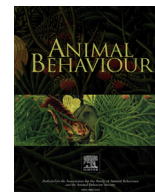




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Volatile odours reflect breeding status but not social group membership in captive Damaraland mole-rats

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In mammals, olfaction plays a key role in social behaviour, for example, in identifying mating opportunities and potential rivals. However, we still have a limited understanding of how social information is encoded in animal odours, including the social determinants of chemical similarity and diversity. Here, we used gas chromatography to analyse the chemical composition of swabs taken from the facial and anogenital regions of Damaraland mole-rats, *Fukomys damarensis*, a highly social subterranean mammal that relies almost exclusively on olfactory and tactile social cues. We found no sign of individual identity across the two body areas sampled; samples from the facial region and samples of the anogenital region from the same individual were not similar to each other, suggesting that these regions carry different information. However, chemical profiles varied significantly by sex and breeding status; female breeders differed from nonbreeders in their anogenital profiles and had higher chemical diversity in their facial profiles compared with both males and nonbreeders. Interestingly, we found no signals of social group identity. Instead, individual identity may be conveyed through signature mixes that are learned through frequent contact, rather than through specific odours associated with genetic kinship or social group membership. Our results highlight the complexity of chemical communication systems in social species and suggest that signals of group level identity are not necessary for behavioural responses based on group membership.

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Chemicals are evolutionarily the oldest and most widespread mechanism of information transfer; hence they are used as a means of communication across the animal kingdom (Müller et al., 2020; Wyatt, 2014a). Chemicals that transfer information either within an individual (i.e. hormones) or among individuals are referred to as infochemicals (Müller et al., 2020). There are two broad types of infochemicals that animals use to communicate. First, pheromones (either a single chemical or a specific combination of chemicals) are evolved chemical signals between members of the same species

that trigger a specific reaction in the responder, and are often used to transfer information about the status of an individual (Wyatt, 2014b). For example, dominant male house mice, *Mus musculus*, scent-mark their territories with urine containing darcin, which stimulates the females to mate (Roberts et al., 2010). Second, signature mixes (a variable mixture of chemicals) can be used for individual recognition or to identify members of a particular social group (Wyatt, 2014b). For example, social insects use a combination of gland secretions and cuticular hydrocarbons present on the exoskeleton to identify their groupmates and exclude foreign intruders (van Zweden & d'Ettorre, 2010). Pheromones by necessity vary little in their composition between individuals because they must reliably trigger a consistent response, whereas signature

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mixes must have sufficient variability between individuals or social groups, and consistency within individuals or groups, to allow for identification and differentiation.

Despite substantial progress having been made in identifying the kinds of information that odours can convey, we still know surprisingly little about the chemical world that animals live in. This is especially the case for vertebrates, where the identification of chemical compounds involved in olfactory communication lags far behind that of invertebrates. For example, among mammals, detailed studies of olfactory communication are heavily biased towards laboratory model systems such as mice, rats and rabbits, with relatively few studies conducted on wild species (Wyatt, 2014a). Nevertheless, behavioural experiments have demonstrated that many mammals can identify and respond to odour cues reflecting mating opportunities, such as species (Caspers et al., 2009), sex (Mitchell et al., 2018; Swaisgood et al., 2000), group identity (Schneeberger et al., 2021), whether females are in oestrus (Gildersleeve et al., 2012; Johnston, 1979; Müller & Manser, 2008), genetic relatedness (Charpentier et al., 2008; Leclaire et al., 2013) and pregnancy status (Mitchell et al., 2017).

Some studies have also used approaches such as gas chromatography - mass spectrometry (GC-MS) to characterize individual 'chemical profiles'. This has allowed the identification of potential pheromones that may communicate sex and reproductive status (Crawford & Drea, 2015; Jemiole et al., 1987; Kean et al., 2011; Theis et al., 2013) as well as chemicals that covary with genetic relatedness (Green et al., 2015; Stoffel et al., 2015). Little is known about the determinants of chemical diversity in odours, although Stoffel et al. (2015) found that the chemical diversity of odours increases with genetic diversity in Antarctic fur seals, *Arctocephalus gazella*. It is also possible that mammals living in larger social groups may have greater diversity in their odours due to the transfer of chemicals during frequent close contact between group members, as appears to occur with cuticular hydrocarbons in ants (Soroker et al., 2003) and has been suggested to occur in naked mole-rats, *Heterocephalus glaber* (O'Riain & Jarvis, 1997).

