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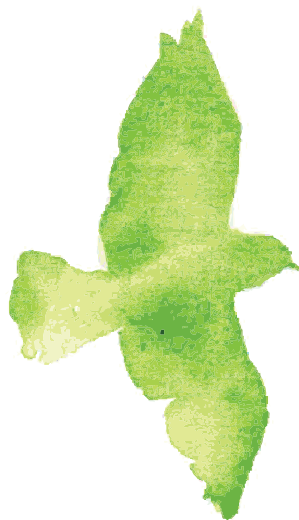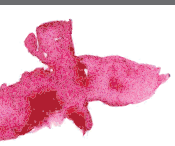
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RECENT ADVANCES IN THE ECOLOGY AND EVOLUTION OF THE BATHYERGIDAE

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Editorial: Recent advances in the ecology and evolution of the Bathyergidae

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Editorial on the Research Topic
Recent advances in the ecology and evolution of the Bathyergidae

Thirty or more species of bathyergid mole-rats exist across sub-Saharan Africa, from naked mole-rats (*Heterocephalus glaber*) in the Horn of East Africa to Cape dune mole-rats (*Bathyergus suillus*) and blesmols (genus *Georychus, Fukomys, Cryptomys, Heliphobus, Bathyergus*) in the Western and Southern Cape at the southern tip of Africa. The family exhibits a broad spectrum of social organization ranging from strictly solitary to eusocial representatives (Bennett and Faulkes, 2000). Early research on the Bathyergidae focused on describing and understanding the social behavior and basic ecophysiology of a few species (e.g., McNab, 1966; Jarvis and Sale, 1971) and subsequently the discovery of eusociality in naked mole-rats (Jarvis, 1981) attracted even more attention and paved the way for an explosion of studies (Begall et al., 2007). In particular, biomedical, behavioral, and ecological research across the bathyergid mole-rats has expanded exponentially in recent years, again with attention focused primarily on naked mole-rats with exceptional findings on their extreme longevity (Buffenstein, 2005) and unusual physiological properties such as the ability to survive relatively long periods of anoxia (Park et al., 2017).

Although their subterranean habitat may protect bathyergid mole-rats from a hostile environment and many types of predators, the constraints of that subterranean environment have led to a host of morphological, physiological and behavioral adaptations which are discussed in these articles. These studies of the Bathyergidae and their adaptations, may also help us understand naked mole-rats by placing them in an evolutionary and ecological context. The broad goal of understanding the Bathyergidae is advanced in part by multi-species comparative analyses (of which we have included six) as well as examining some of the mole-rat species other than *Heterocephalus*

(i.e., Damaraland, Ansell's and Highveld mole-rats). The breadth of work represented in this collection also includes six studies on wild animals in the field and six laboratory based studies.

Some of these articles have benefitted from new experimental technologies (such as Finn, van Vuuren et al.'s and Zöttl et al.'s with the use of RFIDs to quantify underground activity patterns) that we predict will be applied broadly to behavioral work on multiple bathyergid species. Others draw on established techniques and published results to explore particular biological processes across the family, such as Oosthuizen and Bennett's review of biological clocks and Hart et al.'s comparison of metabolic function across the Bathyergidae. Burda has also looked back on published results from 30 years of work with Zambian mole-rats (genus *Fukomys*) to frame a trove of unanswered questions that will keep the next generation of bathyergid researchers busy.

One challenge of studying the behavior of any fossorial animal is that their underground burrows make behavioral observations difficult. While Finn, van Vuuren, et al.'s and Zöttl et al.'s RFID method may change some of this, much of our current understanding of the behavior of these species has come from laboratory observations, which themselves are limited by short artificial burrow systems that provide little opportunity to dig or disperse. Nonetheless, Hite et al.'s modification of the Hebb-Williams maze to assess spatial learning in burrowing species is likely to be repeated on other bathyergid species, and even in two other families of subterranean rodents represented by the genera *Spalax* and *Ctenomys*.

Our characterization of mating system and population structure, in bathyergids and many other taxa, is often based on behavioral observation and inference from findings such as Finn, Thorley, et al.'s discovery that Damaraland mole-rats can disperse more than 4 km from their natal burrows. However, Szafranski et al. went further and used microsatellites to confirm that their captive naked mole-rats are monogamous, whereas Hess et al. used nested clade analysis of mitochondrial genes to explore the role of rivers in gene flow and fragmentation in this species, and they reported the surprising discovery that the current size of a river is not a direct predictor of the divergence between populations separated by the river. Lutermann et al. looked at how parasites can affect how mates are chosen in the Highveld mole-rat (*Cryptomys hottentotus hottentotus*) and Lutermann

also took a step back and reviewed the broader role of parasites in a potential scenario on the evolution of social behavior in animals that are both protected and restricted to an underground burrow.

Anatomical phenotypes, and adaptations to the fossorial niche, were studied by Montoya-Sanhueza et al. who compared limb development across a broad spectrum of bathyergid mole-rats. Begall et al. and Toor et al. examined the behavioral phenotypes or personalities of Ansell's mole-rats (*Fukomys anselli*) and task specialization in naked mole-rats respectively; while the size of the white head patch does not correlate with differences among Ansell's mole-rats, partner preference tests are predictive of social roles in naked mole-rats.

This collection of papers on the behavior, ecophysiology, anatomy and genetics across the Bathyergidae may appear to be an eclectic collection, but it highlights the diversity of adaptations to the subterranean niche in the African mole-rats. It further supports the argument that in order to fully understand the amazing adaptations of naked mole-rats, we must also know how their fellow bathyergid relatives have adapted to a life underground.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

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Seasonal Changes in Locomotor Activity Patterns of Wild Social Natal Mole-Rats (*Cryptomys hottentotus natalensis*)

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Differences in individual locomotor activity patterns may be linked to a number of ecological factors, such as changes in ambient temperature or photoperiod. Observations on subterranean mammals suggest that they exhibit diel rhythms despite the lack of visual cues in their underground burrows, but it is unknown how seasonality and individual characteristics affect their activity. In this study we use RFID technology to monitor daily activity patterns of wild, social Natal mole-rats (*Cryptomys hottentotus natalensis*) during the summer and winter to investigate how their activity varies with season and whether their activity depends on individual characteristics such as body mass, sex and reproductive status. We found that in winter, individuals were more active during the time with the highest soil temperatures, whereas in summer, they showed a bimodal activity pattern during early morning and late afternoon coinciding with cooler soil temperatures. Individual characteristics, including reproductive status, did not affect general activity indicating that reproductive and non-reproductive individuals contribute equally to cooperative behaviors. We suggest that the activity patterns may be a behavioral adaptation to avoid extreme burrow temperatures and a mechanism to maintain a stable core body temperature. We highlight the advantages of RFID technology to study wild small mammal movements.

Keywords: locomotor activity, temperature, subterranean mammal, mole-rat, RFID, circadian rhythm

INTRODUCTION

Animal locomotor activity patterns have been the subject of much interest in the study of animal behavior. These activity patterns may be influenced by endogenous (i.e., intrinsic) or exogenous (i.e., environmental) factors, which may or may not persist under constant conditions (Hazlerigg and Tyler, 2019). When a biological rhythm persists in a near-24-h period under constant conditions (e.g., complete darkness), the circadian rhythm is assumed to "free run," showing evidence for control by a biological clock (Benstaali et al., 2001). Conversely, the lack of an endogenous clock may result in the degradation of rhythmic patterns under constant conditions (Hazlerigg and Tyler, 2019). Biological clocks conform to daily and predictable exogenous cues

known as *zeitgebers* in a process referred to as entrainment in which the clock's rhythm becomes equal to the cycle of the *zeitgeber* (Johnson et al., 2003).

It is widely accepted that the light-dark cycle (i.e., rising and setting of the sun) is the most common *zeitgeber* for the entrainment of circadian rhythms (Benstaali et al., 2001). Ambient temperature may also affect circadian rhythms in both homeothermic (e.g., most mammals) and heterothermic (e.g., birds, squamates, some mammals) vertebrates (Rensing and Ruoff, 2002; Refinetti, 2015). Some mammals dwelling in arid or arctic regions exhibit adaptive heterothermy, where the core body temperature may fluctuate with ambient temperature under certain conditions (Cain et al., 2006; Williams et al., 2012; Allali et al., 2013; Farsi et al., 2020b). For example, camels (*Camelus dromedarius*) exhibit heterothermy when dehydrated (Allali et al., 2013) and arctic ground squirrels (*Urocitellus parryii*) during hibernation (Williams et al., 2012). Recently it has been discovered that ambient temperature instead of the light-dark cycle may entrain circadian rhythms in body temperature, melatonin cycles and locomotor activity in some of these species (Allali et al., 2013; Farsi et al., 2020a,b). While ambient temperature may not be as strong a *zeitgeber* compared to light-dark cycle in homeothermic mammals, many will adjust their activity patterns in response to ambient temperature to avoid exposure in harsh environments (Cain et al., 2006; Roll et al., 2006, however see Farsi et al., 2020b). For example, arid dwelling rodents avoid the hottest period of the day and by becoming nocturnal (Randall, 1993; Roll et al., 2006). Lastly, biotic cues, such as feeding time, food availability, social cues, or predation, may also affect activity patterns (Mistlberger and Skene, 2004; Halle, 2006; Getz, 2009; Refinetti, 2015; Beale et al., 2016). Individuals in a population may synchronize their activity patterns *via* social cues to be active nearly simultaneously even in complete darkness (e.g., under snowfall, Korslund, 2006), and isolated individuals may exhibit asynchronous activity patterns (Getz, 2009; Lövy et al., 2013). It has been suggested that simultaneous activity in voles provides an individual with protection against predation due to "safety in numbers" or possibly confusing predators by a "swarming" effect (Gerkema and Daan, 1985; Inman and Krebs, 1987) and species may alter their activity patterns in response to predation pressure which may act as a weak *zeitgeber* (Getz, 2009; Vlasatá et al., 2017).

In the subterranean ecotope, and other photic void environments, occupants are rarely, if ever, exposed to light. Despite limited exposure to light, many subterranean mammals kept in constant darkness and temperature whilst under laboratory conditions still exhibit strong circadian rhythms of locomotor and physiological activity (e.g., melatonin and body temperature cycles). A large body of work has focused on physiological circadian rhythms and entrainment to photoperiod in many species including African mole-rats (Family Bathyergidae: Riccio and Goldman, 2000; Oosthuizen et al., 2003; van Jaarsveld et al., 2019; Hart et al., 2021), blind mole rats (Family Spalacidae: Rado et al., 1993), coruros (Family Octodonidae: Begall et al., 2002), and tuco-tucos (Family Ctenomyidae: Jannetti et al., 2019; Flôres et al., 2021). However, the majority of these experiments lack the complex

environmental conditions that these animals may experience in the wild, including changes in infrequent light pulses, atmospheric conditions, burrow microclimate, social cues, or predator-prey interactions. Therefore, other *zeitgebers* may be involved in entraining circadian rhythms in these animals (reviewed in Beale et al., 2016). Observations of free-ranging subterranean mammals have revealed that some species may exhibit arrhythmic activity patterns over the 24-h period, such as some pocket gophers (*Thomomys*; Andersen and MacMahon, 1981; Gettinger, 1984), yet many other species exhibit distinct daily peaks of activity, including root rats (*Tachyoryctes*: Jarvis, 1973), African mole-rats (*Heterocephalus*: Jarvis, 1973; *Heliophobius*: Šklíba et al., 2007; *Fukomys*: Šklíba et al., 2014, 2016a), blind mole rats (*Spalax*: Rado et al., 1993; Šklíba et al., 2016b), coruros (*Spalacopus*: Rezende et al., 2003), and tuco-tucos (*Ctenomys*: Jannetti et al., 2019; Flôres et al., 2021). These activity patterns suggest entrainment of a circadian clock, which has led some authors to conclude that temperature may be an essential *zeitgeber* for the entrainment of activity rhythms in subterranean mammals (Benedix, 1994; Šklíba et al., 2014; Hart et al., 2021). Homeothermic regulation of body temperature suggests that homeothermic animals are less sensitive to environmental changes (Rensing and Ruoff, 2002), but some African mole-rats exhibit heterothermy (Buffenstein and Yahav, 1991; Bennett et al., 1993a; Oosthuizen et al., 2021) and therefore, fluctuations in ambient temperature are more likely to affect their activity patterns.

African mole-rats are a unique clade of subterranean rodents, with some species being strictly solitary and others occurring in large family groups (Bennett and Faulkes, 2000). Living underground offers thermal buffering to extreme temperature fluctuations with burrow depth varying with species (Bennett et al., 1988). Soil properties and food availability likely explain why tunnels are constructed at specific depths (Broekman et al., 2006; Lövy et al., 2015). The depth of tunnels and the nests are greatest for mole-rats inhabiting arid regions with sandy soils, where foraging tunnels are between 30 and 40 cm below ground, and nests may exceed 2 m in depth (Lovegrove and Painting, 1987; Bennett et al., 1988). In other soil types foraging tunnels are at more shallow depths of around 10–20 cm with nests between 30 and 60 cm (Hickman, 1979; Spinks et al., 1999; Šklíba et al., 2012; Šumbera et al., 2012). Deep dead-end tunnels may help regulate burrow temperatures or humidity, and these features vary between 78 cm in loamy soil types of the Natal region in South Africa (Hickman, 1979) to over 3 m in the sand of the Kalahari (Lovegrove and Painting, 1987). The deeper tunnels of the Damaraland mole-rat (*Fukomys damarensis*) provide a greater thermal buffer against the extreme daily and seasonal temperatures of the Kalahari compared to the shallow tunnels of common mole-rats (*Cryptomys hottentotus*), which generally occur in more mesic habitats, such as the Natal region in South Africa (Bennett et al., 1988). Even with deep tunnels, the foraging burrows of mole-rats experience seasonal temperature fluctuations, and it has been suggested that temperature may act as a *zeitgeber* for daily activity patterns in mole-rats (Goldman et al., 1997; Šklíba et al., 2007; Hart et al., 2021). The activity patterns and circadian rhythms of mole-rats have been well

studied in laboratory settings (Hickman, 1980; Bennett, 1992; Riccio and Goldman, 2000; Oosthuizen et al., 2003; van Jaarsveld et al., 2019; Hart et al., 2021). In two recent studies under laboratory conditions, homeothermic mole-rat species showed decreased activity during periods of higher temperature and the authors suggested that temperature may drive nocturnal behavior (van Jaarsveld et al., 2019; Hart et al., 2021). The observed activity entrainment by ambient temperature may be due to a thermoregulatory response to avoid over-heating while digging during periods of increased burrow temperatures (Hart et al., 2021). Few studies have been conducted on wild mole-rats (Šklíba et al., 2007, 2014, 2015) and even fewer still on seasonal variation in activity (Šklíba et al., 2007).

This study set out to determine if daily activity patterns fluctuated with seasonal temperature changes in the Natal mole-rat (*Cryptomys hottentotus natalensis*), a social species inhabiting mesic grasslands of southeastern South Africa and Lesotho at altitudes from sea level to the Drakensberg escarpment (ca. 3,000 m). The montane regions they inhabit experience hot summer days and cold winter nights, with snow often accumulating at the highest elevations during winter. Unlike most other bathyergids, Natal mole-rats exhibit heterothermy (Oosthuizen et al., 2021), and they would be expected to respond to changes in burrow temperature to a greater degree in comparison to their homeothermic counterparts (Šumbera, 2019; Wallace et al., 2021). The link between ambient temperature and activity patterns in African mole-rats has not been thoroughly investigated in a field-based study, and never in a heterothermic mole-rat species. Previous laboratory experiments on entrainment of circadian rhythms in Natal mole-rats indicate the entrainment in both melatonin and activity cycles under a light-dark cycle and free-running cycles under constant conditions (Hart et al., 2004). In this study, we used a radio-frequency identification (RFID) reader array to monitor individual activity in free-ranging Natal mole-rats. This non-invasive method allows for automated continuous monitoring of activity patterns in a natural habitat without disturbing the subjects (Korslund, 2006; Francioli et al., 2020). Activity rhythms may show plasticity, where individuals or populations adjust to seasonal changes in the environment (Halle, 1995; Rezende et al., 2003; Halle, 2006; Beale et al., 2016). Therefore, it was predicted that Natal mole-rats would alter their activity patterns seasonally to cope with the fluctuating burrow temperatures due to their shallow tunnels (Hickman, 1979). Additionally, trapping efforts indicated that mole-rats appeared to exhibit reduced activity during periods of rain when compared to clear days. It is possible that mole-rats may be able to respond to the change in air pressure or temperature caused by the rain or cold fronts as seen in other animals (Garthe et al., 2009; Boyer and MacDougall-Shackleton, 2020). Lastly, because Natal mole-rats exhibit only behavioral reproductive suppression compared to that of the Damaraland mole-rats, which exhibit behavioral and physiological suppression (Bennett et al., 1996; Molteno and Bennett, 2000; Oosthuizen et al., 2008), we predicted that locomotor activity differences between the breeders and non-reproductive individuals would be less distinct than in species

with higher reproductive skew. We emphasize that in the current study we did not investigate entrainment of circadian rhythms by environmental *zeitgebers*, but rather investigated the potential role of environmental variables on the diel activity rhythms of colonies of a subterranean mammal during the summer and winter months of the Southern Hemisphere.

MATERIALS AND METHODS

Animal Capture
Natal mole-rats were captured using Hickman live traps during the wet summer (February to March) and dry winter (July to October) seasons from August 2019 to July 2021 at Glengarry Holiday Farm in KwaZulu-Natal, South Africa (–29.322530°, 29.712982°, 1,600 m a. s. l.; **Supplementary Figure 1**). Natal mole-rats are a social species of mole-rat that live in family groups (referred to as colonies) with one reproductive female, a couple of reproductive males, and the remaining non-reproductive group members include the overlapping generations of offspring from the reproductive individuals (Oosthuizen et al., 2021). A total of 13 distinct colonies (including 118 unique animals) were used for the study, with a mean colony size of 8.8 ± 3.2 (range 4–13); 10 of the 13 groups were complete colonies (i.e., included reproductive individuals and all non-reproductive subordinates). Traps were placed in exposed tunnels at each colony, baited with sweet potato, and checked at 2-h intervals from 07 h00 to 22 h00, depending on air temperature. Colonies were recaptured at 6-month intervals. Colony membership of individuals was initially determined by capture location, a lack of aggression between individuals, and later confirmed by recapture of individuals in the same colony (as opposed to capture in neighboring colonies) during subsequent captures. Capture methods have been standardized in many field studies of bathyergids (Jarvis and Bennett, 1993; Šklíba et al., 2007, 2012, 2016a; Lövy et al., 2013; Finn et al., 2018). Captured mole-rats were housed with family members at ambient temperature (15–25°C), provided with wood shavings as bedding and fed sweet potato *ad libitum*. All animals were sexed, weighed to the nearest gram (Pelouze SP5, Rubbermaid, United States), and assigned a reproductive status at capture. Reproductive females were identified by a perforate vagina and prominent teats (Bennett and Faulkes, 2000). Male reproductive status was determined by body mass, presence of testes, and prolonged presence in the colony (Finn et al., 2018). All individuals greater > 20 g were implanted with a subcutaneous passive integrated transponder tag (Trovan Unique, DorsetID, Netherlands) to uniquely identify them. After 48 h without any signs of individuals present in the burrow system (i.e., no blocked tunnels, fresh mounds, triggered traps, or bait eaten), the colony was assumed to be completely captured and the colony was released back into their burrow.

Hourly Climate Data
Hourly air pressure, air temperature, soil temperature, and rainfall during winter 2020 and summer 2021 were obtained for the study site from the global atmospheric reanalysis dataset

TABLE 1 | Variables from ERA5-Land dataset used for analysis.

Variable	Description	Units
2 m temperature (T_{air})	Temperature of the air 2 m above the soil surface	K
Total precipitation (rainfall)	Accumulated liquid and frozen water that falls to the Earth's surface; a mean of daily values	m
Air pressure (P)	Force per unit area of the atmosphere on the surface of the land.	Pa
Soil temperature level 1 (T_{S1})	Temperature of the soil in layer 1 (depth 0–7 cm), taken from the middle of each layer	K
Soil temperature level 2 (T_{S2})	Temperature of the soil in layer 2 (7–28 cm)	K
Soil temperature level 3 (T_{S3})	Temperature of the soil in layer 3 (28–100 cm)	K

The data are freely available from the Copernicus Climate Change Service which holds the intellectual property rights of the raw data.

TABLE 2 | Hourly climate during the study period at Glengarry (mean ± SD).

	Mean	Min	Max
Winter 2020 (August–October, n = 65 days)			
T_{air}	11.1 ± 3.66°C	5.3 ± 3.15°C	17.7 ± 5.08°C
T_{S1}	11.9 ± 2.93°C	7.0 ± 2.78°C	17.7 ± 3.93°C
T_{S2}	11.6 ± 2.38°C	10.3 ± 2.30°C	12.7 ± 2.51°C
T_{S3}	10.9 ± 1.50°C	10.8 ± 1.49°C	11.0 ± 1.50°C
ΔP	37.1 ± 6.84 kPa	3.2 ± 3.08 kPa	95.0. ± 23.25 kPa
Rain	1.0 ± 1.99 mm	0.0 ± 0.19 mm	3.3 ± 4.52 mm
Summer 2021 (February–April, n = 57 days)			
T_{air}	15.9 ± 2.33°C	11.6 ± 1.91°C	20.8 ± 3.60°C
T_{S1}	17.5 ± 1.88°C	13.8 ± 1.66°C	22.0 ± 2.97°C
T_{S2}	17.5 ±1.37°C	16.6 ± 1.33°C	18.4 ± 1.45°C
T_{S3}	17.4 ± 0.62°C	17.4 ± 0.62°C	17.5 ± 0.62°C
ΔP	28.9 ± 3.60 kPa	2.0 ± 3.31 kPa	76.1 ± 17.82 kPa
Rain	2.5 ±4.41 mm	0.1 ± 0.40 mm	9.1 ± 12.34 mm

T_{air} is the air temperature at 2 m above the soil; soil temperatures were measured at depths of 0–7 cm (T_{S1}), 7–28 cm (T_{S2}), and 28–100 cm (T_{S3}); and ΔP is the change in air pressure from 1 h to the next. Rain is hourly precipitation.

ERA5-Land (**Table 1**; Muñoz-Sabater, 2019). The ERA5-Land dataset is a global climate analysis model based on satellite and weather station climate observations allowing for accurate estimates of climate variables at specific locations with a 9 km accuracy (0.1° × 0.1°; Muñoz-Sabater et al., 2021). Users can specify the boundary GPS points of less than 9 km to query data from the database, and we selected a 1 km radius to encompass the study site. The dataset is freely available from the Copernicus Climate Change Service, and holds the intellectual property rights of the raw data. The results contain modified Copernicus Climate Change Service information. We used soil temperature measured at depths of 0–7 cm (T_{S1}), 7–28 cm (T_{S2}), and 28–100 cm (T_{S3}), which correspond to the depths of foraging tunnels (T_{S1} and T_{S2}) and the nest (T_{S3}). A temperature logger (DS1922L iButton, Maxim Integrated Products, Dallas, TX, United States) was buried at the study site at 15 cm below the soil surface in an area of direct sunlight and set to record hourly temperatures from August to October 2020. We compared the soil temperatures from the data logger and T_{S2} from the ERA5-Land dataset. We found that soil temperatures from the data logger were on average 3.67 ± 1.32°C higher than T_{S2}, yet the daily fluctuation in temperature was similar between temperature loggers and ERA5-Land values (**Supplementary Figure 2**). Therefore, we

assumed that soil temperatures from ERA5-Land were a modest representation of actual variability of soil temperatures. It is important to note that the soil temperatures in this study may not accurately represent burrow temperatures, and furthermore, that ambient temperature in tunnels may be higher than the surrounding soil (*sensu* Holtze et al., 2018). The change in hourly air pressure (ΔP) from 1 to 2 h was calculated to determine whether changes in air pressure predicted activity. To determine if climate varied between hour and season the climatic variables were compared by conducting a linear regression with the climate variable as response and season, hour and interaction between season and hour as predictors. Hourly climate variables were then combined with the activity data to determine the environmental effects of daily and seasonal activity patterns.

