
2,6,10-Trimethyldodecane[a]	18.233	3891-98-3	
Pentadecane[c]	18.935	629-62-9	
Hexadecane[c]	20.636	544-76-3	
Heptadecane[c]	22.288	629-78-7	
Octadecane[c]	23.858	593-45-3	
Isopropyl Myristate[b]	23.649	110-27-0	1827
Isopropyl Palmitate[b]	27.008	142-91-6	1981
Tetracosane[b]	31.307	646-31-1	2400
Pentacosane[b]	32.684	630-02-4	2500
Hexacosane[b]	34.319	630-01-3	2600
Aliphatic alkenes			
(E)-Oct-2-ene[b]	4.319	111-67-1	810
(Z)-Oct-2-ene[b]	4.379	7642-04-8	815
X-Octene	4.485	—	—
X-Octene	4.601	—	—
(3E)-1,3-Octadiene[b]	4.784	1002-33-1	827

Non-1-ene[a]	6.215	124-11-8	
(3E)-3-Nonene[a]	6.228	20063-77-8	
(4E)-2,4-Dimethyl-2,4-heptadiene[a]	5.850	74421-05-9	
(3E)-3-Decene [a]	7.773	19150-21-1	
Undec-1-ene[a]	10.734	821-95-4	
(3Z)-3-Dodecene[a]	13.055	7239-23-8	
Tridecene-1[b]	15.060	2437-56-1	1291
Aliphatic esters			
Acetic acid, butyl ester[b]	4.515	123-86-4	813
Butyl n-hexanoate[b]	12.825	626-82-4	1186
Dodecanyl acetate[b]	20.290	122-66-3	1606
Aliphatic ketones			
Pentan-2-one[b]	2.432	107-87-9	690
Acetoin[c]	3.441	513-86-0	
Hexan-2-one[b]	4.029	591-78-6	790
5-Methylhexan-2-one[a]	5.991	110-12-3	
1-Octen-3-one[b]	8.112	4312-99-6	980
beta-Nonanone[b]	10.644	821-55-6	1090
Undecan-2-one[b]	14.953	112-12-9	1292
Dodecan-2-one[a]	14.608	6175-49-1	
Benzenoids			
Toluene[c]	3.726	108-88-3	762
alpha-Methyltoluene[b]	5.561	100-41-4	855
1,4-Dimethyl benzene[c]	5.696	106-42-3	862
Styrene[c]	6.224	100-42-5	895
4-Ethylbenzoic acid, cyclopentyl ester[a]	6.522		
1-Ethyl-3-methylbenzene[b]	7.622	98-82-8	958
Benzaldehyde[c]	7.769	100-52-7	947
Carbamic acid, methyl-, phenyl ester[a]	7.975	1943-79-9	
Trimethyl benzene[b]	8.110	95-63-6	990
Acetophenone[c]	10.137	98-86-2	1062
p-Cresol[c]	10.717	106-44-5	1077
m-Cresol[c]	10.525	108-39-4	
1-Isopropenyl-2-methylbenzene[a]	10.492	7399-49-7	
Phenylethyl Alcohol[c]	11.180	60-12-8	1121
Benzenepropanol[b]	13.401	122-97-4	1237
1-Isopropyl-2-methoxy-4-methylbenzene[a]	13.721	1076-56-8	
3-Propylphenol[a]	14.195	621-27-2	
Benzaldehyde, 3-hydroxy-4-methoxy[a]	16.978	621-59-0	
Diethyltoluamide[a]	20.208	134-62-3	
Miscellaneous compounds			
5-Isopropenyl-2-methylcyclohexanol[a]	7.457	18675-33-7	
(3E)-2,6-Dimethyl-1,3,7-octatriene[a]	7.898	6876-07-9	
1-Propynylcyclohexane[a]	7.930	18736-95-3	
(2E)-3,7-Dimethyl-2-octene[a]	7.951		

5,7-Dimethyl-1,6-octadiene[a]	8.152	85006-04-8	
1-Methyl-3-{2-methyl-2-propenyl)cyclopentane[a]	8.113	75873-00-6	
6-Methyl-5-heptene-2-one[b]	8.232	110-93-0	984
(6E)-2,6-Dimethyl-2,6-octadiene[b]	8.332	2609-23-6	1004
1,2-Dimethyl-1-cyclooctene[a]	8.335	54299-96-6	
1-Isopropyl-2-methyl-3-(1-methylethylidene)cyclopropane[a]	8.451	24524-52-5	
(6Z)-2,6-Dimethyl-2,6-octadiene[a]	8.636	2492-22-0	
(3E)-3-Ethyl-2-methyl-1,3-heptadiene[a]	8.562	61142-35-6	