Furthermore, many mammalian species use multiple sources of odours to convey information (Johnston, 2003). For example, in golden hamsters, *Mesocricetus auratus*, odour sources include urine, faeces, vaginal secretions, flank glands, ear glands, Harderian glands, saliva and glands on the feet (Johnston, 2003). In this species, habituation - dishabituation experiments have shown that different body areas encode different information, with reproductive status being signalled by vaginal and flank odours, while individual identity is encoded in odours emanating from these areas plus the ears, urine and faeces (Johnston et al., 1993). However, it is unclear to what extent this pattern is representative of other species. For example, in house mice, reproductive status is communicated via urine (Achiraman et al., 2010).

African mole-rats (family Bathyergidae) provide an excellent opportunity to advance our understanding of mammalian olfactory communication. These animals spend their entire lives in underground burrows, so olfactory and tactile cues are the primary ways in which they sense their environment. Accordingly, they have been shown to possess a particularly diverse array of olfactory receptor genes (Stathopoulos et al., 2014). Behavioural studies have also shown that African mole-rats engage in conspicuous odour inspections in social situations. For example, reciprocal cheek nuzzling has been observed in many contexts, including when unfamiliar individuals meet, when familiar group members are reunited after short periods of separation and also prior to copulation (Bennett, 1989; Caspar et al., 2022). In line with these behavioural observations, Caspar et al. (2022) found large, specialized sebaceous glands in the corners of the mouth in both sexes of several mole-rat species; *Fukomys mechowii*, *Fukomys*

anselli and *H. glaber*; suggesting that they may be present across the African mole-rat clade. Following experimental and chemical analysis of facial gland secretions in Micklem's mole-rats, *Fukomys micklemi*, Caspar et al. (2022) speculated that these odours may be involved in the communication of sex-related information.

A second odour source that is likely to be important in mole-rats is the anogenital area. Behavioural studies have shown that anogenital inspection often occurs after cheek nuzzling (Bennett, 1989; Caspar et al., 2022), and anogenital swabs elicit a similar degree of interest to facial swabs in behavioural experiments (Caspar et al., 2022). Hagemeyer (2010) found that *Fukomys* mole-rats; (*F. anselli*, *Fukomys kafuensis* and their hybrids; prefer anogenital odours from breeding females compared to nonbreeders, suggesting that these odours convey information about reproductive status. These species also respond differently to the anogenital odours of unfamiliar individuals depending on the degree of genetic relatedness (Hagemeyer, 2010; Heth et al., 2004), suggesting that some species may be able to identify unfamiliar relatives via the phenotype matching of odours. However, other mole-rat species do not seem to use phenotype matching and instead appear to learn the odour of familiar individuals. For example, giant mole-rats, *F. mechowii*, are able to differentiate among familiar group members based on olfactory cues from anogenital odours (Heth et al., 2002). Frequent contact appears to be required to maintain familiarity, and periods of separation as short as 3 weeks can lead to individuals no longer recognizing one another in some species (Burda, 1995). In the highly social naked mole-rat, social group identity appears to be conferred by odour-based signature mixes, with individuals preferring the odour of their own group and responding aggressively to the odour of intruders (O'Riain & Jarvis, 1997). Similarly, Damaraland mole-rats, *Fukomys damarensis*, can use soil-borne odours to distinguish between unfamiliar social groups and single conspecifics, and they can also identify the sex of the latter (Leedale et al., 2021). However, it is not known which odour sources are used to convey information about group membership or size, as substrate odours were used in these studies (Leedale et al., 2021; O'Riain & Jarvis, 1997).

Here, we investigated the chemical composition of facial and anogenital odours across 11 social groups of captive Damaraland mole-rats. We hypothesized that in this species, chemical communication is important in decision making related to breeding, cooperation and dispersal. In the wild, this species lives in groups of between two and 41 individuals (Jarvis & Bennett, 1993) which usually comprise a dominant breeding pair and their subordinate offspring (Bennett & Jarvis, 1988; Burland et al., 2002, 2004). This species breeds cooperatively, with subordinates contributing to burrowing, group defence and pup care (Bennett, 1990; Bennett & Faulkes, 2000; Zöttl et al., 2016). Subordinates show delayed dispersal, remaining on their parents' territory beyond sexual maturity but refraining from breeding in the absence of unrelated breeding partners (Kelley et al., 2019). Hence, kin recognition occurs and is important in maintaining high reproductive skew, but not exclusively as physiological suppression also appears to operate (Bennett et al., 1996; Cooney & Bennett, 2000).