Radio-Frequency Identification Array Placement

We utilized an RFID reader array (LID650/608, DorsetID, Netherlands; **Supplementary Figure 2**) and the methods of Francioli et al. (2020) to monitor mole-rat activity during winter (August–October 2020) and summer (February–April 2021). The array consisted of a scanning panel (Trovan ANT 612) measuring 47.5 × 40 × 4 cm and a decoder box (**Supplementary Figure 3**). The array was powered by a 12 v battery and could be attached to a solar panel to prolong battery life (**Supplementary Figure 4**). The array was programmed to scan continuously for transponder tags within the range with a read delay of 3 s. When an animal with a transponder tag passed within range, the numeric code of the tag was recorded with a time stamp on a USB drive attached to the decoder. Thus, if an animal remained in the range of the panel for longer than 3 s, it would have multiple entries at 3-s intervals.

The array was placed at a mole-rat group and left stationary during each reading session. Mole-rat mounds were probed until the tunnel was located and the reader panel was placed directly over the tunnel. The panel had a detection range of 20–30 cm, which is greater than the depth of mole-rat tunnels at the study site, and therefore the chance of animals passing undetected under the panel is minimal. Reading sessions had a mean duration 3.5 ± 1.59 days (range 2–7). We attempted to repeat reading sessions for each group in both seasons; however, some groups went missing and additional groups were selected to maintain an even sample size. Therefore, the mean number of reading sessions per group was 1.3 ± 0.46.

TABLE 3 | Linear regression results comparing climate variables between seasons and time of day.

Predictor	Estimate	S.E.	p-value
T_{air}			
Intercept	20.41	0.28	<0.001*
Season	−5.48	0.17	<0.001*
Hour	0.03	0.02	0.12
Season * hour	0.03	0.01	0.006*
T_{S1}			
Intercept	22.55	0.23	<0.001*
Season	−6.30	0.14	<0.001*
Hour	0.05	0.02	0.002*
Season * hour	0.04	0.01	<0.001*
T_{S2}			
Intercept	22.75	0.12	<0.001*
Season	−6.18	0.07	<0.001*
Hour	0.05	0.01	<0.001*
Season * hour	0.01	0.01	0.10
T_{S3}			
Intercept	24.20	0.06	<0.001*
Season	−6.91	0.04	<0.001*
Hour	−0.01	0.00	0.23
Season * hour	0.00	0.00	0.10
Δ P			
Intercept	13.25	1.36	<0.001*
Season	12.40	0.82	<0.001*
Hour	0.52	0.10	<0.001*
Season * hour	−0.27	0.06	<0.001*
Rain			
Intercept	0.01	0.18	0.95
Season	0.12	0.11	0.29
Hour	0.19	0.01	<0.001*
Season * hour	−0.06	0.01	<0.001*

denotes significance.

Activity Analysis

Radio telemetry studies have indicated that activity bouts of mole-rats can last for up to 1 h (Lovegrove, 1988), and therefore we assumed that activity bouts lasted for 1 h (see **Supplementary Material** for validation). Due to the nature of the study design, we do not know the exact duration of activity bouts, and the results rather include the times and frequency of individual animal activity. Animals were given an active score of 1 if at least one reading was detected on the array during a 60-min interval, and a score of 0 if not detected during this interval. An animal would not gain a further activity point until detected beyond the activity period. Visual inspection of the raw data indicated that animals rarely remained motionless under the reader array. Individuals captured in the colony but not recorded by the RFID reader array were assigned activity scores of zero. All potential variables affecting daily activity were analyzed by fitting a generalized linear mixed model with negative binomial distribution using the *glmmTMB* package (Brooks et al., 2017) in R 4.0.5 (R Core Team, 2021). The negative binomial distribution (values of 0 or 1 in the response) prevents overdispersion in parameters. Predictors

for the full model included life history variables such as sex, body mass, reproductive status (reproductive/non-reproductive), group size; abiotic factors such as T_{air}, T_{S1}, T_{S2}, T_{S3}, ΔP, rainfall, season (wet/dry), time (as sine and cosine wave expressed in radians—where 0 is 00:01, π is 12:00, and 2π is midnight), and the daily cumulative hours of sunlight from sunrise each hour until sunset (with hours of darkness equal to 0). Non-significant predictor variables were dropped until a model with the lowest AIC was generated (**Supplementary Material**). Predictors for the final model included: interactions between sex and reproductive status; sex and body mass; season and time; season, time, and T_{air}; season, time, and T_{S1}; and season, time, and T_{S2}. Individual identity and reading session were specified as nested random intercepts to control for non-independence among observations. All continuous predictors were scaled, and body mass was scaled within sex because males were heavier than females. The proportion of explained variance was estimated using marginal and conditional R^2. An excess mass test for unimodality using the package *multimode* (Ameijeiras-Alonso et al., 2019, 2021) was used to determine if the peaks of activity were unimodal or bimodal by comparing the hours of activity within each season. This package allows for testing the kernel density of data against the null hypothesis that the number of modes is equal to a set number, and can calculate the exact number of modes in a density plot. The calibrated excess mass test by Ameijeiras-Alonso et al. (2019) is more accurate than previous modality tests, such as Silverman's and Hartigan's methods among others, by allowing the user to set the number of modes to test against the null.

RESULTS

All hourly climate variables except rainfall varied significantly between seasons, and all variables except T_{S3} varied significantly with time of day within each season (**Tables 2**, **3** and **Supplementary Figures 6**, **7**). The greatest daily difference in soil temperatures occurred during winter. The maximum day length was 13.28 h during summer and 12.75 h during winter, with a minimum day length of 11.68 and 11.00 h, respectively.

The dataset included 9,972 raw activity points at 3-s intervals from 77 wild mole-rats (41 individuals were captured before array placement, but not recorded) over 16 reader sessions at independent groups (winter $n = 8$, summer $n = 8$). Assuming an activity period of 1 h, the dataset was reduced to 365 total activity points of detected mole-rats (summer $n = 153$, winter $n = 212$) with individuals being detected a mean of 0.89 ± 1.27 times per day during winter (range 0–7) and 0.88 + 1.02 times per day during summer (range 0–4). The results from the model revealed that body mass, T_{air}, T_{S1} and interactions between those temperatures with time of day and season had the greatest effect on predicting periods of activity (**Table 4**). Hours of daylight, rainfall, T_{S2}, T_{s3}, and ΔP as well as life-history traits (sex, reproductive status, and group size) did not affect the likelihood of activity. Mole-rats were most active when soil temperatures were between 12 and 16°C. Locomotor activity patterns were similar for reproductive and non-reproductive individuals in the colony suggesting no specific specialization
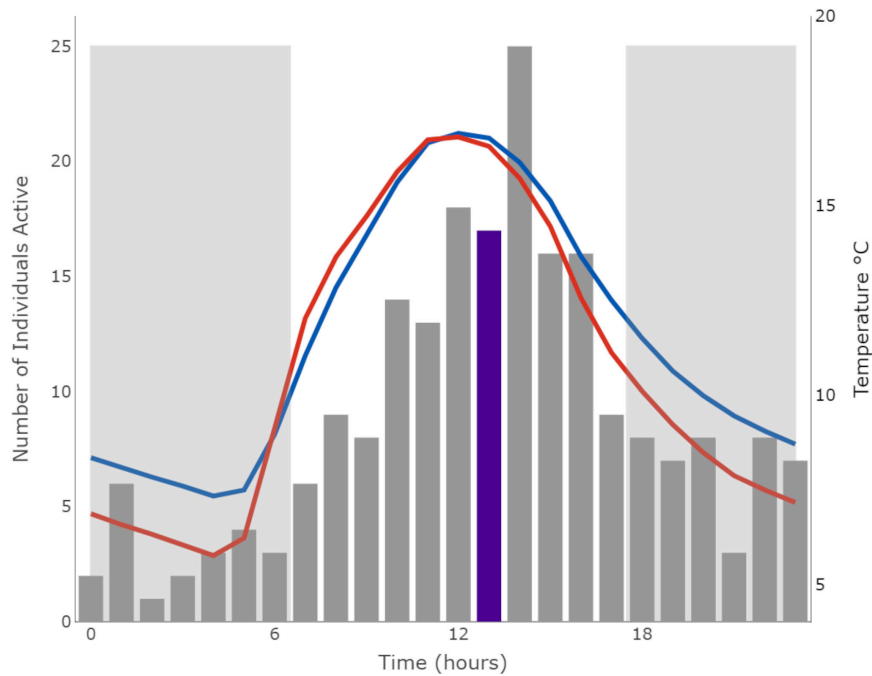
FIGURE 1 | Histogram of daily activity patterns of mole-rats during winter (August–October). Gray bars indicate the total activity points per hour of all animals across the sampled groups during winter. Mean daily air temperature (red) and soil temperature at 0–7 cm (blue) during winter is plotted on the right axis. The median time mole-rats were active is highlighted in purple. Hours of darkness are indicated by the shaded blocks. Climate data were obtained from the Copernicus Climate Change Service, which holds the intellectual property rights of the raw data.
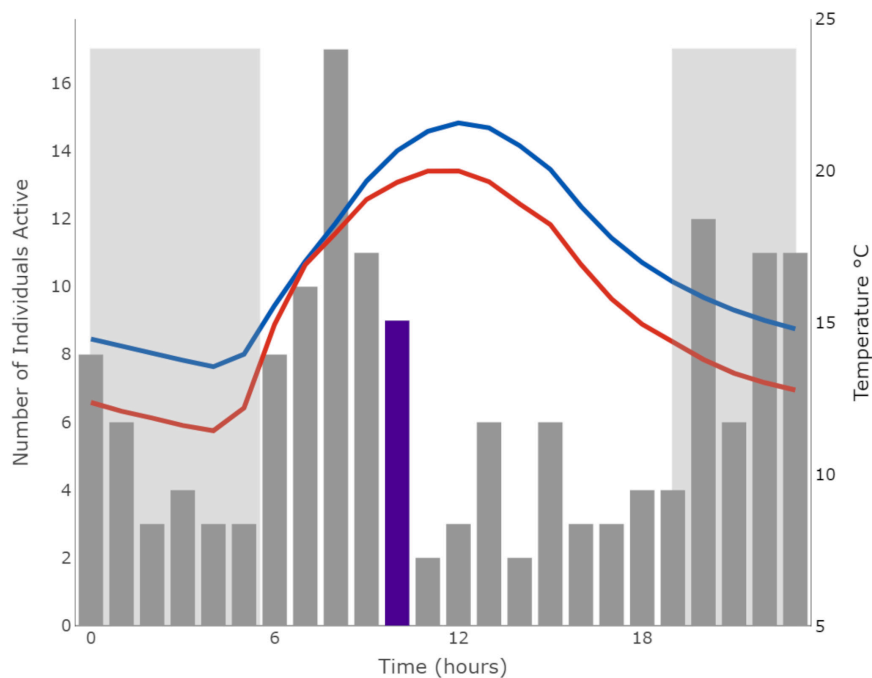
FIGURE 2 | Histogram of daily activity patterns of mole-rats during summer (February–April). Gray bars indicate the total activity points per hour of all animals across the sampled groups during summer. Mean daily air temperature (red) and soil temperatures at 0–7 cm (blue) during summer is plotted on the right axis. The median time mole-rats were active is highlighted in purple. Hours of darkness are indicated by the shaded blocks. Climate data were obtained from the Copernicus Climate Change Service, which holds the intellectual property rights of the raw data.

TABLE 4 | Predictors of daily activity of Natal mole-rat.

Predictor	Estimate	S. E.	P-value
Intercept	−5.38	2.01	0.007*
Body mass	−0.06	0.26	0.02*
Reproductive status	1.14	0.62	0.06
Sex	0.29	0.33	0.38
Body mass * sex	0.46	0.35	0.18
Reproductive status * sex	−0.55	0.78	0.48
Group size	−0.05	0.17	0.76
Sin (time in radians)	1.16	0.26	0.04*
Cos (time in radians)	−0.19	0.30	0.53
Season	0.13	1.03	0.90
Season * sin (time in radians)	−0.95	0.78	0.22
Daylight	−0.89	0.80	0.27
Daylight * season	0.57	0.43	0.19
Daylight * sin (time in radians)	−0.79	0.81	0.33
ΔP	0.00	0.22	0.99
ΔP * season	0.03	0.13	0.83
ΔP * sin (time in radians)	0.42	0.32	0.20
T_{air}	4.49	1.15	<0.001*
T_{air} * season	−2.35	0.67	<0.001*
T_{air} * sin (time in radians)	−4.13	1.34	0.002*
T_{S1}	−4.68	1.68	0.005*
T_{S1} * season	2.71	0.98	0.006*
T_{S1} * sin (time in radians)	6.50	1.86	<0.001*
T_{S2}	−1.98	1.19	0.10
T_{S2} * season	0.78	0.70	0.27
T_{S2} * sin (time in radians)	−0.63	1.12	0.57
T_{S3}	4.09	2.38	0.09
T_{S3} * season	−2.13	1.38	0.12
T_{S3} * sin (time in radians)	−1.40	1.79	0.44
Season * sin (time in radians) * daylight	0.51	0.45	0.25
Season * sin (time in radians) * ΔP	−0.17	0.19	0.36
Season * sin (time in radians) * T_{air}	1.39	0.81	0.09
Season * sin (time in radians) * T_{S1}	−2.67	1.09	0.01*
Season * sin (time in radians) * T_{S2}	0.45	0.65	0.49
Season * sin (time in radians) * T_{S3}	0.27	1.00	0.78

Values are from a general linear mixed model with a negative binomial distribution. AIC = 2859.9, Condition R^2 = 0.02, Marginal R^2 = 0.06. All continuous predictors were scaled to the mean and unit variance for model fitting.
* denotes significance.

of activity based on reproductive status. The total amount of activity did not differ between seasons (i.e., season variable), but the time of day during which individuals were active was significantly different between seasons (i.e., season * time variable). During winter, animals exhibited a single peak in activity during midday when burrow temperatures were highest ($E = 0.02$, p-value = 0.92; **Figure 1**). While during summer, daily activity exhibited a bimodal distribution where animals were more active during the cooler morning and evening temperatures ($E = 0.10$, p-value < 0.001; **Figure 2**). Further linear regression analysis of the relationship between activity and T_{air} as well as T_{S1} indicated a significant relationship in both seasons for T_{S1} (winter: $df = 7,455$, SE 0.0014, p-value < 0.01; summer: $df = 4,616$, $SE = 0.0024$, p-value < 0.001), while the relationship

between activity and T_{air} was only significant in summer (winter: $df = 7,455$, $SE = 0.0012$, p-value = 0.17; summer: $df = 4,616$, $SE = 0.0021$, p-value < 0.001).

DISCUSSION

In this study, soil temperatures at < 7 cm and time of day were the best predictors of activity patterns in the heterothermic Natal mole-rat, with soil temperature varying significantly over the course of a day and between seasons. During summer individuals avoided activity during the hottest part of the day (becoming more crepuscular), while in winter they were more active during this time (becoming diurnal). These patterns are similar to responses observed in other rodents (both heterothermic and homeothermic) living in high altitudes or latitudes (Hinze and Pillay, 2006; Williams et al., 2012; Vlasatá et al., 2017; Flôres et al., 2021). This result also confirms recent work on activity patterns in mole-rats observed in a captive setting (Hart et al., 2021). Oosthuizen et al. (2021) observed that wild Natal mole-rats at the same study site exhibited unimodal body temperature fluctuations in winter and bimodal fluctuations in summer and proposed that increased body temperature coincided with time of activity. Our results may validate this prediction because in summer, Natal mole-rats showed bimodal peaks in activity during the cooler soil temperatures and a unimodal peak in activity during winter when soil temperatures were highest. Oosthuizen et al. (2021) found that mean body temperature was higher during winter than during summer, and this result may have indicated increased activity during winter. In contrast to that suggestion and previous studies on a related mole-rat species (Šklíba et al., 2007), we did not find a significant difference in the frequency of daily activity between seasons.

Previous work on bathyergids, including the Natal mole-rat, has shown that circadian rhythms free-run under constant conditions and both light and temperature may entrain circadian rhythms (Hart et al., 2004, 2021; van Jaarsveld et al., 2019). In laboratory studies, mole rats preferred temperatures between 18 and 22°C while in complete darkness (Hart et al., 2021). Interestingly, we found that even though T_{S1} was consistently below this range during winter (5–17°C, **Supplementary Figure 4**) and rarely greater than 20°C during summer (12–21°C, **Supplementary Figure 5**), mole-rats did not show an even distribution of activity periods over a 24-h period during either of the two seasons. However, we must note that soil temperatures from the ERA5-Land dataset were (1) 3°C lower than temperatures recorded *via* temperature loggers at the study site, and (2) may not reflect actual burrow temperatures. Minute temperature changes may be enough to elicit a change in activity rhythms in subterranean rodents (Goldman et al., 1997; Šklíba et al., 2007, this study). Temperature changes in foraging tunnels may well be a primary driving force in the entrainment of circadian rhythms since bimodal and unimodal activity patterns were found in various subterranean mammals (this study, Benedix, 1994; Rezende et al., 2003; Šklíba et al., 2007). Mole-rats spend over 70% of their time in the nest (Bennett, 1990), which may be at the deepest part of their burrow system where

the microclimate is stable (Bennett et al., 1988). Animals resting inside a nest at a stable temperature may be unable to detect temperature changes to trigger an activity bout (Oster et al., 2002; Šklíba et al., 2014). Temperatures in the nest may be higher than the surrounding burrow system due to increased heat generated through huddling and body heat of animals confined in a small space (Buffenstein and Yahav, 1991; Kotze et al., 2008). Hazlerigg and Tyler (2019) suggested that the better a retreat insulates an animal against environmental extremes, the less information the animal may obtain about external conditions and when to emerge. However, the relatively shallow nests of the Natal mole-rat may experience greater temperature fluctuations despite the thermal benefits of huddling (Marhold and Nagel, 1995). Exact thermal conditions inside mole-rat nests are still unknown and would be of interest to understand thermoregulation and activity patterns.

Seasonal changes in environmental characteristics may also affect locomotor activity such as the availability of food, and specifically in the subterranean ecotope, changes in humidity and gas composition in the microhabitat of the burrows. Decreased food availability during the warmer late dry season caused a related decrease in outside the nest forays in the Ethiopian giant root rat (*Tachyoryctes macrocephalus*) which feeds on aboveground grasses and forbs (Vlasatá et al., 2017). Vlasatá et al. (2017) concluded that food caching and availability may be of minimal importance compared to thermoregulatory demands. The seasonal differences in activity observed in this study are unlikely to be affected by seasonal food availability since mole-rats rely upon the underground storage organs of plants (tubers and corms, collectively referred to as geophytes) for their nutritional needs (Bennett and Faulkes, 2000). These geophytes are available year-round, and bathyergids famously cache food, which root rats tend not to do, and caching is presumed to provide the group with resources through droughts and periods of decreased availability (Bennett and Faulkes, 2000). Seasonal changes in carbon dioxide and oxygen concentrations could potentially affect locomotor activity. The seasonal fluctuation of burrow gasses of bathyergids is understudied and completely unknown at the study site. Levels of carbon dioxide may increase during periods of rain due to increased soil moisture and decreased gas permeability, which may in turn trigger increased digging activity (Burda et al., 2007). While our study did not find an effect of rainfall on locomotor activity, our methods prevented an assessment of digging behavior. A study on seasonal changes in gas composition in the burrows of the giant root rat that lives at a similar elevation, climate, and annual rainfall to our study site found minimal fluctuations in carbon dioxide and oxygen levels between the seasons (Šumbera et al., 2020). Furthermore, humidity was not found to change between seasons in the burrows of the silvery mole-rat (*Heliophobius argenteocinereus*) in Malawi (Šumbera et al., 2004).

Natal mole-rats are among the few heterothermic mammals, exhibiting the lowest body temperatures of bathyergids, the narrowest thermal neutral zone, and one of the highest resting metabolic rates in bathyergids (Bennett et al., 1993b; Šumbera, 2019; Oosthuizen et al., 2021). However, the thermal neutral zone of bathyergids may increase at higher altitudes,

and a lower body temperature has been suggested to be an adaptation to colder environments, enabling animals to expend less energy maintaining their body temperature at lower ambient temperatures (Broekman et al., 2006). We found that soil temperatures in foraging tunnels vary between 5 and 30°C below their thermal neutral zone during summer and winter, respectively, and these physiological adaptations may be an evolutionary response to shallow tunnels and the resultant seasonal climate fluctuations they experience. Body temperature was found to fluctuate with activity periods in laboratory studies, but the authors could not determine if activity caused an increase in body temperature (Haupt et al., 2017; van Jaarsveld et al., 2019). Digging and other burrow maintenance behaviors may increase body temperature in mole-rats (Zelová et al., 2010; Okrouhlík et al., 2015). Mole-rats living in shallow tunnels which experience greater temperature variation may need to be selective in their activity periods to prevent overheating due to their poor heat dissipating abilities (Luna et al., 2020; Wallace et al., 2021). However, increased soil moisture during summer may provide a cooling effect *via* conduction to decrease body temperature during or after activity bouts by pressing their ventral surface against a cooler surface (Okrouhlík et al., 2015; Vejmělka et al., 2021). Seasonal changes in daily activity would be an effective thermoregulatory adaptation to reduce the risk of overheating while digging (Goldman et al., 1997; McGowan et al., 2020). Locomotor activity was concentrated during periods of lower temperatures in laboratory studies (van Jaarsveld et al., 2019; Hart et al., 2021) and in periods of moderate temperature in wild studies (this study, Šklíba et al., 2014, 2016b). It cannot be determined if body temperature fluctuated due to fluctuations in burrow temperature or as a response to activity, or a combination of the two. Therefore, we cannot discern if a drop in body temperature stimulated animal activity to increase core body temperature *via* thermogenesis (Block, 1994) or if body temperature increased due to rising burrow temperatures as seen in naked mole-rats (Buffenstein and Yahav, 1991). While increased body temperature may be associated with higher activity levels in other rodents, circadian rhythms of body temperature may not result from daily activity rhythms (Refinetti, 1999; Refinetti and Kenagy, 2018). Future studies should attempt to determine the correlates of body temperature, tunnel temperature and activity patterns utilizing temperature loggers, RFID technology, or accelerometers (Williams et al., 2020).

Laboratory studies found differences in activity patterns between the sexes (Haupt et al., 2017; van Jaarsveld et al., 2019), yet we did not find sex-biased differences in activity patterns in colonies of free-ranging Natal mole-rats. Our study did identify an effect of body size on activity similar to radio-telemetry studies on wild mole-rats of the genus *Fukomys* which found that larger individuals were less active than smaller ones (Lovegrove, 1988; Lövy et al., 2013; Šklíba et al., 2016a). However, Francioli et al. (2020) found that body mass did not predict activity even though reproductive individuals were overall heavier than non-reproductive individuals. Relatedly, we did not find a difference in activity patterns between reproductive and non-reproductive individuals contrary to studies on the social mole-rats of the genus *Fukomys* which found that reproductive

individuals were less active than non-reproductive individuals (Lovegrove, 1988; Oosthuizen and Bennett, 2015; Šklíba et al., 2016a; Van Daele et al., 2019; Francioli et al., 2020; Houslay et al., 2020). These results may indicate that reproductive individuals of *Fukomys* mole-rats must regulate energy expenditure between reproductive and burrow defense tasks. In Damaraland mole-rats reproductive suppression is controlled by physiological and behavioral mechanisms. Ovulation is blocked in non-reproductive females, but incest avoidance prevents breeding in males (Bennett et al., 1996; Molteno and Bennett, 2000). This suppression remains until the individual disperses from the group or an unrelated individual joins the group. In contrast, Natal mole-rats lack physiological reproductive suppression, and incest avoidance or aggressive interactions appear enough to prevent inbreeding (Oosthuizen et al., 2008). The similarities in activity patterns in Natal mole-rats may indicate more equal contributions to burrow maintenance and other cooperative behaviors between dominant and subordinate group members. It may also indicate that food resources are not as limited or scattered at the study site compared to the Kalahari where Damaraland mole-rats occur. Therefore, reproductive Natal mole-rats may not be required to conserve energy by reducing activity or relying on other group members to collect food. It may also indicate that reproductive individuals may need to be constantly on guard to prevent the intrusion of non-group members since groups are much closer together at this study site (Finn, unpublished data).