Pentylidenecyclopentane[a]	8.869	53366-55-5	
(3E)-3-Ethyl-2,5-dimethyl-1,3-hexadiene [a]	8.922	62338-07-2	
2-Ethylhexan-1-ol[a]	9.060	104-76-7	
m-Menth-3(8)-ene[a]	9.218	13828-34-7	
m-Menth-1(7)-ene[a]	9.223	13837-71-3	
(3-Methylbutylidene)cyclopentane[a]	9.253	53366-51-1	
(5E)-4-Methyl-1,5-heptadiene[a]	9.813	998-94-7	
(6E)-2,6-Dimethyl-2,6-octadiene[b]	9.780	2792-39-4	990
3-Ethyl-1,5-octadiene[a]	9.872		
1-Isopropenyl-2-methylcyclohexane[a]	10.003	15193-25-6	
(2E,6E)-4-Methyl-2,6-octadiene[a]	10.078	74498-94-5	
4,8-Dimethyl-1,7-nonadiene[a]	10.099	62108-28-5	
2,5-Dimethyloctahydropentalene[a]	9.973	28588-55-8	
6,6-Dimethylbicyclo[3.1.1]hept-2-ene-2-carbaldehyde[b]	11.088	564-94-3	1151
2-Ethylcyclohexanone[a]	11.410	4423-94-3	
1,2-Dimethyl-1,3-cyclopentadiene[a]	11.691	4784-86-5	
4-Trifluoroacetoxytridecane[a]	12.404		
p-Menthan-3-one[a]	12.415	1196-31-2	
1-Methyl-4-(1-hydroxy-1-methylethyl)benzene[b]	12.828	1197-01-9	1186
alpha-lonene[a]	13.468	475-03-6	
Hexyl 2-methylbutanoate[b]	13.792	10032-15-2	1234
Guanidineacetic acid[a]	13.845	352-97-6	
6,7-Dodecanedione[a]	13.845	13757-90-9	
Quinoline[a]	13.984	91-22-5	
p-Mentha-6,8-dien-2-one[a]	14.050	2244-16-8	
p-Menth-1-en-3-one[a]	14.278	89-81-6	
(3E)-4-(2-Hydroxy-2,6,6-trimethylcyclohexyl)-3-buten-2-one[a]	15.679	55955-46-9	
Bicyclo[10.1.0]tridec-1-ene[a]	17.092	54766-91-5	
(5E)-2,3,5,8-Tetramethyl-1,5,9-decatriene[a]	17.128	230646-72-7	
1,5,9-Undecatriene, 2,6,10-trimethyl-[a]	17.161	62951-96-6	
6,11-Dimethyl-2,6,10-dodecatrien-1-ol[a]	17.642		
(5E)-6,10-Dimethyl-5,9-undecadien-2-one[a]	17.959	3796-70-1	1445
2-Hexyl-1-decanol[a]	18.126	2425-77-6	
(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrienal[a]	19.137	66408-55-7	
(6E)-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol [a]	19.597	7212-44-4	1561

2,3,6-Trimethylnaphthalene[a]	19.943	829-26-5	1548
1-[2-(Isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate [a]	20.472	74381-40-1	
3,7,11-Trimethyl-1-dodecanol[a]	23.169	6750-34-1	
6,10,14-Trimethyl-2-pentadecanone[a]	24.441	502-69-2	1848
(2E)-3,7,11,15-Tetramethyl-2-hexadecene[b]	24.487	14237-73-1	
1,2-Benzenedicarboxylic acid, dibutyl ester[b]	25.569	84-74-2	1897
1-(Octyloxy)octane[a]	26.179	629-82-3	
(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol[b]	27.708	150-86-7	2112
2-Ethylhexyl (2E)-3-(4-methoxyphenyl)-2-propenoate[a]	28.855	5466-77-3	
(9Z)-9-Octadecenyl (9Z)-9-hexadecenoate [a]	32.353	22393-98-2	
Monoterpenes			
1,7,7-Trimethyltricyclo[2.2.1.0,2,6]heptane[a]	6.983	508-32-7	924
2,7-Dimethyl-1,7-octadiene[a]	7.069	59840-10-7	
alpha-Pinene[c]	7.264	80-56-8	932
3,7-Dimethyl-1,6-octadiene[a]	7.395	10281-55-7	
3,7-Dimethyl-1,6-octadiene[a]	7.541	2436-90-0	
Camphene[b]	7.615	79-92-5	946