In Damaraland mole-rats, both sexes disperse from their natal social group (usually during the rainy season when constraints on establishing a new burrow system are likely to be lower, Young et al., 2010), but dispersal is male biased, with males dispersing slightly earlier and more frequently than females (Finn, 2017; Torrents-Ticó et al., 2018). Research from the southernmost distributional range of the species found that dispersing females usually establish their own burrow system and live alone (sometimes for several years) until joined by a male (Thorley et al., 2023; Torrents-Ticó et al., 2018). In contrast, dispersing males rarely settle on their own and instead either settle with single females or disperse into

established groups (Burland et al., 2004; Thorley et al., 2023; Torrents-Ticó et al., 2018; Young et al., 2015). However, established groups are xenophobic and intruders of both sexes are usually responded to aggressively, particularly by the same-sex dominant and when groups are actively breeding (Cooney, 2002; Jacobs et al., 1998; Jacobs & Kuiper, 2000). Aggression towards intruders can be severe, even resulting in death (Jacobs et al., 1998), so detecting the composition and reproductive status of existing groups is likely to be beneficial to dispersers (Leedale et al., 2021). Similarly, for group members, detecting intruders and evaluating their threat in relation to the breeding status of the group also appears to be important (Jacobs et al., 1998).

We made two key predictions. (1) There will be chemical differences in odour profiles between the sexes and between breeders and nonbreeders. These differences may be more likely to occur in females, as they undergo different stages of reproduction (such as pregnancy and lactation) which have distinct consequences for male–female interactions. Chemical differences associated with reproduction may also be more likely to occur in anogenital samples than facial samples as the anogenital region is inspected immediately prior to mating. (2) Chemical signals of group membership may occur and will most likely be found in the facial region, which is inspected first when individuals meet. Group level odours could reflect high levels of genetic relatedness within groups, or alternatively might arise through frequent physical contact between group members or their olfactory secretions. If physical contact generates group level odours, we expected that larger groups would be more chemically diverse due to the mixing of chemicals from all group members, for example in toilet areas, as suggested by O’Riain and Jarvis (1997).

METHODS

Study Population

Damaraland mole-rats (Fig. 1) were housed at the University of Pretoria animal facility. Each group consisted of one male and one female breeder, plus their offspring. The identity of breeding females was confirmed through their regular pregnancies as well as their prominent axillary teats. As Damaraland mole-rats avoid inbreeding with familiar relatives, we assumed that only the dominant male bred. In three of the 11 groups that we studied, the breeding status of the adults was unclear; one colony consisted of a

single male that had bred previously, and two colonies consisted of an opposite-sex pair, but there had been no signs of breeding for at least 6 months. We therefore excluded these animals from analyses involving breeding status. Diet and bedding were the same for all groups, providing a homogeneous background odour environment.

Sample Collection, Storage and Transport

In June 2018, samples were taken from 40 individuals (23 males and 17 females, of which seven males and eight females were identified as breeders) from 11 social groups. Prior to sampling, each mole-rat was transferred from its home group into a plastic box. Two types of odour samples were collected: anogenital and facial swabs. For the collection of facial swabs, a small piece of cotton wool held with forceps was gently rubbed against the perioral area and cheeks. Their anogenital area was then gently rubbed with a fresh piece of cotton wool, which was also held with forceps. Each piece of cotton wool was then placed inside an individually labelled glass chromatography vial and stored at -80°C . Cotton wool and chromatography vials were precleaned with methanol followed by pentane and were allowed to dry before sampling. Nitrile gloves were worn during sampling. These did not come into contact with the cotton wool and were changed between each individual. Forceps were cleaned before each sample was taken and the plastic boxes were cleaned between successive individuals using 70% ethanol. Care was taken to avoid the cotton wool from coming into contact with any surfaces other than the specified area of the mole-rat and the chromatography vial. Three control samples were also taken, whereby the cotton wool did not come into contact with a mole-rat; otherwise all sampling procedures were the same.

Sample Preparation and Chemical Analyses

The samples were transported on dry ice to the University of Bielefeld for chemical analyses. After defrosting, 100 μl dichloromethane was added to each sample vial, and the sample was vortexed to encourage the release of the volatile chemicals from the cotton wool. The dichloromethane was then squeezed out of the cotton wool with a blunt glass syringe, transferred to a clean insert in a labelled chromatography vial and evaporated at ambient temperature to a maximum of 5 μl per sample, of which 1 μl was injected into the gas chromatograph with a flame ionization detector (GC-FID, GC 2010 plus, Shimadzu, Duisburg, Germany) equipped with a VF-5 ms capillary column (30 $\times$ 0.25 mm ID, DF 0.25, 10 m guard column, Varian, Lake Forest, CA, U.S.A.). Hydrogen was used as the carrier gas with a flow rate of 1 ml/min. The GC settings were as follows: inlet temperature: 280; starting temperature 50 $^{\circ}\text{C}$ for 5 min, followed by an increase of 20 $^{\circ}\text{C}/\text{min}$ until a final temperature of 320 $^{\circ}\text{C}$ was reached, which was held for 14 min. All equipment was precleaned with dichloromethane before coming into contact with the sample. Eleven ‘blank’ dichloromethane samples were also included in the analysis to identify chemicals resulting from potential contaminants of the dichloromethane. Peaks from GC traces were scored automatically, with manual checking of all traces and adjustment performed where appropriate. The retention time and area of each peak were recorded.