Activity patterns in social species may be affected by social cues, and social mole-rats observed singly may exhibit up to double the activity period of those in a complete colony (Hickman, 1980; Riccio and Goldman, 2000; Lövy et al., 2013). The findings of Lövy et al. (2013) particularly highlight the importance of maintaining social groups during observations. The authors found that a solitary female giant mole-rat (*Fukomys mechowii*) in natural conditions was more active during midday when temperatures were highest, compared to complete family groups which were more active at night (Lövy et al., 2013). Social mole-rats conserve heat *via* huddling and are more prone to heat loss during periods of lower burrow temperatures (Šumbera, 2019). Lövy et al. (2013) proposed that being active during the hottest part of the day minimized heat loss because solitary individuals cannot conserve heat *via* huddling. Mole-rats can shed excess heat from their feet, ventral surface, and *via* behavioral adjustments, such as rolling on their back or pressing their belly to the cooler soil of the tunnel (Vejmělka et al., 2021). This dissipation stays relatively constant regardless of ambient temperature. When ambient temperature drops, heat can be conserved through the vascular constriction in their feet, changes in body posture, or huddling with conspecifics (Kotze et al., 2008; Vejmělka et al., 2021). The fur of common mole-rats (*Cryptomys hottentotus*) was found to have better insulating properties compared to related species that live in tropical environments, and this may be an adaptation to greater fluctuations in burrow temperatures (Vejmělka et al., 2021). Behavioral thermoregulation (huddling, changes in body posture, or moving to warmer or colder areas of the burrow) may help mole-rats to maintain body temperature within their thermal neutral zone. It can be assumed that a decrease in body temperature may trigger locomotor activity, forcing an animal to become active to increase its body temperature (Block, 1994). However, mole-rats huddle with other group members in a nest during rest periods to conserve heat (Kotze et al., 2008). Thus, it would be unlikely that body temperature would significantly decrease while huddling. Therefore, mole-rats may time their periods of wakefulness and rest to coincide with daily burrow temperatures. Future studies investigating activity patterns in social mole-rats should maintain group cohesion to account for the effects of social cues on activity patterns. RFID technology can easily be applied in a laboratory setting to track individual activity patterns of group-living animals with the implementation of new open-source and 3D printing technology to decrease costs (Schielke et al., 2012; Habedank et al., 2021).

Predation avoidance has been suggested to affect activity patterns in other small mammals such as voles (*Microtus ochrogaster*, Getz, 2009). Activity periods for black-backed jackals (*Canis mesomelas*) and serval (*Leptailurus serval*), both known predators of Natal mole-rats, were primarily nocturnal during winter and crepuscular during summer (Ramesh and Downs, 2013; Humphries et al., 2015). Our results indicated that mole-rats reduced activity during these periods in winter, but during summer were more active during predator activity periods. In both serval and jackal, mole-rats make up < 5% of their diet and may be a seasonally available prey item (Humphries et al., 2015; Ramesh and Downs, 2015). Therefore, it is more likely that the low instances of predation on mole-rats by serval and jackal are due to a low encounter rate or greater effort required for capture, instead of mole-rats altering their daily activity patterns in response to predation pressure.

van Jaarsveld et al. (2019) argued that tunnel breaches did not occur frequently enough to allow circadian rhythm entrainment *via* photoperiod. However, exposure to light pulses at infrequent intervals was enough to entrain the circadian rhythm in other subterranean rodents (Decoursey and Menon, 1991; Rado et al., 1993). Recent work in South American tuco-tucos (*Ctenomys* aff. *knighti*) has shown that individuals may entrain their circadian rhythms by infrequent exposure to light during surface excursions (Jannetti et al., 2019; Flôres et al., 2021). At the study site, many groups of mole-rats occupied areas covered with a thick carpet of grass which would require chewing through before soil could be extruded from the burrow. Most mounds encountered at this site had a neat circular hole leading into the tunnel, indicating that mole-rats may spend considerable effort to "clean" the tunnel exit (K. Finn, A. Janse van Vuuren, pers. obs). Eloff (1951) noted that *Cryptomys* would "open a hole to the surface [and] put its nose out" during mound construction. At the study site, mole-rats continued to make mounds through the dry season, likely due to increased soil moisture of mesic grasslands in the Natal region compared to more arid regions of South Africa (K. Finn, A. Janse van Vuuren, pers. obs.). Thus, mole-rats may be exposed to light throughout the year during mound construction. A family group of mole-rats may produce between 6 and 7 mounds per day during the rainy season (Genelly, 1965; Jarvis et al., 1998). During mound construction, one individual may lead digging while other group members assist with sweeping excess soil away and most individuals in

a group contribute to digging (Zelová et al., 2010). Since light may penetrate along the tunnel for less than a meter when the surface is breached (Kott et al., 2014), other individuals in the digging chain may be exposed to light instead of just the lead digging animal. Therefore, whether light or temperature is the dominant entrainer of circadian rhythms in mole-rats remains unclear.

This study used RFID technology as a non-invasive method to monitor activity patterns in a wild subterranean mammal. This method can be easily adapted to monitor activity in small mammals in areas that may involve safety risks to researchers, such as African reserves occupied by large predators. Hickman (1980) recommended studies to understand the effects of temperature and other non-photic cues on activity patterns in subterranean mammals. Surprisingly, in the 40 years since this suggestion, few laboratory studies have explored the effects of varying ambient temperatures on activity and only recently started gaining attention (Goldman et al., 1997; Haupt et al., 2017; van Jaarsveld et al., 2019; Flôres et al., 2021; Hart et al., 2021). There have been many field studies in various subterranean species where burrow or soil temperatures and activity patterns were measured (Gettinger, 1984; Cameron et al., 1988; Lovegrove, 1988; Šklíba et al., 2007, 2014, 2015, 2016b; Lövy et al., 2013; Vlasatá et al., 2017; Jannetti et al., 2019), with only a handful combining both laboratory and field observations (Rezende et al., 2003; Flôres et al., 2021). Results from studies on activity may differ significantly between wild and laboratory subjects of the same species due to artificial laboratory conditions (Rezende et al., 2003; Šklíba et al., 2014 and references therein). To date, only two studies have employed daily temperature fluctuations during observation while keeping animals under constant darkness (van Jaarsveld et al., 2019; Hart et al., 2021); however, these studies were on isolated individuals of a social species. One study has mimicked the periodic light pulses from mound construction (Rado et al., 1993), but they maintained a constant temperature during the experiment. Laboratory studies would benefit from maintaining social species in a tunnel-like atmosphere (*sensu* Zöttl et al., 2016) kept under constant darkness with and without infrequent light pulses and exposed to daily temperature fluctuations to simulate natural variations they experience in foraging tunnels during both winter and summer. The ingenious addition of photo-sensors and accelerometers on wild subterranean mammals (Flôres et al., 2021) would provide valuable data on individual daily activity bouts, quantify exposure to light, and shed light on the effects of photoperiod on the entrainment of circadian rhythms in bathyergids.

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AUTHOR CONTRIBUTIONS

KF, DH, NB, and MZ conceived and designed the study. KF, AJ, and TS collected the data. KF and DH analyzed the data. NB and MZ provided funding for research. KF wrote the first draft of the manuscript. All authors contributed to revisions.

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SUPPLEMENTARY MATERIAL

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Parasite-Mediated Mate Preferences in a Cooperatively Breeding Rodent

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Females of many species discriminate among males when choosing a mate and this can bear indirect and direct benefits including the avoidance of parasite transmission from infested males. In rodents, this may be mediated by androgen hormones that affect the expression of urinary odors. Female choosiness may also vary with a female's infestation status, with infested females being less choosy. In the current study we tested the preference of cooperatively breeding highveld mole-rat (*Cryptomys hottentotus hottentotus*) females for male urinary odors from healthy males and those naturally infested with a cestode (*Mathevotaenia* sp.). Thirty females (15 healthy, 15 infested) were allowed to explore a Y-maze with urine samples from healthy and infested males and the frequency of entering choice arms and chambers as well as the duration spend with each odor sample was recorded. Infestation status did neither affect male body mass, urinary testosterone, nor cortisol levels or the body condition of females. Although overall female activity was not affected by infestation status, infested females entered choice arms and chambers significantly less frequently than healthy females. Surprisingly, healthy females preferred odors from infested males while the opposite was true for infested females, independent of male hormone levels. As the study species lives in groups that tend to share the same infestation status, we suggest that highveld mole-rat females may exhibit a preference for unfamiliar odors, possibly as an indicator of genetic diversity, rather than discriminate between infestation status of males. Similar mechanisms may also play a role in other social species.

Keywords: Bathyergidae, mate choice, sociality, parasite, *Mathevotaenia*, androgen

INTRODUCTION

In many species, females actively discriminate between potential mates (Andersson, 1994). Such mate choice decisions can have both indirect and direct benefits for choosing females (Jennions and Petrie, 1997; Kokko et al., 2003). The former include the transmission of genes that increase offspring fitness by increasing their fecundity, survival and/or mating success (Jennions and Petrie, 1997). Direct benefits can include the acquisition of nutritional benefits through nuptial gifts or paternal care, but also preventing parasite transmission by avoiding infested males (Able, 1996).

Female mate choice is often based on elaborate secondary sexual traits as the costs of such traits can only be sustained by high-quality males (i.e., those in good condition and/or without parasites) (Hamilton and Zuk, 1982; Folstad and Karter, 1992). While in many bird, fish and reptile species females may glean information about a male's infestation status from visual signals such as their nuptial coloration and courtship displays, olfactory cues from various glands, feces and

urine replace these in mammalian species (Penn and Potts, 1998). Olfactory signals can provide information about attributes such as sex, reproductive status, individuality as well as body condition and constitute the most important source of social information in rodents (Johnston, 2003; Ferkin, 2018).

Rodent urine contains a variety of volatile and non-volatile compounds that are under androgen control (Kavaliers et al., 2005). Infection-related changes in condition (e.g., body mass) and endocrine status (e.g., testosterone and/or cortisol) (Willis and Poulin, 2000; Zala et al., 2004; Litvinova et al., 2005) can affect the quality and quantity of these volatiles, providing information to potential mates (Kavaliers et al., 2005; Kavaliers and Choleris, 2018). A number of studies in rodents have reported that females avoid odors of males infested with viruses, bacteria, arthropods or helminth (reviewed in Beltran-Bech and Richard, 2014).

Female preference for healthy males is not always apparent, however, and can be modified by a variety of factors including the costs of being choosy, a female's condition as well as social cues or the mating system (Poulin and Vickery, 1996; Jennions and Petrie, 1997; Klein et al., 1999; Buchholz, 2004; Beltran-Bech and Richard, 2014; Kavaliers and Choleris, 2018). In nature, female mate choice may entail energetic costs of traveling to sample several males, but also to the related increased exposure to predators and/or environmentally transmitted pathogens and parasites (Milinski and Bakker, 1992; Jennions and Petrie, 1997; Kokko et al., 2003). Thus, encounter probability rather than infestation status of males may determine female mate choice. However, even under benign laboratory conditions females may exhibit a lack discrimination or even a preference for infested males (Penn et al., 1998; Klein et al., 1999; Moshkin et al., 2002; Gourbal and Gabrion, 2004; Mazzi, 2004; Barthélémy et al., 2005; Dass et al., 2011; Lai et al., 2016). This could be a result of social cues from other individuals and experiments in laboratory mice (*Mus musculus*) have shown that exposure to odor cues from parasitized males prior to mate choice can reduce the avoidance behavior of healthy females (Kavaliers et al., 2003). Alternatively, the concomitant presence of female odors can increase the attractiveness of odors of infested males (Kavaliers et al., 2006).

As parasites obtain access to resources at the expense of their host (Poulin, 2007), infested females may be constrained in their ability to express mate choice (Poulin and Vickery, 1996). Only a limited number of studies have addressed this hypothesis (Beltran-Bech and Richard, 2014). Some that have tested this hypothesis report a lack of discrimination between healthy and infested males by infested females (Poulin, 1994; López, 1999; Mazzi, 2004; Aguilar et al., 2008) while others found a preference for infested males (Pfennig and Tinsley, 2002). Several of the former studies noted lower condition and/or activity levels of infested females suggesting energetic constraints on mate choice for these females (e.g., Poulin, 1994; López, 1999). Alternatively, the sensory capabilities of infested females may be impaired (Poulin and Vickery, 1996; Klein, 2003).

The majority of studies into parasite-mediated female mate choice in rodents have been conducted on classic laboratory animals, particularly mice (Beltran-Bech and Richard, 2014). However, the use of olfactory cues as an information source concerning parasitic infection status in wild rodents is not well understood. Here, we investigated the scent preferences of highveld mole-rats (*Cryptomys hottentotus pretoriae*) a member of the African rodent family Bathyergidae. This subterranean species is restricted to the summer-rainfall highveld regions of South Africa (Bennett and Faulkes, 2000). Highveld mole-rats are cooperative breeders that live in colonies of up to 12 individuals (Bennett and Faulkes, 2000). Non-breeding males are prevented from breeding due to incest avoidance but are physiologically indistinguishable from breeders (van Rensburg et al., 2003). Female highveld mole-rats are reflex ovulators that require the tactile stimulation of copulation to induce ovulation and non-breeding females are physiologically suppressed within their natal colonies (Malherbe et al., 2004). Nevertheless, non-breeding females of several mole-rat species, including highveld mole-rats, readily engage in sexual activity with unfamiliar and/or unrelated males (Bennett et al., 1996, 1997, 2000; Clarke and Faulkes, 1999; Greeff and Bennett, 2000; Clarke et al., 2001; Bappert et al., 2012, Butler and Bennett, personal observation). This is likely an adaptation to the low encounter probability with unrelated males due to high energetic and survival costs of breeding dispersal in these rodents (Jarvis et al., 1994; Hazell et al., 2000). The ability of females to discriminate relatedness based on urinary cues has been investigated for several mole-rat species (Clarke and Faulkes, 1999; Bappert et al., 2012), but to date no study has addressed whether female mole-rats can discriminate between healthy and infested males and exhibit a preference for the former. Hence, the aim of this study was to assess how parasite infestation affects female odor preference and the mechanisms by which this may be mediated. We investigated whether wild-caught female highveld mole-rats displayed a preference for urinary cues from naturally healthy compared to infested males. Furthermore, we evaluated whether such preferences were linked to male body condition (using body mass as proxy) and/or urinary hormone (i.e., testosterone and cortisol) levels. We also assessed the effects of parasitism on the ability of a female to distinguish between odors. Highveld mole-rats of our study population are parasitized by three helminth species with cestodes (*Mathevotaenia* sp.) being the predominant endoparasites (Viljoen et al., 2011). Hence, we used naturally infested individuals in our study that represent conditions of a chronic infestation. We predicted that (1) females would be able to distinguish between males of different infestation status and that (2) healthy females would exhibit a preference for odor cues from healthy males while (3) infested females would have an impaired ability to discriminate (e.g., lower activity levels) and not exhibit strong preferences for olfactory cues from either infested or healthy male. Previous studies have shown that chronic infestation with *Mathevotaenia* sp. results in reduced urinary testosterone levels in male highveld mole-rats, while it did not affect urinary cortisol levels (Lutermann et al., 2012). Consequently, we hypothesized that (4) such preferences would be linked to urinary hormone levels and females would prefer urinary cues from males with high testosterone, but lower cortisol levels.

MATERIALS AND METHODS

Animal Capture and Maintenance

Mole-rats were collected from areas in the suburbs of Pretoria in Gauteng Province, South Africa (25°45′ S, 28°10′E) from October 2008 through to September 2009. Individuals were captured using modified Hickman live traps (Hickman, 1979) baited with sweet potato and checked twice daily. All captured animals were weighed to the nearest 0.1 g (Scout Pro SPU123, Ohaus Corporation, United States) and sexed. Captured animals were housed in their colonies in plastic containers (100 × 75 × 50 cm) in a temperature-controlled room at 25 ± 1°C and under a 12L: 12D lighting regime. Colonies are generally comprised of one breeding female (readily identified by her enlarged teats and perforated vagina), one, sometimes more, breeding males and their non-breeding offspring. Colony sizes ranged from three to seven individuals. Wood shavings were used as bedding and sweet potato, carrots and apple were available on an *ad libitum* basis. In our study population, two nematode species have been recorded at low prevalence (both: 4.4%) and mean abundance (*Protospirura* sp.: 0.08, *Heligmonina* sp.: 0.06) compared to *Mathevotaenia* (prevalence: 71.9%, abundance: 8.04) with the latter also likely to be more damaging due to their substantially larger size (Viljoen et al., 2011). Cestodes require an intermediate host, usually an arthropod, and cannot directly be transmitted. The intermediate host and life-cycle for *Mathevotaenia* sp. is unknown and hence, we could not employ artificial infestation of our study animals. The infestations of wild-caught individuals were identified based on shedding in feces represent chronic infestations. In contrast to acute infestations, hosts with chronic infestations have likely made physiological adjustments to restore homeostasis and minimize the costs of parasitism (Lutermann et al., 2012).

Determination of Infection Status and Urine Collection

For the determination of the infestation status and urine collection (see below), animals were removed from their colonies and placed individually in a spherical plastic container (height: 20 cm; radius: 9.6 cm) with a wire mesh base and a plastic collection tray placed underneath. Droppings deposited on the wire mesh base of these chambers were examined for the presence of proglottids. This method has been shown to allow a high accuracy in the identification of *Mathevotaenia* sp. infestation (Viljoen et al., 2011). To further ensure that individuals classified as healthy were indeed free of cestodes they were injected subcutaneously with 2 µl/g body mass Ivermectin (Noromectin®, Norbrook Laboratories, Centurion, South Africa) while infested individuals received an injection of saline solution. We allowed a 2-week period to elapse before the infestation status was confirmed by an additional examination of feces. Once their infestation status was confirmed urine was collected from 30 males (15 healthy, 15 infested). We provided apples as a source for energy and moisture during urine collection. Traps were checked at hourly intervals and any urine deposited in these trays was collected using pipettes,

transferred to Eppendorf tubes and stored at –20°C until used in the experiments.

Odor Preference Tests

The odor preferences of 15 healthy and 15 infested non-breeding female mole-rats were determined in a clear Perspex Y-maze. The maze consisted of 3 squares boxes (20 × 20 × 20 cm, one start chamber and two choice chambers) connected with Perspex tubing (7.4 cm diameter). The start box, with a removable door, was connected to a 20 cm long tube, which in turn branched into two 50 cm long tubes (choice arms). Both tubes terminated in a choice chamber. Each choice chamber contained a urine sample (1 ml) from either a healthy or infested male applied to 20 g of fresh sawdust.

Only non-breeding females were used and the odors presented to them originated from males that were unfamiliar to them and each female was tested only once. The identity of males providing urine samples was changed for each trial to reduce the risk of individual factors other than parasite load having an effect on odor preference. We alternated between the left and right choice chamber for the infested odor stimulus, to minimize potential side biases. Due to the substantial effort required to capture a sufficient number of infested, unfamiliar individuals, we used urine from each male twice, once for the non-infested and once for the infested females, and retained the same combinations.

All females were separated from their colonies for 6 h prior to the onset of a preference test to allow for habituation to isolation. We transferred females to the start chamber and allowed them to acclimatize for 15 min. Once this time elapsed, we placed urine samples in the choice chambers and opened the start chamber, giving the female access to the maze. Females had unrestricted olfactory and physical contact with the odor samples during trials. We observed female behavior for 20 min and recorded the following variables: (1) Initial choice arm entered; (2) time spent in each arm and chamber; (3) number of times an arm or chamber was entered; and (4) time spent being active (i.e., not motionless for more than 5 s). Between tests, we cleaned the Y-maze thoroughly with soapy water and 70% ethanol.

Hormone Assays

We determined the creatinine concentrations of all urine samples prior to the hormone analyses to correct for urine dilution (Bonney et al., 1982). Since creatinine is a breakdown product of tissue proteins, it is assumed to be released at a constant rate. This provides a means to standardize samples of different dilutions. We dispensed duplicate aliquots (7 µl) of urine or standards into wells in a microplate together with 210 µl freshly prepared alkaline picric reagent (1 volume alkaline triton solution: 1 volume saturated picric acid: 10 volumes deionized distilled water). The microplate was covered and allowed to stand for 2 h to allow for color development. Subsequently, we measured light absorption at 492 nm on a plate reader. The results are expressed as mmol/ml urine. Samples were processed in two separate assays. The average intra-assay coefficient of variation for both assays was 10.2% and the inter-assay coefficient of variation was 17.0%.

We determined the hormone concentrations using commercial radioimmunoassay kits (Coat-A-Count, Diagnostic

Products, United States) that have been previously validated for the study species (Lutermann et al., 2012). The antiserum is highly specific for testosterone, with a cross reactivity for dihydrotestosterone being less than 5%. A series of known calibrators of testosterone were assayed to set up a standard curve. Urine samples (50 µl) and 125I-labeled testosterone (1,000 µl) were added to assay tubes in duplicate and briefly vortexed. Assay tubes were incubated in a water bath (37°C) for 3 h. Bound and free 125I-labeled testosterone were decanted and the bound fraction on the tubes counted on a Cobra gamma counter for a minute. A calibration curve was used to convert the counts into testosterone concentrations. A serial double dilution of a sample containing a high concentration of testosterone paralleled the standard curve (ANCOVA: $F = 2.8$, $P > 0.05$) following log-logit transformation of the data (Chard, 1978). The sensitivity of the assay was 0.04 nmol/l and the intra-assay coefficient of variation was 7.5% and inter-assay coefficient of variation 12.1%.

Cortisol was measured using a Coat-A-Count cortisol kit (Diagnostic Products Corporation, United States) using the same procedure as described above for the testosterone assay. A volume of 25 µl of urine was used. The assay tubes were, however, incubated in the waterbath (37°C) for 45 min. A serial double dilution of the sample containing a high concentration of cortisol paralleled the standard curve (ANCOVA: $F = 1.34$, $P > 0.05$). The sensitivity of the assay was 4.82 nmol/l. The average intra-assay coefficient of variation was 24.71% and the inter-assay coefficient of variation was 22.12%.

Statistical Analyses

We tested for possible effects of cestode infestation on body condition (measured as body mass) and hormone levels and the time females were inactive with t-tests. Hormone data were log-transformed prior to analyses to achieve a parametric distribution. Due to the large variance observed in values for both hormones we evaluated possible sample size limitations on the outcome of these tests by calculating effect sizes in and computing the *post hoc* power in G*Power 3.1.9 (Faul et al., 2007, 2009). In order to determine whether any initial choice preferences existed for healthy and infested females we compared the first-choice chamber entered (i.e., healthy or infested male odor) using a χ^2-test. These statistical analyses were carried out in IBM SPSS (version 25).