beta-Pinene[c]	8.275	127-91-3	980
alpha-Terpinolene[a]	8.722	586-62-9	
2-Carene[a]	8.764	554-61-0	
alpha-Phellandrene[b]	8.898	99-83-2	1007
alpha-Terpine[c]	9.110	99-86-5	
Cymene[a]	9.257	527-84-4	
Limonene[c]	9.405	1461-27-4	1039
3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene[a]	9.939	13466-78-9	
gamma-Terpinen[a]	10.031	99-85-4	
Geranial[a]	10.371	141-27-5	
Linalool[c]	10.637	78-70-6	1096
p-Mentha-1,4(8)-diene[a]	10.668	586-62-9	
1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol[b]	12.591	507-70-0	1174
Nitrogen compounds			
Indole[c]	14.670	120-72-9	1295
Benzyl nitrile[a]	14.822	140-29-4	
3-Methyl-1H-indole[c]	16.846	83-34-1	
Sesquiterpenes			
Allo-Aromadendrene[a]	16.312	25246-27-9	
alpha-Longipinene[b]	16.418	5989-08-2	1353
2,3-Dimethyldodecane[a]	16.441	6117-98-2	
Unknown sesquiterpene[a]	16.555	—	—
Unknown sesquiterpene[a]	16.418	—	—
Unknown sesquiterpene[a]	16.578	—	—
Farnesane[a]	16.666	3891-98-3	
(+)-Cyclosativene[a]	16.733	22469-52-9	
Unknown sesquiterpene[a]	16.766	—	—

gamma-Muurolene[a]	16.813	30021-74-0	
alpha-Copaene[a]	16.817	3856-25-5	1392
alpha-Bourbonene[a]	16.997		
beta-Elemene[a]	17.074	515-13-9	
Unknown sesquiterpene[a]	17.097	—	—
Unknown sesquiterpene[a]	17.123	—	—
Unknown sesquiterpene[a]	17.129	—	—
Unknown sesquiterpene[a]	17.169	—	—
4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene[a]	17.431	118-65-0	1413
Unknown sesquiterpene[a]	17.139	—	—
alpha-Santalene[a]	17.220	512-61-8	1420
Unknown sesquiterpene[a]	17.259		
beta-Caryophyllene[c]	17.715	87-44-5	1440
Unknown sesquiterpene[a]	17.474	—	—
Germacrene D[a]	17.841	23986-74-5	1464
2,6-Dimethyl-6-(4-methyl-3-pentenyl)bicyclo[3.1.1]hept-2-ene[a]	17.459	17699-05-7	1436
Unknown sesquiterpene[a]	17.673	—	—
beta-Gurjunene[a]	17.914	17334-55-3	1427.7
Unknown sesquiterpene[a]	18.065	—	—
Unknown sesquiterpene[a]	17.611	—	—
alpha-Caryophyllene[a]	18.357	6753-98-6	1459
Unknown sesquiterpene[a]	18.427	—	—
Aromadendrene[a]	18.458	109119-91-7	1460
alpha-Muurolene[b]	18.619	10208-80-7	
alpha-Amorphene[a]	18.650	483-75-0	1468
Unknown sesquiterpene[a]	18.716	—	—
beta-Farnesene[a]	18.788	18794-84-8	1458
Unknown sesquiterpene[a]	18.790	—	—
Unknown sesquiterpene[a]	18.855	—	—
Unknown sesquiterpene[a]	18.923	—	—
1-Methyl-4-(5-methyl-1-methylene-4-hexenyl)-1-cyclohexene[a]	18.748	495-61-4	1501.2
Unknown sesquiterpene[a]	18.961	—	—
Unknown sesquiterpene[a]	18.994	—	—
Unknown sesquiterpene[a]	19.058	—	—
Calamenene[a]	19.059	483-77-2	1516
Unknown sesquiterpene[a]	19.252	—	—
Unknown sesquiterpene[a]	19.331	—	—
delta-Cadinene[a]	19.400	483-76-1	
alpha-Panasinsen[a]	19.495	56633-28-4	
Unknown sesquiterpene[a]	19.673	—	—
Unknown sesquiterpene[a]	19.796	—	—
alpha-Calacorene[a]	19.814	21391-99-1	