Statistical Analyses

For the alignment of the peaks, we used the same method as described in Gilles et al. (2024). Briefly, we extracted the peak areas and retention times of each chromatogram using GC Solutions v2.41. Chromatograms were aligned using the GCalinR package (Ottenmann et al., 2018) in R v3.6.1 (R Core Team, 2012). As a data



Figure 1. A Damaraland mole-rat, *Fukomys damarensis*. This species is subterranean, using their teeth for burrowing. They have poor visual capabilities and rely on olfactory and tactile senses. Photo credit: Hazel Nichols.

cleaning step, we removed all peaks we found in blank dichloromethane samples, those that were found in only one sample and those that contributed less than 1% to the chemical profile. We used GC-FID for chemical analysis as this technique is more sensitive than GC-MS in detecting organic compounds. However, GC-FID does not allow the identification of individual chemicals. This means that multiple chemicals can potentially be represented by a single peak, and peaks that were present in control samples may therefore also include chemicals genuinely present in our samples. We therefore retained peaks that were found in both samples and controls in our main analysis. To test whether this methodological decision affected our results, we conducted a supplementary analysis with peaks found in >1 control sample removed (37 peaks). Overall, we found very few differences in our results (see Supplementary Material for details).

Chemical similarity

We calculated the relative contribution (%) of each substance to the total peak area of all substances following [Caspers et al. \(2009\)](#) and [Stoffel et al. \(2015\)](#). This was done to account for the potential differences in the amount of secretion collected. Using these data, we compared the similarity of the samples from the anogenital region and the facial region and also from the two sexes and breeders and nonbreeders. We transformed the aligned data ($\log x + 1$) and calculated a similarity matrix based on Bray Curtis ([Clarke et al., 2006](#)). We then analysed potential differences between a priori defined groups using a multivariate analysis of similarities (ANOSIM) and a permutational MANOVA (PERMANOVA) using Primer 7 (Version 7.0.23; primer-e, <https://www.primer-e.com/software>).

Chemical diversity

Analyses of chemical diversity were carried out in R version 4.0.1 ([R Core Team, 2012](#)) using generalized linear mixed-effect models (GLMMs) in the lmer package ([Bates et al., 2015](#)). To investigate whether chemical diversity (the number of peaks present in each sample) differed by sex and breeding status, we fitted chemical diversity as the response variable, with sex and breeding status, and the interaction between them, fitted as explanatory variables. To investigate whether individuals in larger colonies had more diverse chemical profiles, we fitted chemical diversity as the response variable, with colony size as the explanatory variable. Separate models were fitted for facial and anogenital samples as we predicted contrasting relationships for these different sampling areas. Models investigating chemical diversity were fitted with Poisson distributions, and we included observation level random effects to account for overdispersion (theta parameters varied between 10.696 and 11.96, [Harrison, 2014](#)). We initially fitted social group as a random effect to account for repeat sampling from multiple individuals in the same group. However, this resulted in a singular fit and social group explained negligible variation, likely due to there being a single breeding male and female per group. We therefore elected not to include social group as a random effect.

Ethical Note

All procedures conformed to the [ASAB Ethical Committee/ABS Animal Care Committee \(2023\)](#) guidelines for ethical treatment of animals. Ethical approval for all elements of this study was granted by the animal ethics committees of the University of Pretoria (EC032-18) and Swansea University (280819/168). The most likely potential stressor to the mole-rats came from the handling procedure. To minimize stress, the animals were moved by scooping them in plastic containers, rather than lifting them by the tail, as this has been shown to reduce handling stress in rodents ([Hurst &](#)

[West, 2010](#)). For the collection of anogenital swabs, mole-rats were restrained by the tail and their rear end was lifted off the ground, leaving their forepaws on the ground to minimize stress. We minimized sampling time, taking approximately 3–5 min per individual, after which the animal was transferred back to its home group. Animals did not show signs of stress (for example excessive urination or defecation) during handling and there were no signs of aggression when the mole-rats were returned to their home colony after sampling. All animals were retained in the laboratory after sampling for use in future studies.

Animals were housed in rectangular plastic enclosures (minimum size 50 × 80 cm). Enclosure size was modified according to group size, with larger social groups being housed in larger enclosures. Social groups were maintained in controlled temperature rooms held at 25 °C and were on a 12:12 h light:dark photoperiod, with low-intensity lighting. All social groups were housed in the same room, but groups were not allowed visual or physical contact. All animals were provided with fresh wood shavings and soft paper towelling as bedding, and enclosures included plastic tubing and/or nestboxes for enrichment. Mole-rats were fed ad libitum on sweet potato, supplemented with other fruit and vegetables such as apple, carrot and cucumber. Mole-rats do not drink free water, obtaining all water requirements from the diet ([Bennett & Jarvis, 1995](#)).