To evaluate the effect of female and male infestation on the frequency to enter a choice arm or a choice chamber we employed generalized linear mixed models (GLMMs) with a Poisson distribution and a log-link function. Female and male infestation status (healthy/infested) as well as the 2-way interaction between these variables were added as independent variables in the model. In addition, the concentration of testosterone and cortisol were added as covariates. Female ID as well as the ID of the male combination were included as random effects to control for repeated measurements. We converted the time spent in the choice chambers to proportions before analyzing the effects of female and male infestation status as well as male hormone levels on female preferences using a general linear mixed model.

These analyses were carried out with the statistical software R (version 3.6.1, R Core Team 2019)[1] using the R package lme4 (Aguiar and Sala, 1998). The corresponding p-values were calculated with the R package car (Fox and Weisberg, 2011).[2] We used the R package emmeans for *post hoc* multiple comparisons with Tukey's HSD (Lenth, 2019).[3] We simplified models by sequentially dropping non-significant terms using the drop1 function in R to obtain the most parsimonious model based on the lowest AIC value (Burnham and Anderson, 2002). P-values of ≤ 0.05 were considered to be significant and results are reported as mean $\pm$ standard error (SE).

RESULTS

Male Traits

There was no significant difference between the body masses of healthy (131.5 ± 6.4 g) and infested males (118.1 ± 8.5 g, $n = 30$, $t = 1.25$, df $= 28$; $p = 0.220$). Furthermore, healthy (139.8 ± 21.5 nmol/mmol creatinine) and infested males (105.6 ± 19.92 nmol/mmol creatinine) did not differ significantly in their testosterone levels ($t = -1.22$, df $= 28$; $p = 0.234$). The effect size for this comparison were moderate (Cohen's $d = 0.44$) and the power low at $\beta = 0.282$. Although healthy males tended to have lower cortisol levels (264.4 ± 86.6 nmol/mmol creatinine) than infested males (554.7 ± 153.3 nmol/mmol creatinine) this was not significant ($t = -1.83$, df $= 28$, $p = 0.078$). The effect size for this comparison were fairly high (Cohen's $d = 0.67$) and the power moderate at $\beta = 0.558$. There was no correlation between the testosterone and cortisol levels of males ($R_S = 0.050$, $n = 30$, $p = 0.803$). Differences in testosterone levels between healthy and infested male pairs varied widely (mean $T_{healthy} - T_{infested}$: -34.20 ± 33.52 nmol/mmol creatinine, range: -235.96 to 345.73). Similarly, the difference in cortisol level between healthy and infested males varied widely (mean $T_{infested} - T_{healthy}$: 290.28 ± 188.68 nmol/mmol creatinine, range: -922.21 to 1971.78).

Parasite Effects on Females

Body mass did not differ significantly between healthy (98.7 ± 3.5 g) and infested females (87.3 ± 5.1 g, $t = 1.86$, df $= 28$, $p = 0.074$). Ten of the healthy females and eight of the infested females first entered the chamber with the odor from a healthy male. There was no significant difference in the initial choice to enter a chamber between healthy and infested females ($\chi^2 = 0.456$, df $= 1$, $p = 0.710$).

Female infestation status did not significantly affect the time spent inactive during the preference trials (healthy: $26.75\% \pm 4.88$, infested: $41.34\% \pm 7.11$, $t = -1.694$, df $= 28$, $p = 0.101$). However, the frequency to enter a choice arm was significantly larger for healthy (15.1 ± 1.0) compared to infested females (6.0 ± 0.7, Wald $\chi^2 = 41.7886$, df $= 1$, $p < 0.0001$, **Figure 1**). Conversely, the infestation status of the male had no

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[1]https://www.R-project.org/

[2]http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

[3]https://CRAN.R-project.org/package=emmeans

FIGURE 1 | Frequency of **(A)** entering the choice arm and **(B)** the choice chamber with the odor of a healthy (open bars) or infested male (solid bars) for healthy and infested female highveld mole-rats.
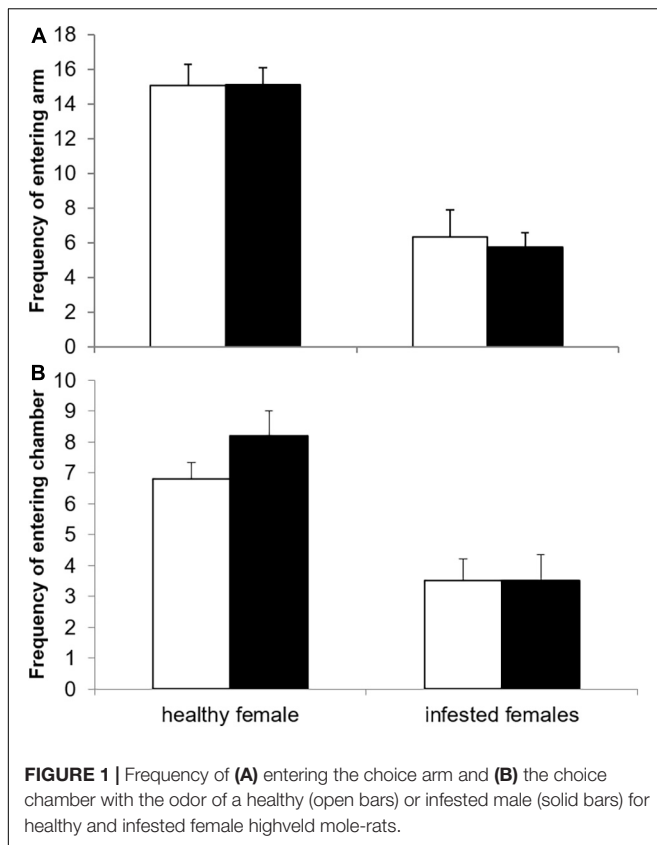
significant effect on the frequency of entering the choice arm and this variable was dropped from the final model. However, the frequency of entering the choice arm decreased slightly, but significantly, with increasing testosterone level (estimate: -0.0013 ± 0.0006, Wald $\chi^2 = 4.1224$, df = 1, $p = 0.042$). The remaining factors including the variable cortisol were all dropped from the final model (**Table 1**).

The infestation status of the female was the only variable retained in the final model for the frequency to enter the choice chamber (**Table 1**). It was significantly higher for healthy females (7.5 ± 0.5) compared to infested females (3.53 ± 0.5, Wald $\chi^2 = 19.391$, df = 1, $p < 0.0001$, **Figure 1**).

Neither the female [$F_{(1, 28)} = 0.00$, $p = 1.00$], nor the male infestation status [$F_{(1, 27)} = 0.74$, $p = 0.396$] had a significant effect on the proportion of time a female spent with an odor sample. However, there was a significant interaction between female and male infestation status [$F_{(1, 27)} = 15.19$, $p = 0.0006$, **Figure 2**]. Healthy females spend significantly more time with the odor of the parasitized male than that of the healthy male ($t = 2.94$, df = 28, $p = 0.032$, **Figure 2**). In contrast, there was no significant difference in the proportion of time spent with an odor of healthy and infested males for infested females ($t = 0.30$, df = 28, $p = 0.990$). As a result, healthy females spent significantly less time with a healthy male's odor compared to infested females ($t = 2.75$, df = 28, $p = 0.050$) while the opposite was true for the odor of an infested male ($t = -2.75$, df = 28, $p = 0.048$). Furthermore, the time spent with a male odor was not affected

significantly by the level of cortisol [$F_{(1, 27)} = 3.90$, $p = 0.059$]. The variable testosterone was dropped from the final model (**Table 1**).

DISCUSSION

In accordance with our first prediction, female highveld mole-rats were able to distinguish between the urinary cues from healthy and infested males. However, contrary to several studies in laboratory mice and rats (Kavaliers and Colwell, 1995a,b; Penn et al., 1998; Willis and Poulin, 2000; Ehman and Scott, 2001) and in contrast to our second prediction healthy females spent more time inspecting odor cues from infested males. Although a lack of distinct preference of healthy rodent females has been reported in several studies (Klein et al., 1999; Gourbal and Gabrion, 2004; Ilmonen et al., 2009), clear preferences for infested male rodents have rarely been observed (Moshkin et al., 2002; Barthélémy et al., 2005; Dass et al., 2011). For mice infested with the protozoan *Toxoplasma gondii*, or the tick-borne encephalitis (TBE) virus such reversed preferences coincided with infection-related increases in male testosterone levels (Moshkin et al., 2002; Dass et al., 2011). Thus, they may be a result of parasite manipulation. Such manipulation would increase pathogen/parasite transmission to a sexual partner (*T. gondii*) or a vector (TBE virus) as a result of testosterone-driven increases in sexual behavior and roaming for mates (Lutermann, 2019). However, a similar mechanism is unlikely to apply in the study species as testosterone levels did not vary systematically with infestation status. In addition, testosterone-driven roaming behavior is severely restricted in the subterranean niche and such behavior would not assist the transmission of a parasite with an indirect life cycle such as *Mathevotaenia*.

It has been suggested that anthelminthic treatment with ivermectin may affect the host microbiome which could affect fecal odor (He et al., 2018). However, although several other studies report an effect of helminth parasites on microbiome communities, they could not find an effect of the drug on microbiome composition (Schneeberger et al., 2018; Peachey et al., 2019; Fujishiro et al., 2020; Hu et al., 2020). Hence, we are confident that our anthelminthic treatment did not unduly affect the urinary odor profiles of the males in our study. Similarly, ivermectin has been shown to affect the olfactory bulb in the brain of laboratory mice when injected (Obenhaus et al., 2016). However, this effect was only temporarily and no longer detectable after 7 days (Obenhaus et al., 2016). Consequently, our treatment is unlikely to have affected the ability of females to distinguish between healthy and infested males as the time period elapsed between treatment and choice experiments substantially exceeded 7 days. This hypothesis is further corroborated by the fact that healthy females (i.e., those treated with ivermectin) clearly distinguished between odors from healthy and infested males.

It has been shown in free-ranging species that infested males may be preferred by females as mates based on their body size (Cramer and Cameron, 2007; Chesh et al., 2012). This could be linked to higher testosterone levels in larger males irrespective of parasite infestation (Lopes and König, 2016).

TABLE 1 | Final models for the effects of female and male infestation status and hormone levels on female *C. h. pretoriae* preferences.

Variable	Frequency entering arm			Frequency entering choice chamber			% time in choice chamber		
	F	df	p	F	df	p	F	df	p
Female infestation status	41.789	1	**<0.0001**	19.391	1	**<0.0001**	0.000	1	1.000
Male infestation status	–	–	–	–	–	–	0.744	1	0.396
Female infestation status ×male infestation status	–	–	–	–	–	–	15.189	1	**<0.0001**
Testosterone	4.122	1	**0.042**	–	–	–	–	–	–
Cortisol	–	–	–	–	–	–	3.898	1	0.059

Significant variables are highlighted in bold.

However, in accordance with a previous study in highveld mole-rats (Lutermann et al., 2012), urinary hormone levels were not correlated with male body mass in our study making this an unlikely mechanism in the study species. This could be related to the social modulation of androgens in social bathyergids.

Ilmonen et al. (2009) showed that genetic variability may play a more important role than infestation status in mate preferences of mice. Consequently, the observed pattern could be an indicator of female preference for genetically different males based either on major histocompatibility complex (MHC) genes or general genetic variability (Penn and Potts, 1998; Ilmonen et al., 2009). The expression of MHC genes also affects the blend of peptides contained in murine urine. As gene expression is modulated by parasite infestation, infestation status can also affect urinary odors (Penn and Potts, 1998; Zala et al., 2004; Stowers and Tsuang-Han, 2015). This could have affected odor attraction of healthy females (Zala et al., 2004; Kumar et al., 2014; Moore et al., 2015). However, a systematic alignment between infestation status and genetic make-up in our sample of males appears to be unlikely. Furthermore, genetic data from the closely related common mole-rats (*Cryptomys hottentotus hottentotus*) suggest that females that choose sub-optimal males with regards to genetic variability seek to compensate for this by engaging in extra-pair matings with males from neighboring colonies (Bishop et al., 2007). Currently, there is no comparable information available for the study species. However, as the sub-terranean niche substantially increases the costs of breeding dispersal a similar scenario likely applies to highveld mole-rats.

Rather than choosing based on infestation status or male quality (e.g., hormone level, body size, genetic quality) it has been suggested for several vertebrate species where healthy females do not discriminate against infested males that females requiring male care may choose males to secure parental care (Klein et al., 1999; Mazzi, 2004). In monogamous California mice parental behavior has been linked to male testosterone levels (Gleason et al., 2012). Such information may be conveyed *via* olfactory cues (Kavaliers et al., 2005; Choleris et al., 2009) and could potentially account for the weak negative correlation between testosterone levels and female preferences observed in the current study.

None of the hypotheses presented above explain why we observed entirely different preferences in infested females that, contrary to our prediction, preferred healthy males. Although healthy and infested females did not differ in their time spent

active, infested females exhibited a significantly lower degree of exploration as evidenced by their lower frequency of entering the choice arms and chambers. This could indicate that infested females do indeed experience constraints to their ability to choose (Poulin and Vickery, 1996; Jennions and Petrie, 1997; López, 1999; Beltran-Bech and Richard, 2014; Kavaliers and Choleris, 2018) while they are able to maintain a similar body mass as healthy females. However, unlike in many other studies this did not result in a lack of discrimination (Beltran-Bech and Richard, 2014), but a preference for healthy males. To the best of our knowledge there are no other examples of such strikingly opposing mate preferences between healthy and infested females in the literature. Since our study species is highly social, we propose that social cues experienced in the natal group may play a crucial role in the observed preferences. The role of social cues for mate choice decisions has previously been addressed in several laboratory studies and it has been shown that pre-exposure to males can modify olfactory preferences of both healthy and infested female mice depending on the infestation status of the odor donor (Kavaliers et al., 2003, 2006). The female highveld mole-rats tested in the current study probably only had limited exposure to potential mates due to the social system of the species and their subterranean life-style. However, as group-living species they may be exposed to the odor of related males which constitute undesirable mating partners. In addition, infestation status is frequently the same
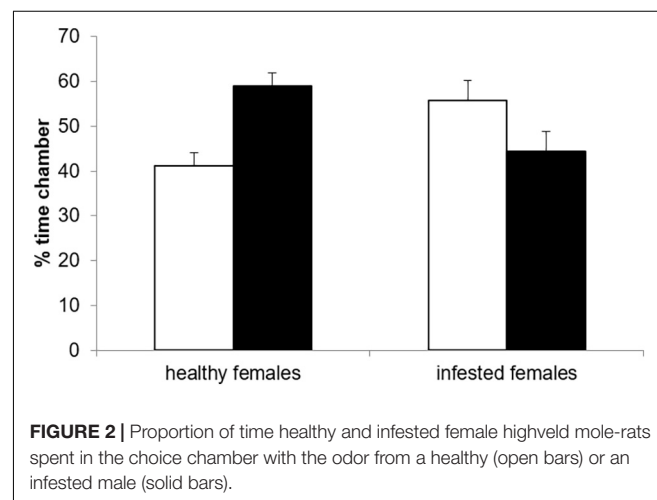
FIGURE 2 | Proportion of time healthy and infested female highveld mole-rats spent in the choice chamber with the odor from a healthy (open bars) or an infested male (solid bars).

among colony members in the Bathyergidae including the study species (Viljoen et al., 2011; Lutermann et al., 2013; Archer et al., 2016). Furthermore, a recent study suggests that in rodents the olfactory profile of healthy individuals living with sick cage mates reflects the health status of the cage mates rather than that of the healthy individual (Gervasi et al., 2018). If similar adjustments of odor profiles are present in highveld mole-rats, the observed odor preferences of both healthy (i.e., living with healthy males) and infested females (i.e., living with infested males) may reflect a preference for unfamiliar male odors (i.e., infested for healthy females, healthy for infested females) rather than a preference or avoidance of odors of infested males. For long-lived species such as African mole-rats securing an unfamiliar and hence, likely unrelated mate that can enable successful breeding dispersal as well as provide parental care may take precedence over parasite avoidance, particularly for parasites that are not directly transmitted such as cestodes. This hypothesis awaits testing in this and other social species in the future. Furthermore, the social life-style of the study species allows for buffering of the costs incurred by parasites in mole-rats (Lutermann et al., 2013). However, odor preferences do not always translate into mate preferences (Clarke and Faulkes, 1999; Gourbal and Gabrion, 2004) and the indiscriminate matings observed in a number of mole-rats including the study species (Bennett et al., 1996, 1997, 2000; Clarke and Faulkes, 1999; Greeff and Bennett, 2000; Clarke et al., 2001; Bappert et al., 2012, Butler and Bennett, personal observation) suggest that although capable of discrimination mole-rats may choose to ignore the cues used to discriminate.

Contrary to our prediction, information about the infestation status is unlikely to be linked to the expression of androgens in the study species. This may be a result of the chronic nature of the natural cestode infestation which contrasts with the acute infestation used in many laboratory studies (Beltran-Bech and Richard, 2014). This is corroborated by a previous study of body mass and hormone levels in highveld mole-rats in response to chronic cestode infestation with testosterone, but not body mass or cortisol levels differing with infestation status while only the latter increased in response to a bacterial challenge (Lutermann et al., 2012). Nevertheless, it may also be a result of sample size limitations. Our power analysis suggests that due to the large variance in testosterone levels larger samples sizes (n = 78) would have been required to identify such differences. Similar limitations applied to a lesser extent for cortisol (required n = 29) due to the greater effect size observed for this hormone. Regardless, the lack of apparent differences in hormone levels between the two groups of males should be treated with caution. The blend of pheromones and peptides contained in urine that are linked to the expression of major urinary proteins (MUPs) and MHC genes in murid rodents (Hurst, 2009; Stowers and Tsuang-Han, 2015) may be the basis for the observed discrimination. However, unlike in murid rodents MUPs concentrations are low in mole-rats (Hagemeyer et al., 2011).

Sensory deprivation in the subterranean environment renders visual and auditory cues less useful and suggests a significant role for olfactory signals in subterranean rodents (Francescoli, 2000). The strength of this selection pressure is illustrated for Bathyergidae by the high genetic variability at loci coding for olfactory receptors that has been reported for this family (Stathopoulos et al., 2014). At the same time, members of the three social genera, *Heterocephalus*, *Fukomys,* and *Cryptomys*, show little postnatal growth of the vomeronasal organ neuroepithelium, but also great within-species variability of the vomeronasal organ neuroepithelium (Dennis et al., 2020). While the current study is the first investigating the role of olfaction in a members of the genus *Cryptomys*, a number of studies in the other social genera have provided evidence for the ability of African mole-rats to discriminate between conspecifics based on olfactory cues (Heth and Todrank, 2007; Toor et al., 2015; Leedale et al., 2021). Although the basis of the observed discrimination in female highveld mole-rat remains to be investigated, the current study demonstrates the ability of a member of *Cryptomys* spp. to discriminate olfactory cues related the infestation status of conspecifics.

CONCLUSION

In conclusion, our study shows that irrespective of infestation status female highveld mole-rats are able to discriminate between healthy and infested male urinary odors. Contrary to other rodent species healthy females prefer the odors of infested males, whereas infested females prefer those of healthy males. In addition, infested females exhibited compromised activity levels. This choice is not based on infestation-related changes in male or females body mass, or male hormone levels. Instead, they may be related to social cues experienced in the natal group and this may also be relevant for other social vertebrate species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the University of Pretoria (EC 14-09) and capture permits were obtained from Gauteng Nature Conservation.

AUTHOR CONTRIBUTIONS

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Clocks Ticking in the Dark: A Review of Biological Rhythms in Subterranean African Mole-Rats

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Biological rhythms are rhythmic fluctuations of biological functions that occur in almost all organisms and on several time scales. These rhythms are generated endogenously and entail the coordination of physiological and behavioural processes to predictable, external environmental rhythms. The light-dark cycle is usually the most prominent environmental cue to which animals synchronise their rhythms. Biological rhythms are believed to provide an adaptive advantage to organisms. In the present review, we will examine the occurrence of circadian and seasonal rhythms in African mole-rats (family Bathyergidae). African mole-rats are strictly subterranean, they very rarely emerge aboveground and therefore, do not have regular access to environmental light. A key adaptation to their specialised habitat is a reduction in the visual system. Mole-rats exhibit both daily and seasonal rhythmicity in a range of behaviours and physiological variables, albeit to different degrees and with large variability. We review previous research on the entire circadian system of African mole-rats and discuss output rhythms in detail. Laboratory experiments imply that light remains the strongest *zeitgeber* for entrainment but in the absence of light, animals can entrain to ambient temperature rhythms. Field studies report that rhythmic daily and seasonal behaviour is displayed in their natural habitat. We suggest that ambient temperature and rainfall play an important role in the timing of rhythmic behaviour in mole-rats, and that they likely respond directly to these *zeitgebers* in the field rather than exhibit robust endogenous rhythms. In the light of climate change, these subterranean animals are buffered from the direct and immediate effects of changes in temperature and rainfall, partly because they do not have robust circadian rhythms, however, on a longer term they are vulnerable to changes in their food sources and dispersal abilities.

Keywords: Bathyergidae, circadian rhythm, seasonal rhythm, rhythmicity, light, temperature, social, solitary

BIOLOGICAL RHYTHMS

Biological timing is measured in the form of cyclical variations in physiological processes and behaviours of organisms. Biological rhythms are ubiquitous in nearly all organisms and range in frequency from milliseconds to many years and at all levels of organisation (Aschoff, 1981; Paranjpe and Sharma, 2005). Biological rhythms can be classified according to the lengths of their periods, ultradian rhythms are shorter than 24 h, circadian rhythms are about 24 h long and rhythms longer

than 24 h are called infradian (Wollnik, 1989). These rhythms are frequently superimposed on one-another, and the integrated multi-frequency timekeeping enables organisms to keep track of their environment and promotes optimal performance and survival (Golombek and Rosenstein, 2010; Kuhlman et al., 2018).

Periodicity under natural conditions does not necessarily demonstrate the presence of a biological clock. Rhythms can be purely exogenous, in which case the rhythm is dependent on the external environment (Aschoff, 1960). However, by far the most common rhythms are the endogenous rhythms which are generated by biological clocks within an organism and continue or free run in the absence of external entraining factors, at least for some time. Endogenous biological rhythms frequently do not run precisely over daily or annual periods and a large number of internal and external factors can influence the rhythm lengths, causing them to deviate from that of external environmental rhythms. To be biologically relevant, endogenous rhythms are synchronised or entrained by periodic environmental rhythms to prevent them from drifting out of phase with the environment (Aschoff and Pohl, 1978). Circadian rhythms are the most widespread biological rhythms and have periods of about 24 h. The daily light-dark cycle is the most predictable cyclical environmental cue and is therefore the most prominent *zeitgeber* used by organisms for entrainment (Amir and Stewart, 1998). Non-photic cues such as temperature, exercise and social cues can also influence biological rhythms, however their effect is usually less potent than that of light (Golombek and Rosenstein, 2010; Refinetti, 2010). However, in nature *zeitgebers* do not act independently, they have compounding effects to generate a more robust entraining effect (Van Jaarsveld et al., 2019).

THE CIRCADIAN SYSTEM

The circadian system can be divided into three fundamental components, (a) the input pathway that collects external timing signals and relays it to the core clock, (b) the central clock that is responsible for the generation of rhythms, and (c) the output in the form or behavioural or physiological rhythms (Agostino et al., 2011). In mammals, photosensitive pigments are found only in the retina of the eye. The rods and cones in the photoreceptor layer of the retina are primarily used for vision, whereas the melanopsins that are present in intrinsically photosensitive retinal ganglion cells (ipRGC) are involved in the circadian system (Berson, 2003). The axons of the ipRGC's form the retinohypothalamic tract (RHT) which projects to the suprachiasmatic nucleus (SCN) that is located in the basal hypothalamus (Moore, 2007). The SCN is the site of the central pacemaker in mammals (Moore, 1983). The neurons of the SCN respond to photic input in a gated fashion, their activation is blocked during the day but not the night. During the night, SCN neurons are activated to different degrees depending on the time at night when the light source is given, and this activation corresponds to periods when the presence of light can also cause behavioural phase shifts for entrainment (Kornhauser et al., 1992; Oosthuizen et al., 2005). Apart from the timing of light, the circadian system is also differentially affected by the quality of the

light in terms of its intensity, duration, and spectrum (Gorman et al., 2003; Duffy and Wright, 2005; Aral et al., 2006; Zubidat et al., 2009, 2010).