Unknown sesquiterpene[a]	19.800	—	—

Unknown sesquiterpene[a]	19.926	–	–
Unknown sesquiterpene[a]	19.797	–	–
Unknown sesquiterpene[a]	20.209	–	–
Unknown sesquiterpene[a]	20.341	–	–
Unknown sesquiterpene[a]	20.137	–	–
Unknown sesquiterpene[a]	20.698	–	–
Unknown sesquiterpene[a]	20.831	–	–
Unknown sesquiterpene[a]	21.133	–	–
Unknown sesquiterpene[a]	21.286	–	–
Unknown sesquiterpene[a]	21.483	–	–
Unknown sesquiterpene[a]	21.598	–	–
Unknown sesquiterpene[a]	21.662	–	–
Unknown sesquiterpene[a]	21.781	–	–
Unknown sesquiterpene[a]	21.952	–	–
Unknown sesquiterpene[a]	22.047	–	–
Unknown sesquiterpene[a]	22.274	–	–
Unknown sesquiterpene[a]	21.897	–	–
Unknown sesquiterpene[a]	22.043	–	–
Unknown sesquiterpene[a]	22.163	–	–
Unknown sesquiterpene[a]	22.284	–	–
Unknown sesquiterpene[a]	22.720	–	–
Unknown sesquiterpene[a]	22.763	–	–
Unknown sesquiterpene[a]	23.164	–	–
Unknown sesquiterpene[a]	23.250	–	–
Unknown sesquiterpene[a]	23.672	–	–
Unknown sesquiterpene[a]	23.830	–	–
Unknown sesquiterpene[a]	23.936	–	–
Unknown sesquiterpene[a]	24.503	–	–
Unknown sesquiterpene[a]	24.457	–	–
Unknown sesquiterpene[a]	24.931	–	–
Unknown sesquiterpene[a]	26.431	–	–
Unknown sesquiterpene[a]	27.863	–	–
Unknown sesquiterpene[a]	29.110	–	–
Unknown sesquiterpene[a]	29.951	–	–
Sulphur compounds			
(Methyldisulfanyl)methane[a]	3.322	624-92-0	745
Isopropyl isothiocyanate[a]		2253-73-8	
3-(Allylsulfanyl)-1-propene[a]	5.441	592-88-1	860.3
1-Isothiocyanato-2-methylpropane[a]	7.353	591-82-2	
Thiacyclopentan-2-one[a]	8.406	1003-10-7	
3-(Allyldisulfanyl)-1-propene[a]	10.250	2179-57-9	1085
Unidentified compounds			
Unidentified	2.460	–	–
Unidentified	5.495	–	–
Unidentified	5.971	–	–

Unidentified	6.090	–	–
Unidentified	6.638	–	–
Unidentified	7.076	–	–
Unidentified	6.351	–	–
Unidentified Terpenoid[a]	7.922	–	–
Unidentified Terpenoid[a]	8.129	–	–
Unidentified Terpenoid[a]	8.245	–	–
Unidentified Terpenoid[a]	8.366	–	–
Unidentified Terpenoid[a]	8.480	–	–
Unidentified Terpenoid[a]	8.497	–	–
Unidentified Terpenoid[a]	8.530	–	–
Unidentified Terpenoid[a]	8.652	–	–
Unidentified Terpenoid[a]	8.766	–	–
Unidentified Terpenoid[a]	8.873	–	–
Unidentified Terpenoid[a]	9.021	–	–
Unidentified	9.795	–	–
Unidentified	10.440	–	–
Unidentified	10.670	–	–
Unidentified	10.881	–	–
Unidentified	10.961	–	–
Unidentified	10.832	–	–
Unidentified	11.197	–	–
Unidentified	11.252	–	–
Unidentified	12.291	–	–
Unidentified	12.492	–	–
Unidentified	12.563	–	–
Unidentified	12.696	–	–
Unidentified	13.045	–	–
Unidentified	13.233	–	–
Unidentified	13.381	–	–
Unidentified	13.721	–	–
Unidentified	14.175	–	–
Unidentified	14.353	–	–
Unidentified	14.357	–	–
Unidentified	14.337	–	–
Unidentified	15.019	–	–
Unidentified	15.721	–	–
Unidentified	15.868	–	–
Unidentified	16.224	–	–