RESULTS

In total across all 80 samples, we found 333 different peaks in our chemical profiles, with individual chemical profiles containing a mean of 52 peaks (range 8–150; see [Fig. S1](#) in the Supplementary Material for example chromatograms). We found no evidence that samples from the anogenital and facial regions of the same individual were more similar to one another than by chance (ANOSIM factors: individual; Global $R = 0.045$; $P = 0.306$), implying that the two different regions do not have similar chemical compositions within each individual. Although levels of chemical diversity were similar for the facial (mean peaks 47.7 ± 4.0 SE) and anogenital swabs (mean peaks 55.7 ± 4.4 SE; GLMM; estimate = -0.189 , SE = 0.121 , $z = -1.56$, $P = 0.119$), the chemical diversity of facial and anogenital regions within each individual was uncorrelated (GLMM; estimate = -0.0002 , SE = 0.003 , $z = -0.077$, $P = 0.939$), suggesting that individuals with more diverse facial profiles were not more diverse in the anogenital region and vice versa. We therefore subsequently analysed samples from the anogenital and facial regions separately.

Chemical Differences Associated with Reproduction

Our analysis of similarity found chemical differentiation between the sexes that depended on breeding status ([Table 1](#), [Figs. 2](#), [S2](#)). Female breeders and nonbreeders differed significantly from

Table 1

Results of permutational MANOVAs investigating chemical similarity based on sex and breeding status

Sample area	Variable	df	SS	Pseudo-F	P
Anogenital	Sex	1	1001.2	0.540	0.85
	Breeding status	1	3441.4	1.856	0.08
	Sex: Breeding status	1	5055.5	2.726	0.02
Facial	Sex	1	2504.4	1.123	0.33
	Breeding status	1	3377.1	1.514	0.20
	Sex: Breeding status	1	1825	0.818	0.49

Modelled separately for anogenital and facial samples. Models contained data from 35 individuals from eight social groups and the resulting P values were determined from 999 permutations of the data.

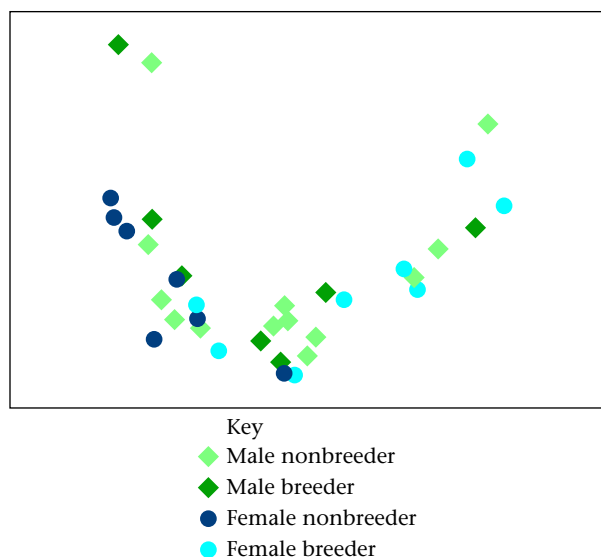


Figure 2. Two-dimensional nonmetric multidimensional scaling (nMDS) plot showing chemical similarity of anogenital samples from Damaraland mole-rats. Female breeders: $N = 8$; female nonbreeders: $N = 7$; male breeders: $N = 7$; male nonbreeders: $N = 13$. In an nMDS plot, axes are dimensionless and the scales of the axes are arbitrary; the closer the symbols appear on the plot, the more similar the samples are in their composition.

one another, but there was little difference between male breeders and nonbreeders. In agreement with our first prediction, this pattern was found in anogenital samples, but not in facial samples (Table 1). We also found that chemical diversity was higher in the anogenital swabs of nonbreeders (mean 63.1 peaks $\pm$ 6.18 SE) compared to breeders (mean 50.7 peaks $\pm$ 7.8 SE; Table 2, Fig. 3). For facial samples, there was a marginally significant interaction between sex and breeding status, with breeding females having higher chemical diversity (mean 63.9 peaks $\pm$ 10.7 SE) than nonbreeding females (mean 37.9 peaks $\pm$ 10.2 SE), while breeding status had little effect on chemical diversity in males (breeding males: mean 42.3 peaks $\pm$ 7.9 SE; nonbreeding males: mean 48.8 peaks $\pm$ 5.2 SE; Table 2, Fig. 4).