GENERAL MECHANISM OF THE CIRCADIAN CLOCK

All circadian clocks have a genetic basis and are driven by delayed transcription-translation feedback loops (TTFL) and post translational modifications (Sharma, 2003). The molecular genetics of circadian clocks have been described in several organisms ranging from prokaryotic organisms such as cyanobacteria (Kondo and Ishiura, 2000; Nakajima et al., 2005) to many kinds of eukaryotic organisms including *Neurospora* (Correa et al., 2003; Dunlap et al., 2007), plants (Gardner et al., 2006), insects (Bargiello and Young, 1984; Williams and Sehgal, 2001; Rosato et al., 2006), and mammals (King and Takahashi, 2000; Reppert and Weaver, 2001; Sato et al., 2004; Ko and Takahashi, 2006). Oscillations of roughly 24 h are generated by the expression, accumulation and degradation of positive and negative clock genes and their products to form loops (Patke et al., 2019). Although the genes involved in the clock mechanisms of different organisms differ, they have a similar functionality in terms of the feedback loops under which they operate. In mammals, two interlocked feedback loops drive the circadian clock at the cellular level (Brown and Doyle, 2020). The core feedback loop, positive element proteins CLOCK and BMAL1 form a dimer and binds to an Ebox region to initiate the transcription of negative element genes *Period* (*Per*) and *Cryptochrome* (*Cry*). After translation, PERIOD and CRYPTOCHROME form heterodimers and translocate back to the nucleus to act on CLOCK:BMAL1 and suppress their own transcription (Ko and Takahashi, 2006). The second regulatory loop is also activated by CLOCK:BMAL1 acting on a promotor region to activate transcription of the retinoic acid-related orphan nuclear receptors, *Rev-erbα* and *Rorα*, which are subsequently translated to their respective proteins. These proteins aid in the regulation of *Bmal1* by controlling the rate of its transcription (Ko and Takahashi, 2006). This secondary, or auxiliary loop is thought to stabilise oscillations (Brown and Doyle, 2020). The stability and period of the circadian oscillations are also affected by post translational modifications such as phosphorylation and ubiquitination, which contribute to the stability of the proteins and is involved in nuclear translocation (Ko and Takahashi, 2006).

Nearly all mammalian cells and tissues contain circadian clocks that can generate autonomous circadian rhythms that persist in isolation and free run at their own innate period (Yoo et al., 2004; Husse et al., 2015). Since the central pacemaker in the SCN receives external photic input, it can synchronise to the external environment, and in turn it maintains temporal synchrony of the downstream peripheral clocks with the environment. Circadian clocks are also involved in the regulation of seasonal rhythms *via* the hormone melatonin. In mammals, pineal melatonin synthesis is under the control of the SCN (Maronde et al., 1999). Melatonin expression peaks at

night and is suppressed during the day, and can thus provide photoperiodic information as the daylength varies across seasons (Hardeland et al., 2006). Seasonality is frequently effected by seasonal changes in hormones from the anterior pituitary. The photoperiodic effects of melatonin on endocrine function is mediated by the *pars tuberalis* of the pituitary gland, a region rich in melatonin receptors (Morgan and Williams, 1996).

EVOLUTION OF RHYTHMICITY AND ITS FUNCTIONAL IMPORTANCE

Due to the ubiquitous nature of rhythmicity in prokaryotic and eukaryotic organisms, the evolution of a temporal order is thought to have originated with early life forms (Hastings et al., 1991; Paranjpe and Sharma, 2005). In primitive organisms, rhythmicity serves to segregate photophilic and photophobic processes essential for survival (Stal and Krumbein, 1985; Nikaido and Johnson, 2000). The evolution of homeothermy in early eutherian mammals enabled them to exploit the nocturnal niche (Crompton et al., 1978) and thereby avoid predation and interspecific competition with dinosaurs (Walls, 1942; Gerkema et al., 2013). Other physiological and biochemical rhythms such as body temperature and metabolism usually show corresponding peaks compared to locomotor activity, indicating that internal rhythms are synchronised (Refinetti, 1999; Riccio and Goldman, 2000a). Similar to daily rhythmicity, animals may also show seasonal rhythmicity. Many animals are exposed to annual fluctuations in their environment, and organisms tend to restrict their energetically expensive processes, such as reproduction, to times of the year when food is abundant and other environmental factors are most favourable. Other seasonally timed behaviours include migration, hibernation and colour changes of pelages to blend in with seasonal environmental colouring.

Rhythmicity provides organisms with both intrinsic and extrinsic fitness benefits. The intrinsic adaptive value of circadian rhythms refers to the temporal coordination of internal processes, for example to segregate incompatible processes or synchronise others. Rhythms also allows organisms to keep track of external time, providing an extrinsic adaptive value (Sharma, 2003). Both of these processes are crucial for survival in natural environments (Paranjpe and Sharma, 2005). Rhythmicity enables animals to keep track of proximate factors such as light and temperature in order to predict and prepare for ultimate factors such as predation risks, food availability and mating opportunities, thereby providing an adaptive advantage (Helm et al., 2013).

The importance of the circadian timing system and its entrainment by light-dark cycles is best demonstrated by the prevalence of increased health risks and in some cases disturbances within ecological systems, which emerge from disruptions of the circadian clock network and desynchronization in timing of the different biological rhythms (Bird et al., 2004; Navara and Nelson, 2007; Rotics et al., 2011; Haim and Portnov, 2013). In modern society there are many artificially induced disruptions of the circadian system that have implications for both humans and animals that have received much attention both in the laboratory and more recently also in field studies. Animals are most severely affected by light pollution and human interference with ecosystems (Longcore and Rich, 2004).

When the internal clock mechanisms of organisms are not appropriately aligned with the external environment, many physiological processes are compromised. Effects of this misalignment include reduced longevity and accelerated aging, increased risk of cancer, metabolic, cardiovascular as well as reproductive disorders and immune dysfunction (Evans and Davidson, 2013). Laboratory studies indicate that alterations to the LD cycle increase mortality of animals (Halberg and Cadotte, 1975; Penev et al., 1998; Davidson et al., 2006; Vinogradova et al., 2009). In a natural habitat, animals with ablated SCNs have a higher mortality as a result of increased predation (DeCoursey et al., 1997; DeCoursey and Krulas, 1998). Artificial light at night (ALAN) contributes to a higher prevalence of several forms of cancer, accelerates tumour growth and increases oxidative stress (Dauchy et al., 1999; Vinogradova et al., 2009), with the disruption of the melatonin rhythm thought to play a crucial role (Shah et al., 1984; Blask and Hill, 1986; Reiter et al., 2000; Baydas et al., 2001; Schernhammer and Schulmeister, 2004). Circadian disruption can lead to a host of adverse metabolic effects such as increased weight gain, obesity and glucose intolerance which may result from altered feeding behaviours (Oishi, 2009; Karatsoreos et al., 2011; Varcoe et al., 2011), and an increased risk of cardiovascular disorders (Knutsson and Boggild, 2000; Ruger and Scheer, 2009). The immune system may also be adversely affected by circadian disruptions, with infections and inflammations arising that can act synergistically with other health consequences that are associated with disturbances in rhythmicity (Scheiermann et al., 2013; Philips et al., 2015; Comas et al., 2017). External disturbances of the circadian system appear to have overall negative effects on organisms.

The changing climates also cause shifts in seasons, which can have serious implications for organisms as their phenological environments are altered (Visser et al., 2010). Animals are adapted to certain environmental factors in their habitats, and they time processes such as reproduction, hibernation and migration accordingly. When seasonal changes occur at times that are different from those animals anticipate and prepare for, mismatches and mistiming occur between the animal and its environment. This effect often spans several trophic levels, for example when shifts in food sources occur there is a disruption in the food chain (Visser et al., 2006). This in turn can have direct implications on the reproduction of animals (Visser et al., 2009). Phenological mismatches in migrating animals can also have severe fitness consequences (Saino et al., 2010), and similarly a recent publication described a phenological mismatch between sexes in a hibernating rodent following a heatwave (Kucheravy et al., 2021). In species that undergo seasonal pelage colour changes, temperature shifts can cause snow to arrive earlier or melt sooner, causing a mismatch between the animal and its environment (Mills et al., 2013). Proper timing of biological events is crucial for the continued survival and fitness of species.

RHYTHMICITY IN CONSTANT HABITATS

Most organisms inhabit highly rhythmic environments where daylight and temperature fluctuate daily or seasonally. There are, however, certain habitats that experience minimal daily and seasonal fluctuations in ambient conditions. While the significance of rhythmicity may be clear-cut for animals that are frequently exposed to environmental fluctuations, it is less obvious for animals that inhabit relatively constant environments. In the absence of rhythmic external cues some organisms may not benefit from entraining their internal clocks to regulate rhythmic behaviour that might be deemed to be less advantageous. In such conditions, rhythmicity may be actively selected against, in particular to preclude the maintenance of energetically expensive input pathways (eyes) and clock mechanisms (Niven and Laughlin, 2008; Porter and Sumner-Rooney, 2018). There is indeed evidence of regression in species that utilise constant habitats, with a gradient of morphological adaptations dependent on the extent of habitat utilisation. Some organisms return to a rhythmic environment periodically and could therefore show lower degrees of adaptation to constant environments. Specifically with regards to vision, adaptations range from animals with slightly reduced eyes to others that are completely eyeless, with all degrees of variation in between (Menna-Barreto and Trajano, 2015). Since photic information reaches the SCN *via* the eyes in mammals, a regressed visual system has implications for the circadian system. Species that utilise constant habitats but are morphologically more similar to species that do not, i.e., possessing acute vision and functional eyes, should display distinct rhythms, whereas eyeless species with absent visual structures should not express circadian rhythmicity, and intermediate species probably exhibit a mosaic of characteristics.

Polar regions are by no means subject to stable environmental conditions since they are subject to large seasonal fluctuations in both temperature and photoperiod. However, changes in photoperiod are so extreme that during the middle of the summer the sun does not set and in winter it does not rise (Bloch et al., 2013). Animals are thus exposed to periods of constant light or dark during mid-summer and winter, during which mammals inhabiting these regions cannot use light to entrain their biological rhythms, resulting in significant variation in rhythmicity amongst animals. Some animals lose rhythmicity completely during this time and may become almost constantly active (Swade and Pittendrigh, 1967; Reierth et al., 1999; Van Oort et al., 2005; Lu et al., 2010), some animals lose rhythmicity intermittently (Arnold et al., 2018), while others retain rhythmicity, but at a lower amplitude (Swade and Pittendrigh, 1967; Hau et al., 2002; Ashley et al., 2014; Ware et al., 2020). Polar animals are still exposed to rhythmic environmental conditions for the remainder of the year, during which they exhibit rhythmicity in behaviour and physiological functions.

More than 60% of the Earth's surface is covered by deep oceans, and deep seas of more than 200 m have long been considered as arrhythmic environments (Mat et al., 2020). Although water temperatures do not show daily variations within a few meters from the surface (Kawai and Wada, 2007), seasonal differences are still noticeable for depths of up to 1 km (Talley, 2011). Light can also penetrate water for up to 1 km (Warrant and Locket, 2004). Internal tides as a result of hydrothermal vents occur at all depths (Mat et al., 2020). Although this biome is poorly studied due to its inaccessibility and technical constraints, rhythmic behaviour has been observed in deep sea organisms. In deep sea organisms that have been brought to the surface, circadian feeding rhythms (Maynou and Cartes, 1988; Modica et al., 2014) and lunar rhythms in melatonin secretion (Wagner et al., 2007) have been detected. Video recordings of deep-sea invertebrates and mussels in their natural environment also reveal circadian rhythmicity in their activity and behaviour (Cuvelier et al., 2014; Mat et al., 2020). Functional melanopsin receptors have been identified in some deep-sea fish, suggesting the potential for photic stimulation of the circadian clock (Davies et al., 2012). Candidate clock genes have also been discovered (Mat et al., 2020), indicating that the circadian clocks of these animals are still functional.

Caves probably have the most stable microclimates of all arrhythmic habitats, as they are completely isolated from light and temperature fluctuations (Beale et al., 2016). Cave dwelling organisms show large variation in their use of the caves as well as in the expression of their biological rhythms. Many species, such as bats, use caves to sleep in, but forage outside, and have robust circadian rhythms (Marimuthu and Chandrashekaran, 1985). Some other organisms are facultative cave dwellers and are strongly specialised to their habitat, with some species even regressing their eyes completely. Despite this, many of these organisms have retained the ability to display circadian rhythms, albeit dampened and with evidence for clock gene mutations, and they do not persist long in constant conditions (Cavallari et al., 2011; Beale et al., 2013, 2016).

The subterranean niche is characterised by constant darkness and otherwise dampened short term climatic fluctuations (Bennett and Faulkes, 2000). A large number of vertebrates, including some 300 mammalian species from different taxa, have evolved fossorial or subterranean lifestyles (Nevo, 1979; Bennett and Faulkes, 2000; Lacey et al., 2001; Peichl et al., 2004). As with cave dwelling species, fossorial species range from species using burrows purely for shelter to others that permanently live underground and surface very infrequently, if at all (Nevo, 1979; Mason and Narins, 2001). Technically, animals that use underground tunnels to sleep in, but forage aboveground are classified as fossorial (Camin and Madoery, 1994; Tomotani et al., 2012). However, since they are frequently exposed to daily environmental light and temperature fluctuations, they do not permanently live in a constant environment and usually display robust rhythmicity.

Many species have evolved to show specific adaptations to a life underground. These adaptations include both morphological and physiological features, including adaptations to the visual system. Eye sizes vary considerably in subterranean animals, with this variation reflecting differences in selection pressures of the various lifestyles (Borghi et al., 2002). Strictly subterranean mammals that very rarely emerge aboveground frequently have reduced or no visual capabilities. These animals can have microphthalmic external eyes, subcutaneous eyes or a complete

loss of eyes (Nevo, 1979; Burda et al., 1990; Cooper et al., 1993a). Animals with small external or subcutaneous eyes usually have regressed or malformed retinal structures, and brain areas that are associated with vision are reduced or absent, however, their circadian systems appear to be intact (Cooper et al., 1993a; Peichl, 2005; Němec et al., 2008a,b; Vega-Zuniga et al., 2017).

MOLE-RATS

African mole-rats from the family Bathyergidae are endemic to sub-Saharan Africa, and all species are subterranean. There are six genera within this family, and a total of around 30 described species, three of the genera contain solitary species and three social species (**Figure 1**; Van Daele et al., 2007; Faulkes and Bennett, 2013). Solitary species are confined to mesic habitats, whereas social species occur throughout much of the continent south of the Sahel.

Mole-rats are believed to have evolved from surface-dwelling to a subterranean existence about 20 million years ago, in the early Miocene (Nevo, 1995; Lavocat, 2013). They have since adapted very successfully to their habitat and show several morphological and physiological adaptations to their environment. These include a regressed visual system which can pose obvious challenges for the circadian system and rhythmicity. Despite their reduced visual system, all mole-rat species investigated to date show some form of rhythmicity, albeit to varying degrees. This suggests that their circadian systems are intact and functional.

Naked mole-rats (*Heterocephalus glaber*) are of particular interest for a variety of medical applications including aging, cancer and pain research and have therefore been the focus of many diverse studies over the past decades (Buffenstein, 2005; Park et al., 2008; Shepard and Kilssil, 2020). Hence, much of the research on mole-rats, specifically the molecular aspects of the circadian clock and its output, were conducted on this species. More recently, there was a spike in interest also in the other mole-rat species in the family Bathyergidae for comparative research in an evolutionary and ecological sense. This family occurs in a wide range of habitats, and exhibits a continuum of sociality, ranging from strictly solitary (*Bathyergus*, *Georychus*, and *Heliophobius*) to social (*Cryptomys*) and highly social (*Fukomys* and *Heterocephalus*). In addition, the species in this family show interesting thermoregulatory properties, some species are homeothermic, some more heterothermic and the naked mole-rat has been described as poikilothermic (Buffenstein and Yahav, 1991; Bennett et al., 1993, 1994b; Boyles et al., 2012; Oosthuizen et al., 2021). Finally, the circadian biology of African mole-rats has received much attention over the past two decades.

MOLE-RAT CIRCADIAN SYSTEM

Input Pathways

As with other mammals, photic information can only enter the circadian system the *via* eyes in mole-rats (Němec et al., 2004; Crish et al., 2006). African mole-rats have small superficial

eyes, and initially it was suggested that mole-rats were not completely blind (Sclater, 1900). Most researchers, however, were in agreement that mole-rats cannot see Eloff (1958); Jarvis (1973). Eloff (1958) reported that the eyes of mole-rats (*Cryptomys* and *Bathyergus* species) are insensitive to light and supported this by the apparent lack of response to bright light being applied to the eye, the lack of pupillary reflex and certain anatomical deviations of the visual system, such as a thin optic tract and the apparent absence of circular muscle fibres around the lens. A thickened cornea was also noted by Eloff (1958). The general consensus amongst researchers studying bathyergids was that they are blind (Peichl et al., 2004), and several, more recent publications referred to 'blind' African mole-rats (Lovegrove et al., 1993; Kössl et al., 1996; Brückmann and Burda, 1997; Oelschlager et al., 2000; Heth et al., 2002). The eyes and visual system of mole-rats have subsequently been investigated in more detail. Although microphthalmic, the architecture of mole-rat eyes is similar to that of surface-dwelling rodents (Peichl et al., 2004; Němec et al., 2008a). Although considerable species variation is obvious, the structural organization seems to be less regular (Nikitina et al., 2004; Peichl et al., 2004). The eye lens is small in most species, except the naked mole-rat, where it is very large, uneven in shape and free floating (Nikitina et al., 2004). The retina is well developed, although in the naked mole-rat, it appears to be folded (Nikitina et al., 2004; Němec et al., 2008a). Structurally, it has all the layers expected in a sighted animal, with rod and cone photoreceptor cells, and multiple types of horizontal, bipolar, amacrine and ganglion cells (Mills and Catania, 2004; Peichl et al., 2004). As a result of the small eye size, mole-rats have low overall numbers of photoreceptors, but like most other mammals, the retina is rod dominated. Nevertheless, a surprisingly high proportion of cones are present, most of which are S-opsin immunoreactive (Peichl et al., 2004). The structural organisation of the eye negates the ability for image formation, but that of the retina suggests that these animals retained the ability to discriminate between light and dark (Nikitina et al., 2004). Indeed, behavioural studies in several mole-rat species revealed severe visual deficits in terms of image formation, visuomotor integration and depth perception (Kott et al., 2016).

In addition to structural constraints, certain genes in the naked mole-rat eye display substitutions that constitute retinal degeneration (Zhou et al., 2020). These genes include CRX, a photoreceptor specific transcription factor, and mutations that are associated with dominant rod-cone dystrophy and Leber's congenital amaurosis (Sohocki et al., 1998), and RPE65 whose encoded protein is part of the vitamin A cycle of the retina, mutations cause retinal degeneration and is also associated with Leber's congenital amaurosis (Wang et al., 2020). Both of these genes have been implicated with the maintenance of circadian function (Gamse et al., 2001; Doyle et al., 2008). This is consistent with the poor vision displayed by the naked mole-rat, and likely other mole-rats as well.

Retinal ganglion cells are fewer in number, the optic tract is thin and contains more unmyelinated fibres compared to surface dwelling rodents such as mice and rats (**Supplementary Figure 1**; Omlin, 1997; Negroni et al., 2003; Němec et al., 2004, 2008a). The optic tract projects to all the usual visual

FIGURE 1 | Images of a representative animal of each of the six genera of the family Bathyergidae. **(A)** *Georychus capensis*; **(B)** *Cryptomys hottentotus pretoriae*; **(C)** *Heliophobius emeni*; **(D)** *Fukomys damarensis*; **(E)** *Bathyergus suillus*; **(F)** *Heterocephalus glaber*. Panels **(A,C,E)** are solitary species, while panels **(B,D,F)** represent social species.
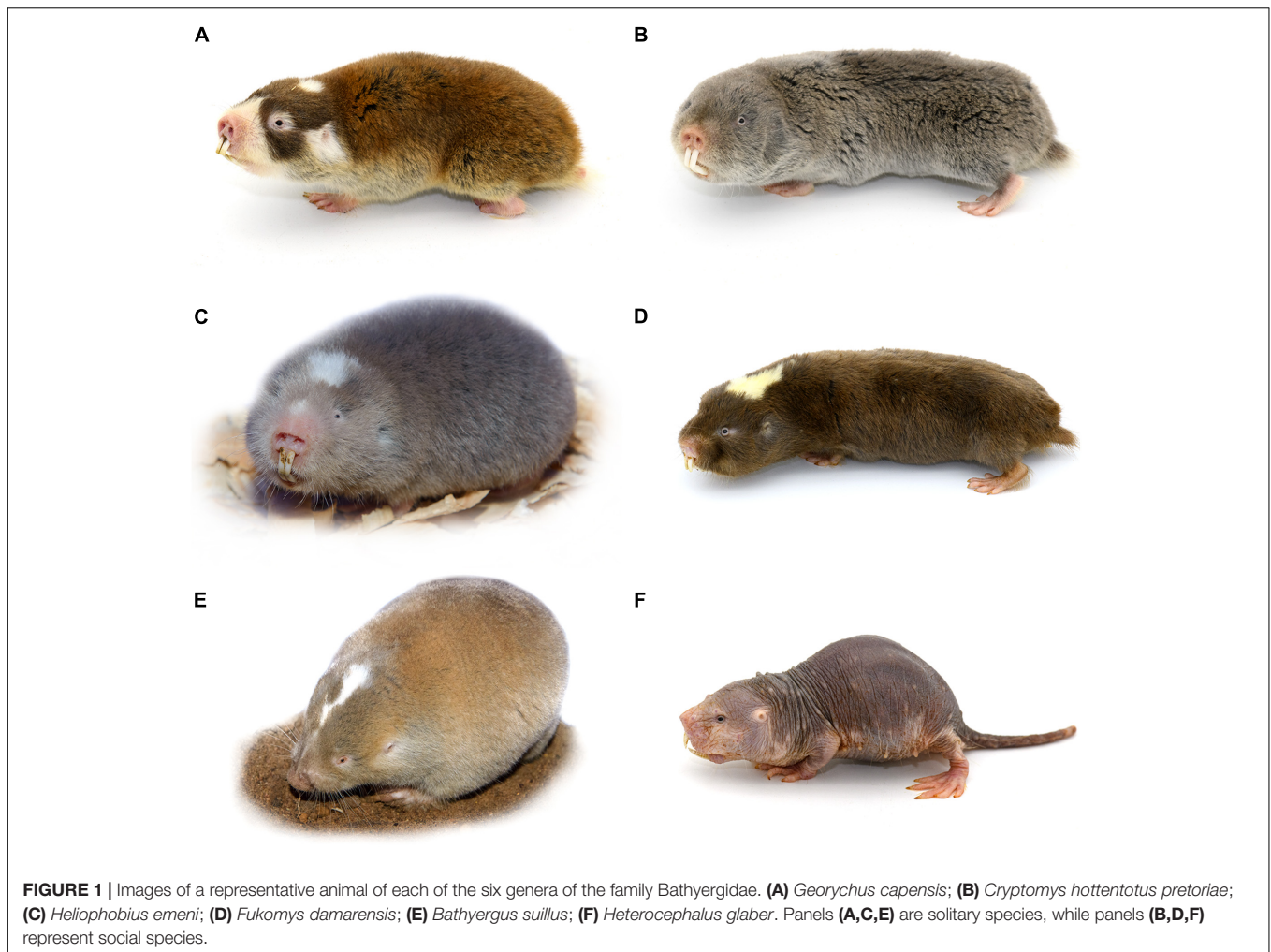
structures in the brain, but compared to surface dwelling rodents, both the brain structure sizes, and proportions of the innervations differ. The SCN is well developed and receives dense bilateral projections, proportionally larger compared to surface dwelling rodents, whereas all other visual structures are reduced and receives proportionally smaller projections (Negroni et al., 2003). Light reaches retinorecipient regions of the brain, as indicated by immunohistochemical labelling (Oelschläger et al., 2000). Structures involved in brightness discrimination are better developed than those that regulate coordination of visuomotor reflexes, as is also indicated by the structure of the eye (Peichl et al., 2004; Němec et al., 2008a). Correspondingly mole-rats have low visual acuity, reflecting their reliance on other sensory senses in their habitat.

The circadian system of the blind mole-rat, *Spalax ehrenbergi*, a subterranean rodent from another family, has been much more extensively studied. It shows a more severe regression of the visual system, with subcutaneous eyes, a complete loss of vision and corresponding small or absent retinal projections to visual structures, as in African mole-rats (Bronchti et al., 1991; Cooper et al., 1993b). The blind mole-rat have less than a thousand retinal ganglion cells, almost all of which contain melanopsin, suggesting

a functional circadian system (Hannibal et al., 2002; Esquiva et al., 2016). The mole-rat visual system suggests selective progressive and regressive evolution of the structural attributes, driven by selection forces of their subterranean habitat.

The Clock and Its Mechanism

In mole-rats, as with other mammals, the central pacemaker is located in the SCN and receives bilateral innervation from a subset of retinal ganglion cells (Němec et al., 2008a). Light sensitivity of the SCN has been tested in several mole-rat species by measuring the expression of the immediate early gene *c-fos*, which is a marker of light activated neuronal activity in the SCN (Oosthuizen et al., 2005). The SCN neurons appear to react to light in most of the species tested, however, the response is variable (Oosthuizen et al., 2005, 2010a). In rodents, Fos expression is dependent on the phase of the circadian clock, with low Fos induction during the day and higher during the night (Rose et al., 1999). This is also the case in the Cape mole-rat (*Georychus capensis*), a solitary species, whereas gating according to the phase of the day is not apparent in the highveld mole-rat (*Cryptomys hottentotus pretoriae*), a social species (Oosthuizen et al., 2005). Fos expression in the SCN

of rodents is directly proportional to the number of photons in the light stimulus. Increasing light intensities or longer light pulse durations evokes higher Fos expression in the SCN (Nelson and Takahashi, 1991; Dkhissi-Benyahya et al., 2000). The solitary Cape mole-rat exhibits responses similar to other aboveground rodents, light induces a significant Fos response in the SCN that increases with increasing light intensities (Oosthuizen et al., 2010a). In social species, this response is rather different, large variability is obvious in the common mole-rat (*Cryptomys hottentotus hottentotus*) and the Damaraland mole-rat (*Fukomys damarensis*), both within and between the groups exposed to specific light intensities. The naked mole-rat does not appear to display any Fos expression in response to light illumination (Oosthuizen et al., 2010a). The lack of responsiveness of the naked mole-rat may be in part as a consequence of the size of their eyes. Naked mole-rats are one of the smallest mole-rats, as are their eye sizes. The small size of the eyes limits the number of photoreceptors and the amount of light that can enter. There appears to be a gradient of light sensitivity in the African mole-rats in parallel with their social structures, with solitary species more sensitive to light compared to the more social species (**Figure 2**).

FIGURE 2 | An illustration of the light responsiveness of the SCN comparing aboveground rodents to mole-rats with different degrees of sociality. Aboveground dwelling rodents have a short integration time of light to the SCN and show gating of the circadian clock. Solitary mole-rats show similar gating of light compared to aboveground dwelling rodents but have a longer integration time/higher threshold to light. Social mole-rats do not show gating of the circadian clock and have higher and more variable thresholds to light. Naked mole-rats do not exhibit any Fos expression in the SCN in response to light.

Of the African mole-rats, only the naked mole-rat circadian clock mechanism has been investigated. In addition, it was studied in liver tissue, not the SCN, and patterns observed in the liver could therefore be different from that in the SCN. Ghosh et al. (2021) compared several aspects of the circadian clock between naked mole-rats and mice. All the core circadian

clock genes (*Bmal1*, *Clock*, *Per1/2*, *Cry1/2*) as well as the nuclear receptor genes, *Rev-Erbα* and β, and three *Ror-s*, were identified in the naked mole-rat. In addition, some cytoplasmic Casein kinases were also detected in the naked mole-rat genome (Ghosh et al., 2021). The circadian clock proteins appear to be highly conserved among mammalian species. The presence of the appropriate clock genes does not necessarily guarantee robust rhythmicity. To produce and coordinate overt rhythms, different clock gene expression peak at different times of the day. Positive clock genes (*Bmal1*) normally peak in the morning whereas the negative clock genes (*Per* and *Cry*) peak in the evening according to when certain genes are suppressed or not. In the naked mole-rat, the positive and negative clock genes all peak at the same time in the early morning. These differences may potentially stem from variations in the promotors of clock genes. While the regulatory elements of the promotor regions are highly conserved between species, the number of promotors for specific genes differ between mice and mole-rats and could affect the secondary feedback loops (Ghosh et al., 2021). These results could suggest that the naked mole-rat circadian oscillator is organised differently to that of surface-dwelling rodents, or that evolutionary pressures are driving a loss of circadian function in this species.

The clock genes of the blind mole-rat are much better characterised than those of the bathyergid mole-rats. The two positive element genes *Clock* and *Mop3* (*Bmal1*) have been cloned and sequenced, *MOP3* expression shows circadian variation but not *Clock*. The CLOCK/MOP3 dimer appears relatively conserved but less effective at driving transcription than other mammalian species (Avivi et al., 2001). Three period genes have also been cloned and sequenced, and oscillate with 24 h periodicity in the SCN, retina and peripheral clocks (Avivi et al., 2002). Given the similarity in habitat and selection pressures on blind mole-rats and African mole-rats, it is likely that the other bathyergid mole-rats would also show conserved, functional clock components, although this remains to be discovered.

Output

The output of the circadian clock is manifested in the countless biochemical, physiological and behavioural rhythms displayed by animals. Output rhythms are frequently the only way to determine the phase of the circadian clock. In African mole-rats, several circadian rhythms, including locomotor activity, body temperature, melatonin and metabolism, have been investigated. Locomotor activity received the most attention while other rhythms are rather poorly studied in only a few species.

DAILY RHYTHMICITY IN MOLE-RATS

Melatonin

In mammals, melatonin is primarily produced in the pineal gland and is involved in the regulation of the sleep-wake rhythm and body temperature (Cagnacci et al., 1992). The naked mole-rat melatonin signal appears to be disrupted at several steps along its pathway. The pineal gland is atrophied (Quay, 1981) and although the genes involved in the synthesis of melatonin

are intact, the expression of some of the genes are very low or undetectable (Kim et al., 2011). In addition, the melatonin receptors have mutations that introduce premature stop signals (Fang et al., 2014). In rodents, melatonin has two high-affinity receptors, MTNR1a and MTNR1b. In naked mole-rats, both of these receptors are non-functional, whereas the Damaraland mole-rat has an intact MTNR1a receptor while the MTNR1b is inactive (Fang et al., 2014). The MTNR1a receptor alone has been shown to be sufficient to maintain photoperiodic responses in the hamster (Prendergast, 2010), suggesting a functional melatonin pathway for at least some of the mole-rat species. Indeed, several species from the genus *Fukomys*, the Damaraland (*F. damarensis*) and Mashona mole-rats (*Fukomys darlingi*), as well as the genus *Cryptomys*, the highveld (*C.h. pretoriae*) and Natal mole-rats (*C.h. natalensis*), display daily rhythms of plasma melatonin concentrations (Richter et al., 2003; Gutjahr et al., 2004; Hart et al., 2004; Vasicek et al., 2005a). Plasma melatonin rhythms have apparently not been investigated in the naked mole-rat, probably because of the evidence of interruption in the pathway.

Metabolism

It appears that rhythms of metabolism have only been examined in the naked mole-rat. Genes involved in the metabolism of glucose and fructose were investigated in mice and naked mole-rats. For both pathways, the mole-rat genes show circadian rhythmicity and appear to be more synchronised than mouse genes (Ghosh et al., 2021). Similarly, the mTOR signalling pathway, that senses the cellular environments and a major regulator of mammalian metabolism and physiology, displays highly synchronised expression in the naked mole-rat, but not in mice (Ghosh et al., 2021). Consistent with this, circadian rhythms of metabolic rate were measured in the naked mole-rat, which increases corresponding to increased body temperature and wheel running activity (Riccio and Goldman, 2000a). Stringent control over metabolic pathways may be essential for the survival in challenging subterranean environments where food may be limited and/or energetically expensive to retrieve. Since the naked mole-rats and other African mole-rats share similar habitat constraints, this is likely also the case in the other genera.

Body Temperature

Most mammals show daily variations in body temperature (T_b), which is usually closely related to locomotor activity, Tb is higher when animals are active (Refinetti, 1999). This is also the case in mole-rats (**Figure 3**). The T_b rhythms of several species have been investigated in the laboratory and in the field, and all of those species show rhythmic T_b fluctuations (**Table 1**; Lovegrove and Muir, 1996; Riccio and Goldman, 2000a; Streicher et al., 2011; Haupt et al., 2017; Van Jaarsveld et al., 2019; Okrouhlík et al., 2021; Oosthuizen et al., 2021). Mole-rats, like many other fossorial rodents, overall have lower T_b compared to aboveground dwelling rodents (Fioretti et al., 1974; Wollnik and Schmidt, 1995; Castillo et al., 2005; Gordon, 2017). Solitary species have mean T_b of around 35.5–36°C (Lovegrove and Muir, 1996; Okrouhlík et al., 2021) whereas the social species have even lower T_b's of between 34°C and 35°C (Riccio and Goldman, 2000a; Streicher et al., 2011; Haupt et al., 2017; Oosthuizen

et al., 2021). The amplitude of T_b rhythms in mole-rats is also smaller compared to other rodents (Castillo et al., 2005; Haupt et al., 2017; Okrouhlík et al., 2021; Oosthuizen et al., 2021). Both the lower body temperatures and rhythm amplitudes are likely thermoregulatory adaptations to the subterranean ecotope (Lovegrove, 1986). Nevertheless, T_b patterns conformed to that of the activity rhythms in mole-rats that have been investigated in the laboratory (Lovegrove and Muir, 1996; Riccio and Goldman, 2000a; Haupt et al., 2017; Van Jaarsveld et al., 2019). Hence, T_b can be used as a proxy for activity when the measurement thereof is challenging, for example in underground dwelling animals.

Locomotor Activity

Much of the early literature claims that there is no locomotor rhythmicity in mole-rats, with initial behavioural studies reporting dispersed activity patterns for both solitary and social species (**Table 1**; Genelly, 1965; Jarvis, 1973; Hickman, 1980; Lovegrove, 1988; Bennett, 1992). Interest in the circadian biology of African mole-rats started to gain traction in the 1990s and Lovegrove was the first to show evidence of locomotor activity rhythms in both a solitary (Lovegrove and Papenfus, 1995) and a social mole-rat species (Lovegrove et al., 1993). Subsequently, locomotor activity rhythms have been investigated in several species in the laboratory, and all species exhibited rhythmicity, albeit to different degrees and with a great deal of variability both within and between species (**Figure 3** and **Table 1**; Riccio and Goldman, 2000b; Oosthuizen et al., 2003; Hart et al., 2004; Vasicek et al., 2005b; Schöttner et al., 2006; de Vries et al., 2008). Intraspecific variability is evident from the proportion of rhythmic and arrhythmic chronotypes in mole-rat species. Overall, there are many animals that are arrhythmic. Differences in sleep has been identified between rhythmic and arrhythmic animals, arrhythmic animals spend a larger proportion of their time awake compared to rhythmic individuals (Bhagwandin et al., 2011b). These differences are also reflected in the numbers of orexinergic cells present in the animals. Orexin promotes wakefulness, and indeed, the arrhythmic animals of several mole-rat species display more orexinergic cell bodies compared to rhythmic chronotypes (Bhagwandin et al., 2011a).

Potential sources of variation in locomotor activity studies in the laboratory include the method of recording, the housing conditions of the animals and the ambient conditions. Locomotor activity can be measured in several different ways, for single animals, passive infrared captors are quite popular, they work reasonably well, but inter- and intra-experimental variation can occur if the sensitivity of the sensors is different. Running wheels can also be used for singly housed animals, and usually render the 'cleanest' results, but have the disadvantage that general activities when the animal is not on the wheel, are not recorded. In addition, not all animals run on wheels. Another option is the use of implanted e-mitters, although these devices are quite expensive and record every movement of the animal, it can render rather noisy results. The recording activity of multiple animals while maintained in a colony is tricky, video recordings can be used if the animals are individually identifiable from above,
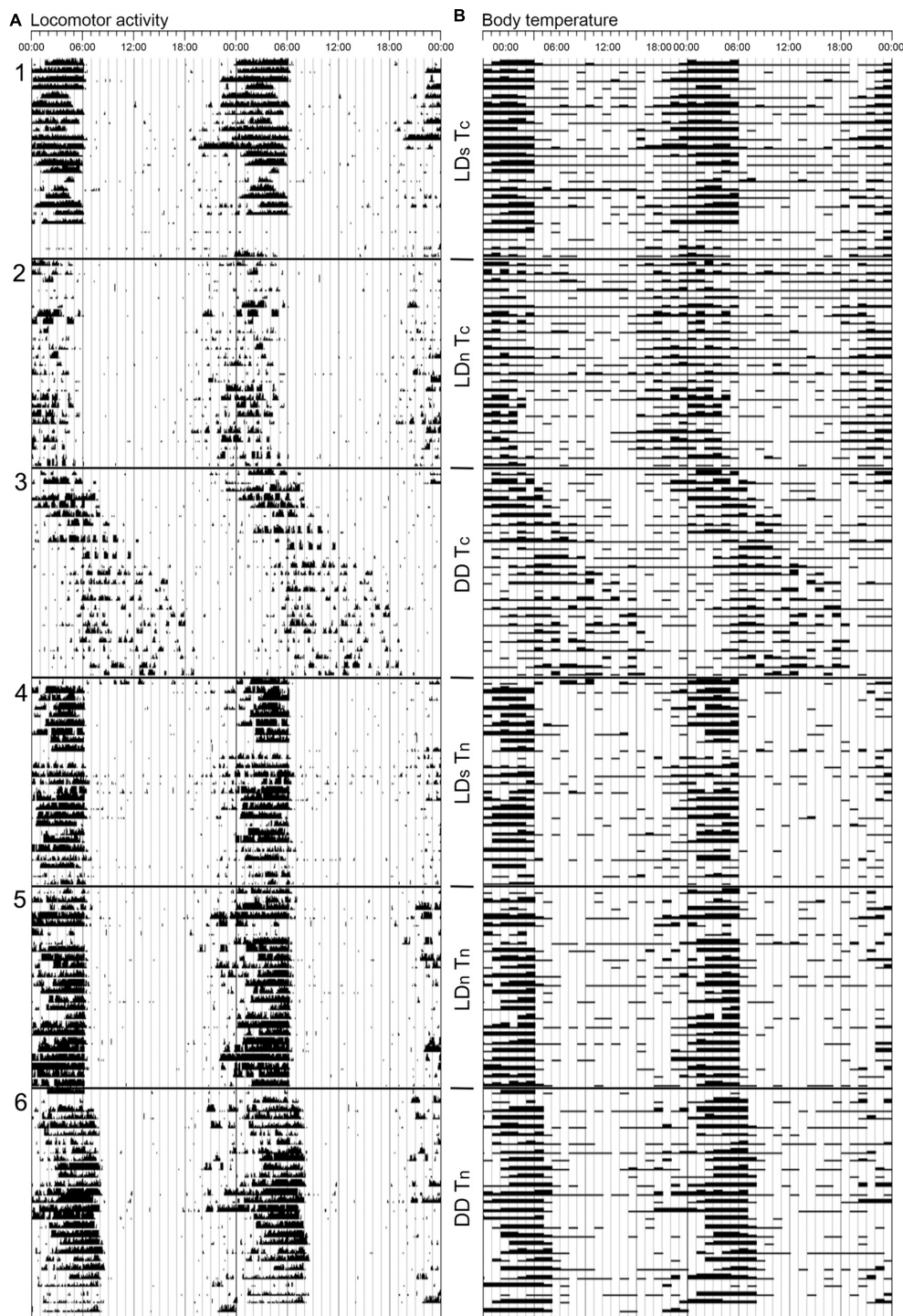
FIGURE 3 | An example of actograms for **(A)** locomotor activity and **(B)** body temperature of a Mahali mole-rat (*Cryptomys hottentotus mahali*) that was subjected to six consecutive light and temperature regimes. The light cycle order was a square wave cycle (12L:12D), simulated dawn and dusk natural cycle (1 h each, 12 h complete darkness) and DD, repeated twice (cycles 1–3, and 4–6). The first three cycles (1–3) had a constant ambient temperature of 25°C, and the last three (4–6) were subjected to a temperature cycle varying between 18 and 28°C over 24 h. The actograms show the close relationship between locomotor activity and body temperature. Cycles 4–6 show more robust rhythmicity, implying the supportive role of ambient temperature to entrainment. A comparison between the DD cycles shows free-running under constant ambient temperature (cycle 3) while activity remains entrained but slightly shifted with a temperature cycle (cycle 6). Actograms are double plotted with the time on the *X*-axis. The number of days is on the *Y*-axis and the experimental conditions are illustrated between the two actograms. LD$_s$, square wave; LD$_n$, natural wave; T$_c$, constant ambient temperature; T$_n$, natural ambient temperature cycle. (Modified from Van Jaarsveld et al., 2019, with permission from Elsevier).

TABLE 1 | A summary of the literature on daily rhythms of African mole-rats.

Species	Literature	S/C	Rhythm details	#	L/F
Georychus	Lovegrove and Papenfus, 1995	S	Nocturnal activity, free-running in DD and LL, splitting in LL	7	L
			Cycles: 12L:12D, LL, DD		
	Lovegrove and Muir, 1996	S	Activity and T_b rhythms present, T_b may not be endogenous	6	L
			Cycles: LD, DD		
	Oosthuizen et al., 2003	S	Mostly nocturnal activity, some diurnal, re-entrain and show free-running, variable	11	L
			Cycles: 12L:12D, forward and backward shifts, DD, short and long days		
	Okrouhlík et al., 2021	S	T_b rhythms in summer and winter, distinct peaks in activity	2	F
			Field: Winter/summer		
	Hart et al., 2021a	S	Activity entrainment to T_a cycles	12	L
			Cycles: DD with T_a variations		
Bathyergus	Herbst and Bennett, 2006	S	*B. janetta* – Mound production at night or early morning	9	F
			Field: winter/summer		
	Okrouhlík et al., 2021	S	*B. suillus* – T_b rhythms in summer and winter, peaks during the day	6	F
			Field: Winter/summer		
Heliophobius	Jarvis, 1973	S	Dispersed activity	1	F
	Šklíba et al., 2007	S	Activity at any time of the day, timing related to T_a, amount related to rainfall	11	F
	Ackermann et al., 2017	S	Diurnal activity, very slow entrainment	13	L
			Cycles: 12L:12D, 12 h shift, DD, Ta changes, long and short day		
Cryptomys	Hickman, 1980	S, C	Intermittent activity of single and groups of animals, slightly more at night	6	L
			Cycles: Natural light in combination with 9L:15D		
	Bennett, 1992	C	Activity at all times of the day, no distinct nocturnal/diurnal pattern	8	L
			Cycles: LL		
	Oosthuizen et al., 2003	S	Mostly nocturnal activity, some diurnal	7	L
			Cycles: 12L:12D, 12 h shift, DD, LL, long and short day		
	Hart et al., 2004	S	Nocturnal, good entrainment, free running under DD and LL	12	L
			Cycles: 12L:12D, 12 h shift, DD, LL		
	Gutjahr et al., 2004		*C.h. pretoriae* – Melatonin rhythms in LD, suppressed in LL	138	L
			Cycles: 12L:12D, DD, LL		
	Schöttner et al., 2006	S	Nocturnal activity, slow entrainment, free running, variable	12	L
			Cycles: 12L:12D, 12 h shift, DD		
	Oosthuizen and Bennett, 2015	S	Nocturnal, level of activity dependent on T_a, breeding status differences	16	L
			Cycles: 12L:12D Ta variations		
	Haupt et al., 2017	S	Nocturnal, activity and T_b rhythms, T_b inversely related to T_a	16	L
			Cycles: 12L:12D, DD, Ta variations		
	Van Jaarsveld et al., 2019	S	Nocturnal, square wave better entrainment, free run, more robust rhythms under cyclic T_a	16	L
			Cycles: Light square wave, dawn/dusk, constant T_a, cyclic T_a		
	Oosthuizen et al., 2021	C	Summer – Bimodal rhythms, winter – unimodal rhythms, T_a related	39	F
			Field: summer/winter	(23/16)	
	Hart et al., 2021a	S	Activity entrainment to T_a cycles	12	L
			Cycles: DD with T_a variations		
Fukomys	Genelly, 1965	C	*C. hottentotus* (*F. darlingi*) Active at all hours of the day and night	7, ?	L/F
			Cycles: Not specified, probably 12L:12D?		
	De Graaf, 1972	C	*F. damarensis* – Unknown, indications of increased activity around dawn and dusk	?	F
			Field: Time not specified		
	Lovegrove, 1988	C	*F. damarensis* – Equally active during day and night	5	F
			Field:		
	Lovegrove et al., 1993	C	*F damarensis* – LD: diurnal activity, long day – distinct activity spike, fast entrainment, free-running	2 col	L
			Cycles: 12L:12D, 16L:8D, DD	(5/4)	
	Oosthuizen et al., 2003	S	*F. damarensis* – Mostly diurnal, some nocturnal, entrainment, free-running	8	L
			Cycles: 12L:12D, 12 h shift, DD, LL, long and short day		

(Continued)

TABLE 1 | (Continued)

Species	Literature	S/C	Rhythm details	#	L/F
	Richter et al., 2003	L	*F. damarensis* – Clear melatonin rhythms in LD, modified by photoperiod	24	L
			Cycles: 12L:12D, 14L:10D, 10L:14D		
	Vasicek et al., 2005b	S	*F. darlingi* – Most nocturnal, some diurnal, variable, entrainment, free-running, 2 entrained to masking cycle	12	L
			Cycles: 12L:12D, 12 h shift, DD, LL, masking (3 h cycles)		
	Vasicek et al., 2005a	S	*F. darlingi* – Melatonin rhythms under LD cycles, DD and LL	12	L
			Cycles: 12L:12D, DD, LL		
	de Vries et al., 2008	S	*F. anselli* – Mostly nocturnal	11	L
			Cycles: 12L:12D, 12 h shift, DD		
	Streicher et al., 2011	C	*F. damarensis* – Variable, 24 h and 12 h rhythms, multiple, arrhythmic	23	F
			Field: Summer/winter	(8/15)	
	Schielke et al., 2012	C	*F. anselli* – Tendency towards rhythmicity	47	L
			Cycles: Natural light conditions		
	Lövy et al., 2013	C	*F. mechowii* – Nocturnal, variable depending on social status	6	F
	Šklíba et al., 2014	C	*F. anselli* – 1 diurnal daily peak, T_a related, social cues may disrupt Ta related activity rhythms	17	F
	Oosthuizen and Bennett, 2015	S	*F. damarensis* – Nocturnal, activity level depends on Ta	16	L
			Cycles: 12L:12D with T_a variations		
Heterocephalus	Davis-Walton and Sherman, 1994	C	No colony level circadian rhythmicity	2 col	L
			Cycle: Dim red light (DD)	19/28	
	Riccio and Goldman, 2000a	S	Mostly nocturnal, variable, LD: entrain/freerun/arrhythmic	15*	L
			Cycles: 12L:12D varying intensities, 6 h shift, T cycles		
	Riccio and Goldman, 2000b	S	Rhythms of T_b and metabolism, increases coincided with increased activity	4	L
			Cycles: 12L:12D		

Twenty-seven animals were introduced in this experiment but only 15 used the running wheels.