Signals of Group Identity

Contrary to our second prediction, we found that chemical profiles from individuals in the same social group were no more similar to each other than expected by chance; there was no signal of social group identity in either the facial (ANOSIM factor: group identity; Global $R = -0.114$; $P = 0.95$) or anogenital samples (ANOSIM factor: group identity; Global $R = -0.021$; $P = 0.57$). We also found no impact of colony size on chemical diversity in either the facial (GLMM; $N = 40$ individuals across 11 social groups,

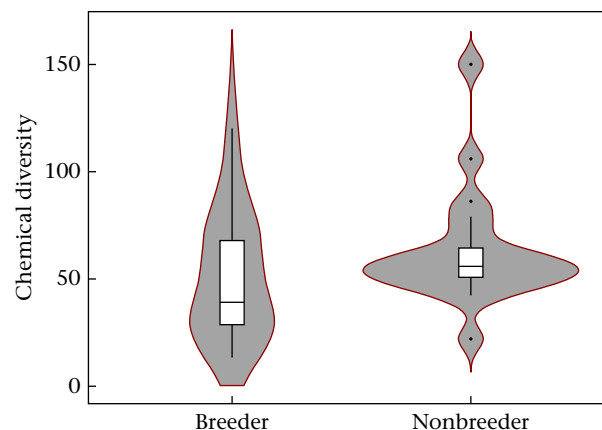


Figure 3. The chemical diversity of anogenital profiles of nonbreeders ($N = 20$) and breeders ($N = 15$). The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers. Violin plots show kernel probability densities of the data.

estimate = -4.97×10^{-4} , SE = 0.031, $z = -0.016$, $P = 0.987$) or anogenital samples (GLMM; $N = 40$ individuals across 11 social groups, estimate = 7.04×10^{-4} , 0.026, $z = 0.027$, $P = 0.978$).

DISCUSSION

Sex and Breeding Status Differences

In accordance with our predictions, we found sex and breeding status differences in the chemical composition of Damaraland mole-rat odours. Our analysis of similarity found that anogenital odours conveyed information on sex and reproductive status, with breeding females being significantly different from nonbreeding females in their chemical profiles, while males did not show a significant difference. Furthermore, the average chemical diversity of anogenital odours was lower in breeders than nonbreeders, regardless of sex. Signals of sex and breeding status are consistent with observations of anogenital sniffing preceding mating in mole-rats (Bennett & Faulkes, 2000; Caspar et al., 2022), where males may assess reproductive opportunities through determining the reproductive state and/or quality of their prospective mates. Sex- and breeding-related differences in the chemical composition of odours appear to be common in mammals, having been found across the clade (for example in lemurs, delBarco-Trillo et al., 2012; humans Penn et al., 2007; giant pandas, *Ailuropoda melanoleuca*, Hagey & MacDonald, 2003; rats, Zhang et al., 2008, fossa, *Cryptoprocta ferox*, Vogler et al., 2008; and bats, Voigt et al., 2008). Furthermore, sex-associated odours are also known to impact mating decisions in mammals (Gildersleeve et al., 2012; Harrington, 1977; Johnston, 1979; Müller & Manser, 2008).

Table 2
Results of GLMMs of the effect of sex and breeding status on chemical diversity

Sample area		Estimate	SE	z	P
Anogenital	(Intercept)	3.610	0.165	21.883	$<2 \times 10^{-16}$
	Sex (male)	0.367	0.238	1.538	0.124
	Status (nonbreeder)	0.561	0.237	2.365	0.018
	Sex (male), status (nonbreeder)	-0.514	0.319	-1.612	0.107
Facial	(Intercept)	4.056	0.192	21.179	$<2 \times 10^{-16}$
	Sex (male)	-0.405	0.283	-1.432	0.152
	Status (nonbreeder)	-0.639	0.285	-2.241	0.025
	Sex (male), status (nonbreeder)	0.762	0.384	1.983	0.047

Modelled separately for anogenital and facial samples. Models contained data from 35 individuals from eight social groups.

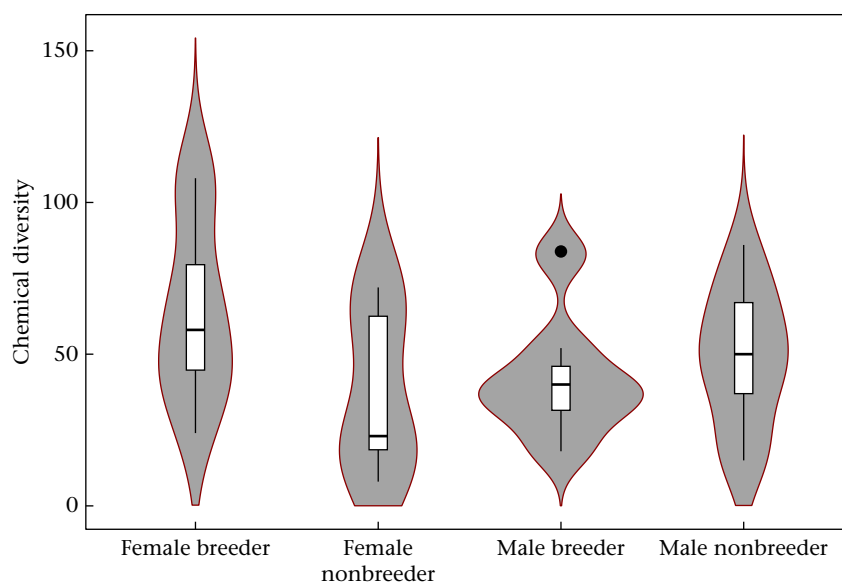


Figure 4. The chemical diversity of facial chemical profiles of female breeders ($N = 8$), female nonbreeders ($N = 7$), male breeders ($N = 7$) and male nonbreeders ($N = 16$). The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers. Violin plots show kernel probability densities of the data.

Damaraland mole-rats therefore fit in with broad mammalian patterns in terms of anogenital odours.

Facial odours also revealed patterns related to breeding status and sex, with female breeders showing greater chemical diversity than female nonbreeders, but there being little difference in relation to breeding status in males. This may be because female breeders undergo frequent changes in their reproductive cycle (for example moving between sexual receptivity, pregnancy and lactation), which could lead to them producing a greater variety of odours. Interestingly, while clear patterns were present in terms of chemical diversity, we did not find any evidence of chemical differences in facial swabs between the sexes or in relation to breeding status. One possibility is that such differences are instead communicated through anogenital odours, which are assessed immediately prior to mating. However, our results differ from those of Caspar et al. (2022) who found that the chemical composition of facial odours differed between the sexes in a related species, the giant mole-rat. This difference could potentially be related to our sampling methodology; Caspar et al. (2022) analysed fur clippings rather than using swabs, which may have captured different suites of chemicals. Alternatively, there may be genuine differences between species, whereby information on reproductive status and sex is conferred by the anogenital region in Damaraland mole-rats, but the facial area in giant mole-rats. Such species level differences have been found in other rodents. For example, house mouse urine indicates reproductive status (Achiraman et al., 2010) whereas hamster urine appears not to (Johnston et al., 1993).

Our results indicating odour differences based on sex and breeding status in Damaraland mole-rats align with a recent behavioural study of the same species by Leedale et al. (2021). They found that both sexes exhibit a preference for sniffing or digging in substrate that had been in contact with opposite-sex solitary individuals, and that mole-rats can distinguish between substrate from solitary individuals and breeding groups. Our results suggest that anogenital and facial odours are likely involved in the communication of sex and breeding status, being assessed directly when individuals meet and/or via substrate after odour transfer during tunnel maintenance activities. However, it is also possible that additional chemicals that we did not measure, such as those

found in urine or faeces, might also convey this information, potentially in conjunction with body odours. In the wild, Damaraland mole-rats of both sexes disperse from their natal group and, while females often settle alone, males appear to seek single females with which to establish new breeding groups (Torrents-Ticó et al., 2018). Dispersal likely happens above ground, so the odour profiles of waste substrate deposited on the surface during tunnel maintenance may be an important source of odours for dispersers seeking a mate (Leedale et al., 2021).

Absence of Colony Level Differences

Contrary to our prediction, we did not find any evidence that odours conveyed a signal of social group identity. Furthermore, we did not find that individuals from larger social groups had more complex chemical profiles. A lack of olfactory cues of group membership is perhaps surprising as the mixing of chemicals from all group members may be expected (O'Riain & Jarvis, 1997), especially when kept under laboratory conditions where close contact between individuals may occur more frequently than it does in the extended burrow systems found in the wild. It therefore seems likely that there was insufficient transfer of chemicals between individuals to be detectable using our methods, which identify small volatile and semivolatile substances, including those that are present in nonaqueous solutions, such as lipids. However, nonvolatile substances such as proteins, which were not investigated in our study, may transfer between individuals or onto substrate and could confer information about group size and membership. Such substances have been found in other rodents, for example house mice, which have been shown to recognize individuals and respond to relatedness cues using major urinary proteins (MUPs; Green et al., 2015; Roberts et al., 2018). MUPs do not appear to be present in mole-rats, but other nonvolatile substances could potentially fulfil a similar role (Hagemeyer et al., 2011).

The lack of a group level odour signature suggests that information on kinship might not be conveyed through volatile odours from the anogenital or the facial region in Damaraland mole-rats (as social groups contained parents, offspring and siblings). This is in contrast to patterns found in some other mole-rat species,

which respond differently to the odours of unfamiliar individuals dependent on genetic relatedness (Hagemeyer, 2010; Heth et al., 2004). Nevertheless, the context of these response differences is unclear as courtship and mating behaviours are just as likely to occur between unfamiliar siblings as unfamiliar nonkin (Heth et al., 2004). Alternatively, information on group identity may be present in Damaraland mole-rats but could be masked by the chemical differences we found between breeders and nonbreeders. However, behavioural studies show that Damaraland mole-rats appear to use familiarity alone when making mating decisions, and treat all unfamiliar individuals as nonkin, even when they are siblings (Leedale et al., 2021). It is therefore likely that kinship information is either undetectable in Damaraland mole-rats or is insufficiently informative to govern mating decisions. The use of familiarity to identify kin is common in species that maintain stable social groups over sustained time periods, such as many cooperatively breeding species, and is likely to be sufficient to avoid inbreeding in most circumstances in the wild (Nichols, 2017). As Damaraland mole-rats can disperse over relatively large distances (up to 4.8 km, Finn, 2017) and rarely settle adjacent to their natal group (Thorley et al., 2023), inbreeding may be sufficiently rare following dispersal that there is little selection pressure for the evolution of additional kin recognition mechanisms above and beyond recognizing familiar groupmates. Supporting this notion, the mean relatedness of breeding pairs across 16 wild colonies assessed using 10 microsatellites was found to be close to zero (Burland et al., 2004), although formal assessments of inbreeding rates in wild Damaraland mole-rats are currently lacking.

Behavioural experiments in several mole-rat species (including Damaraland mole-rats) have demonstrated that group members can individually identify each other based on odours (Burda, 1995; Heth et al., 2002; Jacobs & Kuiper, 2000), although this recognition is short-lived and siblings 'forget' each other after a short period of separation (Jacobs & Kuiper, 2000). Moreover, individuals still forget each other when both have maintained contact with their social group (but not each other), suggesting that individual level, rather than group level cues are used to determine group membership (Jacobs & Kuiper, 2000). Our chemical analyses of odour profiles support this possibility, as no group level odours were identified. We did not investigate chemicals associated with individual identity in our study, but future work taking multiple samples from each individual would provide a meaningful measure of individual variability and may be able to identify individual identity cues in mole-rat odours, should they occur.

Finally, it is possible that nonolfactory signals could be used to assess group membership. For example, naked mole-rats have recently been shown to exhibit group-specific vocalizations that can be used to differentiate between home and foreign groups (Barker et al., 2021). Such signals are culturally transmitted and are learned during development and yet are sufficiently flexible to be modified should the breeding female be replaced (Barker et al., 2021). Vocal signals may be particularly useful in establishing group membership when animals are in close contact with each other. However, they are less likely to be useful when animals are spatially separated. This could be important in the context of dispersal, when dispersing mole-rats are more likely to first come into contact with soil that has been excavated from burrow systems rather than with the individuals themselves (Leedale et al., 2021). Here, olfaction may be important, and it is possible that solitary individuals seeking a mate may produce attractant pheromones. However, our animals were housed in social groups, so we were unable to test this possibility here. Future work investigating the

odour profiles of dispersers in the wild would shed light on this possibility.

Conclusion

Here, we analysed the chemical composition and diversity of odours obtained from Damaraland mole-rat facial and anogenital regions. We found sex and breeding status differences in chemical diversity and composition. Together with behavioural experiments on the same species by Leedale et al. (2021), our results suggest that odours (conveyed by close contact or via substrates) are used to assess potential breeding opportunities. However, we found no evidence of group level signature mixes that could be used to convey information on group membership. Although initially surprising, this pattern is consistent with behavioural experiments by Jacobs and Kuiper (2000) that suggest that Damaraland mole-rats likely learn the odour profiles of all of their group members, which, in the wild, may include over 40 individuals (Jarvis & Bennett, 1993). The lack of a group level odour also supports behavioural studies showing that individual level familiarity may be used to make breeding decisions (Leedale et al., 2021) and to repel potential intruders (Jacobs & Kuiper, 2000), while the transfer of odours from multiple group members on to substrates such as sand and soil may convey information about group size (Leedale et al., 2021). Our results highlight the complexity of chemical communication systems in social species and suggest that signals of group level identity are not necessary for behavioural responses based on group membership.

Author Contributions

Barbara A. Caspers: Writing – review & editing, Visualization, Validation, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Hazel J. Nichols:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Joseph I. Hoffman:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Kevin Arbuckle:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Nigel C. Bennett:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. .

Data Availability

Data and R code supporting this paper are available at Figshare: <https://doi.org/10.6084/m9.figshare.26271457>.

Declaration of Interest

The authors declare no conflicts of interest.

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at <https://doi.org/10.1016/j.anbehav.2024.10.029>.

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