The S/C column indicates whether the animals were housed singly or in colonies. The sample size is in the # column, numbers in brackets indicate summer and winter samples, or the number of animals in colonies. Studies conducted in the Lab or Field are indicated in the L/F column. T_b, body temperature; T_a, ambient temperature.

but it takes very long to analyse subsequently. Alternatively, RFID tags can be implanted in the animals and tag readers can be placed strategically over a laboratory tunnel system. These tags and readers are also costly and do not provide a continuous activity feed.

Housing conditions can also potentially affect activity of animals, firstly the actual housing containers mole-rats are frequently maintained in are square box containers while they live in tunnels in their natural habitat. In addition, social animals are frequently housed in isolation to record locomotor activity of a single animal as a result of recording constraints mentioned above. Social entrainment of rhythms has been implicated for social mole-rat species (Šklíba et al., 2014), therefore rhythmicity of animals may differ according to whether they are isolated or in their natal colonies.

The ambient conditions in the laboratories can also influence activity rhythms of animals. Light is the primary cue for circadian rhythmicity in most animals, and mole-rats are fully capable of perceiving it. In the Highveld mole-rat, *C.h. pretoriae*, it has been shown that these animals are sensitive to the intensity of light, activity is masked by light at higher intensities (above 10 lux), and animals show higher levels of activity at lower light intensities (below 10 lux), although the temporal profile remains relatively similar. Ambient temperature also affects the level of activity, with mole-rats being less active at higher temperatures that approaches the thermoneutral zone (around 30°C), and more active at cooler temperatures (Oosthuizen and Bennett, 2015; Haupt et al., 2017).

LABORATORY VS. FIELD

The results of several field studies indicate that many mole-rat species have distinct peaks in their daily activity (Šklíba et al., 2007; Streicher et al., 2011; Okrouhlík et al., 2021; Oosthuizen et al., 2021; Finn et al., 2022). These peaks appear to be related to the burrow temperatures, which is not surprising as temperatures in the burrows fluctuate daily and seasonally (Bennett et al., 1988; Šklíba et al., 2007; Lövy et al., 2013). Whether these rhythms are in fact circadian or merely in response to thermoregulatory challenges would require further investigation. Most laboratory studies include light in the testing regimes, and although mole-rats can perceive light this is not a natural situation for them. In their natural habitat, mole-rats are not exposed to light on a regular basis, but they are subjected to slight temperature fluctuations in their burrows. It is therefore to be expected that mole-rats would be more sensitive to temperature changes and also be able to entrain to rhythmic

temperature cycles. Entrainment to temperature cycles has in fact been tested in the laboratory, it seems that light still overrides temperature as a *zeitgeber*. When both light and temperature cycles are presented, temperature cues play a supportive role but if light is not present, animals entrain their activity to temperature cues (**Figure 3**; Van Jaarsveld et al., 2019; Hart et al., 2021b).

Interestingly, early studies found no difference between laboratory and field activity in mole-rats (Jarvis, 1973; Hickman, 1980) but noted that the type of activity in the two environments may differ. More recent studies under more controlled and stringent laboratory conditions, and thus more unnatural set-ups, may result in the larger differences observed between laboratory and field rhythmicity.

DIURNALITY VS. NOCTURNALITY

Most animals are adapted to a specific temporal niche and arrange their physiology and behaviour accordingly to display diurnal, nocturnal or crepuscular rhythms of activity. From an evolutionary perspective, rodents are thought to share a nocturnal ancestor, and indeed the majority of modern rodents are nocturnal, while diurnality has evolved secondarily in the order Rodentia (Roll et al., 2006). Mole-rats are no exception and many display distinct activity rhythms that correspond with day or night. However, the activity patterns displayed by mole-rats show large inter and intraspecies variation, and a significant proportion of animals show arrhythmic activity (Oosthuizen et al., 2003; Hart et al., 2004; de Vries et al., 2008; Oosthuizen and Bennett, 2015; Ackermann et al., 2017; Haupt et al., 2017).

In aboveground dwelling animals, the visual environments and light levels of nocturnal and diurnal animals differ markedly, and over time, the eyes of animals have adapted to the amount and quality of light they are exposed to in their particular environments (Hall and Ross, 2006). The anatomy of the eye is therefore usually a good indicator of the temporal niche that animals occupy (Gerkema et al., 2013). The general morphology of the eye (size and shape) and types of photoreceptors present differ between nocturnal and diurnal animals (Schmitz and Motani, 2010). Nocturnal animals frequently have large eyes, large pupils to allow more light to enter the eye, and large corneas and lenses relative to eye size as an adaptation for increased visual sensitivity, whereas the opposite is true for diurnal animals (Hall et al., 2012). The retinas of nocturnal animals typically contain greater numbers of rod photoreceptors for higher light sensitivity, while diurnal animals have more cone photoreceptors for colour vision and have a higher threshold for bright light (Kelber, 2018). Commonly, nocturnal rodents have cone populations in the order of 0.5–3% of the total photoreceptor complement whereas diurnal species have much higher proportions of cones in their retina (Feldman and Phillips, 1984; Szél and Röhlich, 1992; Calderone and Jacobs, 1995; Peichl and Moutairou, 1998;

Bobu et al., 2008; Gaillard et al., 2008; Kryger et al., 2018; Van der Merwe et al., 2018).

Surprisingly, the eye morphology of mole-rats resembles that of diurnal rather than nocturnal animals in several aspects. While the eye size of all mole-rats is microphthalmic (1.3–3.5 mm range), most species (except the naked mole-rat) have small lenses compared to their eye sizes (Nikitina et al., 2004; Němec et al., 2008a). In addition, the photoreceptor proportions are more similar to diurnal rodents than nocturnal rodents. Although the retina is rod dominated, mole-rats possess in the vicinity of 10% cones, which is very high in comparison to nocturnal rodents [mouse: 3% (Jeon et al., 1998); rat: <1% (Szél and Röhlich, 1992; Peichl et al., 2004; Němec et al., 2008a)]. In reality, some mole-rat species show diurnal activity [Damaraland mole-rat (Lovegrove et al., 1993; Oosthuizen et al., 2003), Silvery mole-rat (Ackermann et al., 2017)], and some others display nocturnal activity (Cape mole-rat (Lovegrove and Papenfus, 1995; Oosthuizen et al., 2003), all *Cryptomys* species investigated (Hart et al., 2004; Schöttner et al., 2006; Haupt et al., 2017), Ansell's mole-rat (de Vries et al., 2008) and the naked mole-rat (Riccio and Goldman, 2000b). In some cases, animals from a species display different temporal activity in different experiments, for example Damaraland mole-rats displayed diurnal activity in the study by Lovegrove and Papenfus (1995), whereas Oosthuizen and colleagues found most animals to be diurnal, but also some nocturnal animals in 2003, and in 2015, all 16 animals displayed nocturnal activity (Oosthuizen et al., 2003; Oosthuizen and Bennett, 2015).

Laboratory conditions, however, are very unnatural for mole-rats. In their natural habitat, their burrow systems are sealed and devoid of light, therefore the animals are not routinely exposed to light for entrainment (Bennett and Faulkes, 2000). Even if a burrow is opened, very little light penetrates to the burrow, and it dissipates very rapidly (Kott et al., 2014). Hence, temporal activity patterns of mole-rats are likely more strongly influenced by their social environment and conditions in their microenvironment. Social entrainment is generally not regarded as a strong *zeitgeber*, but in the absence of light, it may play a larger role in synchronising activity of animals (Mistlberger and Skene, 2004). Some studies indicate that free-living mole-rats in colonies are active at the same time (Šklíba et al., 2014; Oosthuizen et al., 2021), whereas another suggests that individual activity patterns are not synchronised (Lövy et al., 2013). Social synchronisation of activity is probably also to some degree dependent on the body size of the animals and ambient temperature in the burrow systems. Species with smaller body sizes may be more synchronised than larger-bodied species for thermoregulatory purposes, as animals would huddle together the nest when it is colder (Šumbera, 2019). There are daily and seasonal temperature fluctuations in the burrows, albeit dampened compared to aboveground (Bennett et al., 1988; Lovegrove and Knight-Eloff, 1988; Lövy, 2011; Šklíba et al., 2014). Mole-rats seem to be very sensitive to these changes and have been shown to entrain their locomotor activity to temperature rhythms in the laboratory (Van Jaarsveld et al., 2019; Hart et al., 2021b). Under field conditions, in the absence of light, ambient temperature

could substitute as the primary cue for daily entrainment (Oosthuizen et al., 2021).

SEASONAL RHYTHMICITY IN MOLE-RATS

Many animals exhibit seasonal changes in their behaviour and physiology, especially ones that inhabit temperate regions with large annual environmental changes (Prendergast et al., 2002). The pineal hormone, melatonin, plays a crucial role the mediation of seasonality. The pineal gland activity is under circadian control, such that melatonin is secreted exclusively during the night and is suppressed by light (Wehr, 1997; Richter et al., 2003). The duration of the nocturnal melatonin peak is used for photoperiodic information. The melatonin rhythm is closely associated with the T_b rhythm and can also be modulated by non-photic cues such as exercise (Wyatt et al., 1999; Yamanaka et al., 2014). Animals use proximate factors such as day length and temperature to time ultimate processes such as reproduction, hibernation and migration, all of which require prior anticipation and preparation.

Locomotor Activity and Body Temperature

Mole-rats do not hibernate, although some species are more heterothermic (Lovegrove, 1986; Bennett et al., 1993; Marhold and Nagel, 1995; Boyles et al., 2012; Oosthuizen et al., 2021), and a recent study shows evidence of occasional torpor events in one of the social *Cryptomys* species. These torpor bouts were not frequent, did not show any rhythmic pattern and were not related to T_a (Oosthuizen et al., 2021).

Several laboratory studies have investigated photoperiodic changes in locomotor activity, however these studies invariably subjected animals to long and short-day light conditions without taking ambient temperature into account. All laboratory studies indicate that both solitary and social mole-rats can adjust their activity periods according to different day lengths, thus effectively synchronising to long and short-day light cycles (Lovegrove et al., 1993; Oosthuizen et al., 2003; Ackermann et al., 2017). Since it is difficult to recreate authentic seasonal changes in the laboratory, seasonal variations in locomotor activity and T_b can best be observed in the field. The measurement of locomotor activity of free-living mole-rats can be challenging and labour intensive, but radiotelemetry and implanted data loggers have been used successfully in the field (Šklíba et al., 2007; Streicher et al., 2011; Lövy et al., 2013; Okrouhlík et al., 2021; Oosthuizen et al., 2021). Data loggers measure body temperature variations, and locomotor activity can be inferred from increases and decreases in body temperature (Refinetti, 1999; Oosthuizen et al., 2021). Mound production has also been used to assess activity of animals (Genelly, 1965; Herbst and Bennett, 2006), however, this is not a very accurate measure of circadian or seasonal activity. Although mole-rats may create more mounds during certain times of the day, they may be active during other times as well (Hickman, 1980). Seasonally, mound production is highly dependent on rainfall, several species have been reported to be more active after rainfalls (Genelly, 1965; De Graaf, 1972; Lovegrove, 1988; Buffenstein et al., 2012). Mole-rats can still extend burrows during the dry season, but instead of digging through the hard top layers of soil, old tunnels are filled up while no aboveground activity is visible (Jarvis et al., 1998; Šumbera et al., 2003b). Some of the solitary species change their burrow architecture seasonally by changing burrowing strategy, but in most species, burrow systems are not significantly modified according to season (Šumbera et al., 2003b; Thomas et al., 2012a,b, 2013, 2016).

Field data shows that there are seasonal shifts in locomotor activity and T_b in several species of mole-rats (**Table 2**; Šklíba et al., 2007; Streicher et al., 2011; Okrouhlík et al., 2021; Oosthuizen et al., 2021; Finn et al., 2022). Locomotor activity and T_b of the smaller bodied social species, and solitary animals seem to be correlated with burrow temperature, whereas the large bodied social species appear to be arrhythmic in the field and activity has no relation to burrow temperatures (Lövy et al., 2013). These shifts in activity could again be related to thermoregulatory constraints of smaller animals, and it is not known whether the rhythms exhibited are endogenous or in response to environmental conditions.

Seasonal Breeding

Solitary subterranean mammals frequently breed seasonally (Nevo, 1961; Bennett and Faulkes, 2000; Sol Fanjul et al., 2006; Katandukila et al., 2013), with the breeding season typically associated with rainfall and food availability to ensure that young are born at the optimal time of the year (Katandukila et al., 2013). This is also true for African mole-rats, all solitary mole-rat species are seasonal breeders (Jarvis, 1969b; Van der Horst, 1972; Bennett and Jarvis, 1988a; Bennett et al., 1991; Šumbera et al., 2003a; Herbst et al., 2004; Hart et al., 2006a,b). Solitary mole-rats are notoriously difficult to breed in the laboratory, thus all information on the seasonality of breeding originates from field studies. However, laboratory studies have confirmed that all of the solitary species do have the potential for opportunistic breeding should environmental conditions allow it (Herbst et al., 2004; Hart et al., 2006a,b, 2008; Oosthuizen and Bennett, 2007, 2009; Oosthuizen et al., 2008a; Ngalameno et al., 2017).

Solitary mole-rats are usually aggressive and territorial, in particular outside the breeding season (Nevo, 1979; Šumbera, 2001; Bennett et al., 2006). They also typically have long gestation times (*Bathyergus* ∼50 days, *Georychus* ∼46 days and *Heliophobius* ∼90 days) (Bennett and Faulkes, 2000), which suggests that they would have to anticipate the breeding season well in advance and require a proximal environmental cue other than light. Most of the solitary bathyergids inhabit mesic areas with relatively predictable rainfall seasons (Bennett and Faulkes, 2000), and indeed, the breeding of solitary mole-rats appear to be strongly linked with the rainy seasons (Šumbera et al., 2003a; Herbst et al., 2004; Hart et al., 2006a).

TABLE 2 | A summary of the literature on the presence or absence of seasonal rhythms in African mole-rats.

Genus	Literature	Rhythm details	L/F
Georychus	***Activity/T_b***		
	Oosthuizen et al., 2003	*G. capensis* entrains to long and short days	L
	Thomas et al., 2012a	Seasonal differences in burrow structure	F
	Okrouhlík et al., 2021	T rhythms – Small seasonal shifts in T_b rhythms	F
	Reproduction		
	Bennett and Jarvis, 1988a	Breeds seasonally	L/F
	Oosthuizen and Bennett, 2007	Potential for opportunistic breeding (GnRH challenges)	L
	Němec et al., 2008a	No seasonal differences in GnRH containing neurons	L
	Oosthuizen and Bennett, 2009	No seasonal differences in sex hormones	L
Bathyergus	***Activity/T_b***		
	Herbst and Bennett, 2006	*B. janetta* – Seasonal difference in mound production, more in wet season	F
	Thomas et al., 2012b	*B. suillus* – No seasonal changes in burrow geometry and digging activity	F
	Okrouhlík et al., 2021	*B. suillus* – T rhythms – small seasonal shifts in T_b rhythms	F
	Reproduction		
	Jarvis, 1969a	*B. suillus* – Seasonal breeder, histology	L
	Van der Horst, 1972	*B. suillus* – Seasonal breeder, histology	L
	Bennett et al., 1991	*B. suillus and B. janetta* – Seasonal breeders	L
	Herbst et al., 2004	*B. janetta* – Breed seasonally, elevated sex hormones in breeding season	F
	Hart et al., 2006a	*B. suillus* – Seasonal differences in gonadal morphometrics and sex hormone concentrations	L
	Hart et al., 2006b	*B. suillus* – No seasonal difference in pituitary potential	L
	Hart et al., 2008	*B. suillus* – No seasonal difference in GnRH-ir cells, smaller in females in non-breeding season	L
Heliophobius	***Activity/T_b***		
	Šumbera et al., 2003b	Seasonal burrow changes	F
	Šklíba et al., 2007	Mostly unimodal activity, seasonal phase shifts in activity	F
	Ackermann et al., 2017	Entrains activity to long and short days	L
	Reproduction		
	Jarvis, 1969a	Seasonal breeder	L/F
	Šumbera et al., 2003a	Seasonal breeding	L/F
	Ngalameno et al., 2017	Seasonal breeding, increase in gonadal geometrics and hormones during breeding season	F
Cryptomys	***Activity/T_b***		
	Thomas et al., 2013	*C.h. hottentotus* – No seasonal difference in burrow geometry	F
	Oosthuizen et al., 2021	*C.h. natalensis* – Seasonal shifts in temporal Tb	F
	Finn et al., 2022	*C.h. natalensis* – Seasonal shifts in temporal activity	F
	Reproduction		
	Bennett, 1989	*C.h. hottentotus* – Breed seasonally	L
	Spinks et al., 1997	*C.h. hottentotus* – Breed seasonally, ♂ – no seasonality in testicular morphology	L
	Spinks et al., 1999	*C.h. hottentotus* – Breed seasonally, ♀ – continued reproductive function in non-breeding season	L
	Spinks et al., 2000	*C.h. hottentotus* – No seasonal difference in LH response to GnRH challenge	L
	Van der Walt et al., 2001	*C.h. pretoriae* – No seasonal difference in gonadal histology or LH response to GnRH challenge	L
	Janse van Rensburg et al., 2002	*C.h. pretoriae* – Seasonal breeder, retain reproductive potential in non-breeding season	L
	Du Toit et al., 2006	*C.h. pretoriae* – GnRH system is not affected by season	L
	Oosthuizen et al., 2008b	*C.h. natalensis* – Aseasonal breeders, no seasonal difference in LH response to GnRH challenge	L
	Oosthuizen et al., 2010b	*C.h. natalensis* – Gonadal histology and hormones do not differ seasonally	L
	Hart et al., 2021b	*C.h. mahali* – Aseasonal breeder	L
Fukomys	***Activity/T_b***		
	Genelly, 1965	*F. anselli* – Mounds produced during the rainy season	F
	De Graaf, 1972	*F. damarensis* – More active after rain, indications of increased activity around dawn and dusk	F
	Lovegrove, 1988	*F. damarensis* – Increased mound production after rains	F
	Lovegrove et al., 1993	*F. damarensis* – Activity phase shifts according to different photoperiods	L
	Oosthuizen et al., 2003	*F. damarensis* – Entrain activity to long and short days	L
	Streicher et al., 2011	*F. damarensis* – Large variation, winter 1 activity peak, summer frequently 2 peaks	F
	Reproduction		
	Shortridge, 1934	*F. damarensis* – Fixed breeding season suspected	
	Bennett and Jarvis, 1988b	*F. damarensis* – Breed aseasonally	L

(Continued)

TABLE 2 | (Continued)

Genus	Literature	Rhythm details	L/F
	Burda, 1989	*C. hottentotus* (*F. amatus*) – Breed aseasonally	L
	Bennett et al., 1994a	*F. darlingi* – Breeds aseasonally	L
	Bennett and Aguillar, 1995	*F. mechowii* – Aseasonal breeder	L
	Sichilima et al., 2008	*F. mechowii* – Breeds aseasonally	L
	Sichilima et al., 2011	*F. anselli* – Breed aseasonally	L
Heterocephalus	*Activity/Tb*		
	Buffenstein et al., 2012	Digging to extend burrows during the breeding season	F
	Reproduction		
	Jarvis, 1969b	Seasonal breeder	F/L
	Jarvis, 1991	Aseasonal breeder	
	Brett, 1991	Aseasonal breeder	

Laboratory or field studies are indicated in the L/F column. T_b, body temperature.

Mole-rats have also been shown to be very sensitive to small fluctuations in burrow temperatures (Bennett et al., 1988; Šklíba et al., 2007; Lövy et al., 2013). It is likely that solitary mole-rats use burrow temperature as a proximate factor to anticipate the rainy season with its more abundant food and dispersal opportunities.

Some social mole-rat species have also been described as seasonal breeders (Shortridge, 1934; Jarvis, 1969a; Bennett, 1989; Janse van Rensburg et al., 2002), with more recent studies contradicting some of the older ones (Bennett and Jarvis, 1988b; Brett, 1991). Nevertheless, the majority of the social species breed aseasonally, with pregnant females captured at all times of the year (Bennett and Jarvis, 1988b; Burda, 1989; Brett, 1991; Bennett et al., 1994a; Bennett and Aguillar, 1995; Oosthuizen et al., 2008b; Sichilima et al., 2008, 2011; Hart et al., 2021a). In both seasonally and aseasonally breeding species, there are no seasonal differences in gonadal histology, gonadal hormones, LH responses to GnRH challenges, or the GnRH system in the brain (Spinks et al., 1997, 1999, 2000; Van der Walt et al., 2001; Du Toit et al., 2006; Oosthuizen et al., 2008b, 2010b).

Once in an established colony, social mole-rat species have the advantage that they do not have to search for breeding partners. The seasonally breeding *C.h. hottentotus* lives in a mesic habitat with a predictable rainfall pattern, occurring sympatrically with two of the solitary species. This species has a long gestation period of about 2 months (Bennett, 1989), suggesting the need for an external cue for the onset of the breeding season. This species probably also responds to burrow temperature changes to trigger breeding activity. Although aseasonally breeding social species also have long gestation periods, ranging from around 60–110 days (Bennett and Jarvis, 1988b; Burda, 1989; Jarvis, 1991; Bennett et al., 1994a; Bennett and Aguillar, 1995; Bennett and Faulkes, 2000; Hart et al., 2021a), they breed throughout the year, independent of external factors. For most of the social mole-rat species, the only seasonal consideration would be dispersal. Depending on their habitat, many of the species are confined to their natal colonies during drier periods and can only disperse after rainfall when the soil is soft enough to dig.

Timing of dispersal does not require an endogenous rhythm; it is most likely a direct response to rainfall and triggered by softer soils.

DO MOLE-RATS REALLY NEED RHYTHMS?

The ubiquitous nature of the circadian clock suggests an adaptive significance, and whilst this may be true for many species, it may be less obvious for animals that inhabit stable habitats. All mole-rats show a regression of the visual system with small eyes and reduced structures that are related to vision. Their circadian systems are functional, but also appear to show some degree of regression, although not as severe as for the visual system. Mole-rat circadian systems are less sensitive to light compared to aboveground dwelling rodents, nevertheless light still seems to override other environmental *zeitgebers* when it is present. In the absence of light, mole-rats are responsive to non-photic cues such as ambient temperature and to some extent, also social entrainment. The extent of regression of the visual system and circadian system in the various mole-rat appears to reflect the need or dependence on daily and seasonal rhythms. Daily variations in the activity and physiology of mole-rats do not require an endogenous rhythm *per se*, it can purely be a response to environmental conditions that can vary from day to day. In terms of seasonalbreeding, mole-rats have long gestation times and hence would require advance warning for the onset of the breeding season. The breeding season of all the solitary mole-rat species occurs at the end of the rainy seasons, both in South Africa and in East Africa (Bennett and Faulkes, 2000; Ngalameno et al., 2017; Okrouhlík et al., 2021), they can therefore respond to rainfall and moister soil to initiate reproductive behaviour. Anticipation of the breeding season may be most pertinent for the Namaqua dune mole-rat (*B. janetta*), which occurs in more arid habitats where rainfall may be less regular, unless they simply refrain from breeding when it is too dry. Overall, it appears that the survival and fitness of African mole-rats do not depend heavily on endogenous circadian or seasonal rhythmicity. It is

likely that their circadian systems are in the process of regression and disappearance, although at a different speed compared to the rest of the visual system.

ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

Climate change is one of the biggest threats to biodiversity currently (Williams et al., 2008). It brings about unpredictable and extreme changes in environmental conditions such as temperature and rainfall (Huber and Gulledge, 2011). Aboveground dwelling species are perhaps more directly and immediately affected by these threats, while the subterranean environment largely buffers its inhabitants against environmental extremes. Nevertheless, both temperature and rainfall are important *zeitgebers* for mole-rats to time their daily and seasonal activities. Responding directly to environmental conditions instead of having very robust endogenous rhythms may be viewed as an advantage in these conditions since rapid shifts in daily activity would not affect the animals adversely. Nevertheless,

changes in temperatures and rainfall can influence the food sources and the availability thereof, as well as the hardness of soil for extending burrows and dispersal. A considerable disadvantage of being strictly subterranean is that animals cannot escape their habitat easily should it become uninhabitable. Climate change is therefore a very real threat for African mole-rats, although their particular vulnerabilities are associated with their habitat and differ from those of aboveground dwelling rodents.

AUTHOR CONTRIBUTIONS

MO wrote the manuscript. NB reviewed the manuscript. Both authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

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Naked Mole-Rat Social Phenotypes Vary in Investigative and Aggressive Behavior in a Laboratory Partner Preference Paradigm

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Here we employed the partner preference test (PPT) to examine how naked mole-rat non-breeding individuals of different behavioral phenotypes make social decisions. Naked mole-rats from six colonies were classified into three behavioral phenotypes (soldiers, dispersers, and workers) using a battery of behavioral tests. They then participated in a 3 h long PPT, where they could freely interact with a tethered familiar or tethered unfamiliar conspecific. By comparing the three behavioral phenotypes, we tested the hypothesis that the PPT can be used to interrogate social decision-making in this species, revealing individual differences in behavior that are consistent with discrete social phenotypes. We also tested whether a shorter, 10 min version of the paradigm is sufficient to capture group differences in behavior. Overall, soldiers had higher aggression scores toward unfamiliar conspecifics than both workers and dispersers at the 10 min and 3 h comparison times. At the 10 min comparison time, workers showed a stronger preference for the familiar animal's chamber, as well as for investigating the familiar conspecific, compared to both dispersers and soldiers. At the 3 h time point, no phenotype differences were seen with chamber or investigation preference scores. Overall, all phenotypes spent more time in chambers with another animal vs. being alone. Use of the PPT in a comparative context has demonstrated that the test identifies species and group differences in affiliative and aggressive behavior toward familiar and unfamiliar animals, revealing individual differences in social decision-making and, importantly, capturing aspects of species-specific social organization seen in nature.

Keywords: affiliation, aggression, behavioral phenotype, eusocial, naked mole-rat, partner preference

INTRODUCTION

Animals show significant variation in social behavior, both within and between species, including selectiveness for interactions with familiar or novel conspecifics. In rodents, some species live in small social groups and show a preference for interactions with familiar group members. For example, prairie voles (*Microtus ochrogaster*) are socially monogamous, tending to live in mate pairs or small groups with extended family (Carter and Getz, 1993; Getz et al., 1993). Once a pair bond is

established, both female (Williams et al., 1992; Carter and Getz, 1993) and male (Blocker and Ophir, 2016) prairie voles prefer their partner to an opposite-sex stranger and show increased aggression toward unfamiliar conspecifics as a form of territorial or mate defense behavior (Carter and Getz, 1993; Young et al., 2011). Other species live in larger, more dynamic groups that vary based on environmental conditions, leading to greater interaction with out-group members (Berdoy and Drickamer, 2007; Ebensperger et al., 2012). For example, degus (Octodon degus) are highly social with significant female peer affiliation, where females have greater fitness in large same-sex groups (Ebensperger et al., 2016; Insel et al., 2020). Due to breeding limitations and environmental risks like predation, these groups will readily accept strangers because breeding females benefit from greater access to mates and communal breeding (Ebensperger et al., 2012; Insel et al., 2020). Thus, how animals make decisions about which individuals to approach vs. avoid and whether to be affiliative vs. aggressive is intimately intertwined with the evolution of their species-specific social organization and also has implications for individual differences in social behavior.

Within rodents, the African mole-rats (family Bathyergidae) provide an excellent opportunity to compare different social structures within closely related species. Among the 30+ species in the family, there are examples of eusocial, social, and solitary living (Faulkes and Bennett, 2021). Our study species is the naked mole-rat (Heterocephalus glaber), which is a eusocial member of the family. Naked mole-rats reside in expansive subterranean colonies with a reproductively active breeder caste consisting of a single queen and 1–3 male consorts, and a non-breeding subordinate caste (Jarvis, 1981; Brett, 1991; Jarvis et al., 1994). Most naked mole-rats exhibit lifelong philopatry by remaining in their natal colony, rarely interacting with foreign conspecifics (Burda et al., 2000). They are xenophobic and sensitive to foreign colony scent, so if an interaction with unfamiliar animals does occur, aggression is common and may result in the intruder's death (Lacey and Sherman, 1991; O'Riain and Jarvis, 1997). Rapid social decisions during acute interactions are essential and commonplace within a naked mole-rat colony. When meeting in a tunnel, naked mole-rats will make quick decisions for which animal will pass over the other, which is directly related to their social hierarchy (Clarke and Faulkes, 1997, 1999; Toor et al., 2015). Behavioral phenotypes have been described within the non-breeding subordinates, although whether they are discrete phenotypes or continuous traits is still being explored (Mooney et al., 2015; Gilbert et al., 2020; Holmes and Goldman, 2021). In the present study, we classified naked mole-rat subordinates as one of three behavioral phenotypes based on their aggression and motivation to explore. Soldiers were categorized by their high aggression toward novel animals in a one-on-one interaction, dispersers were categorized by their curiosity and willingness to leave the colony as reported previously using the dispersal paradigm, and workers were categorized as non-aggressive and non-exploratory individuals (Lacey and Sherman, 1991; O'Riain et al., 1996; Toor et al., 2020).

Here we employed the partner preference test (PPT) to examine how naked mole-rat non-breeding individuals of different behavioral phenotypes make social decisions. The PPT (Figure 1) is a standardized laboratory social choice paradigm where the experimental animal is placed in a 3 chamber apparatus and has 3 h to explore and interact with a familiar conspecific (e.g., mating partner or sibling) in one chamber and an unfamiliar conspecific in the other (Williams et al., 1992; Beery, 2021). Since naked mole-rats are potentially at risk of losing their colony scent and becoming unrecognizable to colony-mates over time (O'Riain and Jarvis, 1997), we were also interested in investigating if a shorter time in the PPT would be consistent with the full 3 h test. The PPT has commonly been used to study opposite-sex mate preferences in prairie voles but also to test for same-sex alliances in prairie voles, meadow voles (Microtus pennsylvanicus), and degus (DeVries et al., 1997; Beery et al., 2008; Insel et al., 2020). Generally speaking, animals that form selective social relationships with a specific mate or non-mate peers such as prairie voles and meadow voles spend more time with familiar vs. unfamiliar conspecifics (DeVries et al., 1997; Beery et al., 2018; Lee et al., 2019). In contrast, degus—which form flexible, non-kin based social groups—spend more time in social chambers vs. alone, but do not show a preference for familiar or novel conspecifics (Insel et al., 2020). Gregarious species such as rats, mice, and spiny mice show a lack of familiarity preferences and sometimes even show novelty preferences (Moy et al., 2004; Smith et al., 2015; Beery and Shambaugh, 2021; Fricker et al., 2022). By comparing soldier, disperser, and worker naked mole-rats in this paradigm, we tested the hypothesis that the PPT can be used to interrogate social decision-making in this species, revealing individual differences in behavior that are consistent with discrete social phenotypes.

MATERIALS AND METHODS

Animals

Six captive naked mole-rat colonies (a total of 178 animals) maintained in the University of Toronto Mississauga vivarium were used in this study. Experimental animals ranged from 12 months to 156 months of age. Considering that naked mole-rats reach adulthood within approximately 1 year and can live for over 30 years, all experimental animals were relatively young adults (Buffenstein and Craft, 2021). Each colony was housed in polycarbonate caging comprised of a medium (46 cm × 24 cm × 15 cm high) and small (30 cm × 18 cm × 13 cm high) cage connected via polycarbonate tubing, lined with corncob bedding, crinkle paper, and added tubing within the caging. The habitat size was kept constant for the duration of the study. Animal housing rooms were kept between 27 and 28°C with 50% humidity, and were on a 12 h light:dark cycle with lights on at 7:00 a.m. Animals were fed hydrated sweet potato daily and wet Teklad mouse chow three times a week. At 6 months of age, all animals were implanted with a subcutaneous microchip (Avid, Cat. No. 2,125, 12 mm) for individual identification. Animals were uniquely marked with a permanent marker for every testing session to allow visualization on video recordings. All testing took place between 12:00 P.M. and 5:00 P.M., and all work was

FIGURE 1 | The partner preference test chamber with two tethered stimulus naked mole-rats. One side of the chamber contains the familiar conspecific and the other end contains the unfamiliar conspecific. Tethered animals are blurred in the image because they rarely, if ever, remain still.

approved by the University Animal Care Committee (protocol numbers 20011632 and 20011695). All behavioral scoring (see below) was performed by an experimenter blind to the familiarity status of stimulus animals as well as sex and phenotype, but not colony, of the experimental animals.

Behavioral Phenotype Categorization

A battery of behavioral paradigms was conducted to categorize the phenotypes of all individuals within each colony. First, animals were tested using the dispersal paradigm. This testing procedure was adapted from O'Riain et al. (1996) and Toor et al. (2020). Animals were fed hydrated sweet potato approximately 2 h before testing to minimize hunger as motivation to leave the colony. A single hole was opened on the side of the larger cage, and a plastic platform (22.86 cm x 30.48 cm) was placed directly under the hole so that animals could easily explore the opening and its surrounding area. When an animal fully exited, its identification was recorded, and it was immediately returned to its colony in the cage farthest from the hole. Each trial lasted 30 min, and 3 dispersal trials (one per day for three consecutive days) were performed. Animals who exited 3 or more times during the entire disperser test (combined score across the 3 trials) were considered dispersers unless they showed aggression during the out-pairing test (see below).

Next, an out-pairing test was administered to determine each individual's aggressive phenotype. The experimental animal was paired for 10 min with an unfamiliar opposite-sex animal from a different colony with similar or less weight. The out-pairing was conducted in a 46 cm × 24 cm × 15 cm high cage (medium size). Interactions in which the focal animal punctured the stimulus animal's skin were immediately stopped. In rare cases when the stimulus animal was aggressive to the focal animal, the trial was immediately stopped and then repeated with a different

stimulus animal on the same day after completing all other remaining trials.

Soldiers were defined as any animal exhibiting aggression in the out-pairing test. Dispersers were defined as any animal who exited a cumulative of three or more times across three trials of the disperser test and was not aggressive during the out-pairing. Individuals who neither exited the colony during the disperser test nor exhibited aggression during the out-pairing test were considered workers. It is important to note that workers were not classified based on working type behaviors but rather the absence of aggression or dispersal-like behavior since working differentiation between phenotypes remains elusive (Gilbert et al., 2020; Toor et al., 2020).

Partner Preference Test

The PPT was conducted to study whether different phenotypes preferentially affiliate with familiar or unfamiliar animals. A 76 cm × 21 cm × 30 cm height cage divided into three equal-sized chambers (21 cm × 25 cm × 30 cm high) lined with corncob bedding was used (see **Figure 1**). A stimulus subordinate naked mole-rat was tethered using a soft shoelace and a spring stop toggle to either side of the testing apparatus; one stimulus animal came from the same colony as the experimental animal and the other from an unfamiliar colony. Both tethered animals were of the same sex and similar or smaller in size (if possible) to the experimental animal. The tethered stimulus animals were only used once as either a "familiar" or "unfamiliar" individual during the experiment. The stimulus animals were habituated to tethering in the chamber, while the experimental animal was habituated in a separate, empty chamber for 10 min. The experimental animal was then released into the center chamber of the apparatus, and was recorded for 180 min (3 h) using a GoPro Hero 3 camera. At the end of each trial, the corncob bedding

was changed, and the apparatus and shoelaces were disinfected. Experimental animals and stimulus animals are generally active for the entire 3 h and never huddle. Therefore, the following behaviors were scored: time spent in each chamber, time spent investigating each stimulus animal, and time spent aggressive toward each stimulus animal. A total of 20 soldiers (9 male, 11 female), 17 workers (10 male, 7 female), and 16 dispersers (8 male, 8 female) were used in this paradigm. Animals that exhibited aggression causing skin punctures were immediately removed, and the trial was ended (3 female soldiers, 4 male soldiers) (**Table 1**).

Statistical Analysis

Statistical analyses were conducted using SPSS (SPSS Statistics for Windows, v.21.0, I.B.M. Corp., Armonk, NY, United States). Preference scores were calculated as: [(time with familiar) – (time with unfamiliar)]/(total time spent interacting). Preference scores were calculated separately for (1) total time spent within a familiar or unfamiliar individual's chamber, (2) direct investigative interactions, and (3) aggressive interactions. Changes in behavior across time were investigated by comparing the 0–10 min, 10–30 min, 30 min–1 h, and 1–3 h intervals. For each preference score, a separate Repeated Measures ANCOVA was used to interrogate changes in preference across time with sex and phenotype as the independent variables and with colony as a covariate, using only the individuals who completed the entire session (i.e., were *not* removed due to aggression; $n = 46$).

Differences in behavior between phenotype groups were investigated separately at the 10 min and 3 h time points. As behavior was not being compared across time and preference scores represent a proportion of time calculated relative to each individual, we included those animals that had been removed due to aggression prior to test completion (therefore $n = 53$). For each preference score, a MANCOVA was conducted between the sexes and the three phenotypes with colony as a covariate. Main effects were explored using pairwise comparisons with LSD confidence interval adjustment for between-phenotype comparisons.

Finally, we explored whether (a) naked mole-rats prefer to spend more time in a chamber with a conspecific or alone (neutral

TABLE 1 | Summary of all individuals used for PPT.

Colony	Soldiers		Dispersers		Workers	
	F	**M**	**F**	**M**	**F**	**M**
L ($n = 27$)	1	0	0	0	0	1
I ($n = 27$)	3	2	2	1	1	2
M ($n = 34$)	2	2	2	2	1	2
F ($n = 24$)	1	3	2	2	0	2
X ($n = 25$)	3	2	1	1	2	2
E ($n = 41$)	1	0	1	2	2	2

Not all colonies had male and/or female representatives from each phenotype. Conversely, some colonies had additional individuals that met our phenotyping requirements but were not used in order to facilitate balance within and between colonies. Only those individuals that were used in the PPT are listed here. n = total number of animals in colony, including breeders.

chamber) and (b) if there was a general partner preference as revealed by spending more time in either the familiar or unfamiliar animal's chamber compared to the other animal's chamber. To do this, we used a separate Repeated Measures ANCOVA for each phenotype as well as for all animals together, followed by pairwise comparisons with LSD confidence interval adjustment for between-chamber comparisons. These analyses were done separately for the 10 min time point and the entire 3 h test.

RESULTS

When exploring changes in behavior across time, no significant effects of time interval [$F(3, 37) = 0.394$, $p = 0.758$, Wilks' $\Lambda = 0.969$], time interval × phenotype [$F(6, 74) = 1.741$, $p = 0.123$, Wilks' $\Lambda = 0.768$], time interval × sex [$F(3, 37) = 1.119$, $p = 0.354$, Wilks' $\Lambda = 0.917$], or time interval × phenotype × sex [$F(6, 74) = 0.906$, $p = 0.495$, Wilks' $\Lambda = 0.868$] were found on the preference scores for time spent in chamber (**Figure 2A**). Similarly, no significant effects of time interval [$F(3, 37) = 1.762$, $p = 0.171$, Wilks' $\Lambda = 0.875$], time interval × phenotype [$F(6, 74) = 1.031$, $p = 0.412$, Wilks' $\Lambda = 0.852$], time interval × sex [$F(3, 37) = 0.024$, $p = 0.995$, Wilks' $\Lambda = 0.998$], or time interval × phenotype × sex [$F(6, 74) = 0.387$, $p = 0.885$, Wilks' $\Lambda = 0.940$] were detected on investigative preference scores (**Figure 2B**). Finally, the same holds true for aggressive preference scores, where no significant effects of time interval [$F(3, 37) = 0.513$, $p = 0.676$, Wilks' $\Lambda = 0.960$], time interval × phenotype [$F(6, 74) = 1.205$, $p = 0.313$, Wilks' $\Lambda = 0.830$], time interval × sex [$F(3, 37) = 0.897$, $p = 0.452$, Wilks' $\Lambda = 0.932$], or time interval × phenotype × sex [$F(6, 74) = 0.447$, $p = 0.845$, Wilks' $\Lambda = 0.931$] were detected (**Figure 2C**).

When testing for differences in behavior across phenotype groups, significant effects of phenotype on the chamber, investigative, and aggressive preference scores were detected at the 10 min time point. Phenotype significantly influenced chamber preference score [$F(2, 46) = 8.583$, $p = 0.001$], where workers had significantly higher preference scores than soldiers and dispersers ($M_W = 0.397 \pm 0.119$, $M_S = -0.156 \pm 0.108$, $p = 0.001$; $M_D = -0.237 \pm 0.119$, $p < 0.0005$) (**Figure 3A**). Phenotype also influenced investigative preference scores [$F(2, 46) = 3.79$, $p = 0.03$], where workers had higher preference scores than soldiers and dispersers ($M_W = 0.1 \pm 0.137$, $M_S = -0.280 \pm 0.125$, $p = 0.048$; $M_D = -0.415 \pm 0.138$, $p = 0.011$) (**Figure 3C**). Furthermore, phenotype influenced aggressive preference scores [$F(2, 46) = 7.804$, $p = 0.001$], where soldiers had lower aggressive preference scores than did workers and dispersers (the negative value indicating preference for aggression toward unfamiliar conspecifics) ($M_S = -0.596 \pm 0.112$, $M_W = 0.054 \pm 0.123$, $p < 0.0005$; $M_D = -0.191 \pm 0.124$, $p = 0.019$) (**Figure 3E**). No significant effects of sex were found for chamber preference score [$F(1, 46) = 1.995$, $p = 0.165$], investigative preference score [$F(1, 46) = 1.231$, $p = 0.273$], or aggressive preference score [$F(1, 46) = 0.749$, $p = 0.391$]. Phenotype effects were reduced following the full 3 h test. No significant effects of phenotype [$F(2, 46) = 0.648$, $p = 0.528$] or sex

FIGURE 2 | Mean chamber, investigative, and aggressive preference scores over four time intervals in the partner preference test. **(A)** Mean chamber preference score (positive score indicates preference for time spent in the familiar animal's chamber). No significant differences were found. **(B)** Mean investigation preference score (positive score indicates preference for time spent investigating the familiar animal). No significant differences were found. **(C)** Mean aggression preference score (positive score indicates preference for time spent being aggressive toward the familiar animal). No significant differences were found. $n = 17$ workers; $n = 16$ dispersers; $n = 13$ soldiers and only includes individuals who completed the full 3 h session.

$[F(1, 46) = 0.404, p = 0.528]$ were detected for chamber preference score (**Figure 3B**). Similarly, no significant effects of phenotype $[F(2, 46) = 0.443, p = 0.645]$ or sex $[F(1, 46) = 2.282, p = 0.138]$ were detected for investigative preference score (**Figure 3D**). Phenotype marginally influenced aggressive preference scores in the full 3 h test $[F(2, 46) = 3.110, p = 0.054]$, where soldiers showed a greater preference for aggression toward unfamiliar animals than did workers ($M_S = -0.568 \pm 0.155$, $M_W = 0.01 \pm 0.17$, $p = 0.017$) (**Figure 3F**). No significant effect of sex on aggressive preference score was detected $[F(1, 46) = 1.402, p = 0.243]$.

For time spent in each type of chamber at the 10 min time point, a marginal effect of chamber type was detected for all animals combined $[F(2, 50) = 2.702, p = 0.077$, Wilks' $\Lambda = 0.902]$. Significant pairwise effects revealed that animals spent significantly more time in the familiar animal's chamber than alone in the neutral chamber ($M_F = 210.039 \pm 19.072$, $M_N = 134.230 \pm 10.207$, $p < 0.0005$) and also significantly more time in the familiar animal's chamber than the unfamiliar animal's chamber ($M_U = 145.036 \pm 13.813$, $p = 0.015$) (**Figure 4A**). A similar pattern was observed for workers where, although no significant main effect of chamber type was detected $[F(2, 14) = 2.314, p = 0.135$, Wilks' $\Lambda = 0.752]$, significant pairwise effects were found: workers spent significantly more time in the familiar animal's chamber than alone in the neutral chamber ($M_F = 306.476 \pm 26.694$, $M_N = 158.522 \pm 14.749$, $p = 0.001$) and also significantly more time in the familiar animal's chamber than the unfamiliar animal's chamber ($M_U = 134.998 \pm 24.565$, $p = 0.003$) (**Figure 4C**). For dispersers, no significant effect of chamber type was detected $[F(2, 13) = 1.468, p = 0.266$, Wilks' $\Lambda = 0.816]$ though significant pairwise tests revealed dispersers spent significantly more time in the unfamiliar animal's chamber than in the familiar animal's chamber ($M_U = 291.262 \pm 32.341$, $M_F = 170.006 \pm 23.507$, $p = 0.045$) and alone in the neutral chamber ($M_N = 138.731 \pm 12.821$, $p = 0.003$) (**Figure 4E**). No significant effect of chamber type was detected for soldiers $[F(2, 17) = 0.408, p = 0.671$, Wilks' $\Lambda = 0.954]$, nor were there any significant pairwise comparisons (**Figure 4G**).

For time spent in each type of chamber for the full 3 h test, a significant effect of chamber type was detected for all animals combined $[F(2, 50) = 16.609, p < 0.000005$, Wilks' $\Lambda = 0.601]$ where animals spent more time in the familiar and unfamiliar animal's chamber than alone in the neutral chamber ($M_F = 4042.328 \pm 328.715$, $M_N = 1666.747 \pm 121.301$, $p < 0.000001$; $M_U = 3307.923 \pm 316.385$, $p < 0.000001$) (**Figure 4B**). For workers, a significant effect of chamber type was detected $[F(2, 14) = 14.830, p < 0.0005$, Wilks' $\Lambda = 0.321]$, where workers spent significantly more time in the familiar and unfamiliar animal's chamber than in the neutral chamber alone ($M_F = 4995.890 \pm 472.222$, $M_N = 2057.136 \pm 127.765$, $p < 0.00005$; $M_U = 3654.394 \pm 464.352$, $p = 0.007$) (**Figure 4D**). Likewise, a significant effect of chamber type was detected for dispersers $[F(2, 13) = 12.718, p = 0.001$, Wilks' $\Lambda = 0.338]$, where dispersers spent significantly more time in the familiar and unfamiliar animal's chamber than in the neutral chamber alone ($M_F = 4136.554 \pm 438.357$, $M_N = 2154.663 \pm 145.426$, $p = 0.001$; $M_U = 4277.147 \pm 462.692$,
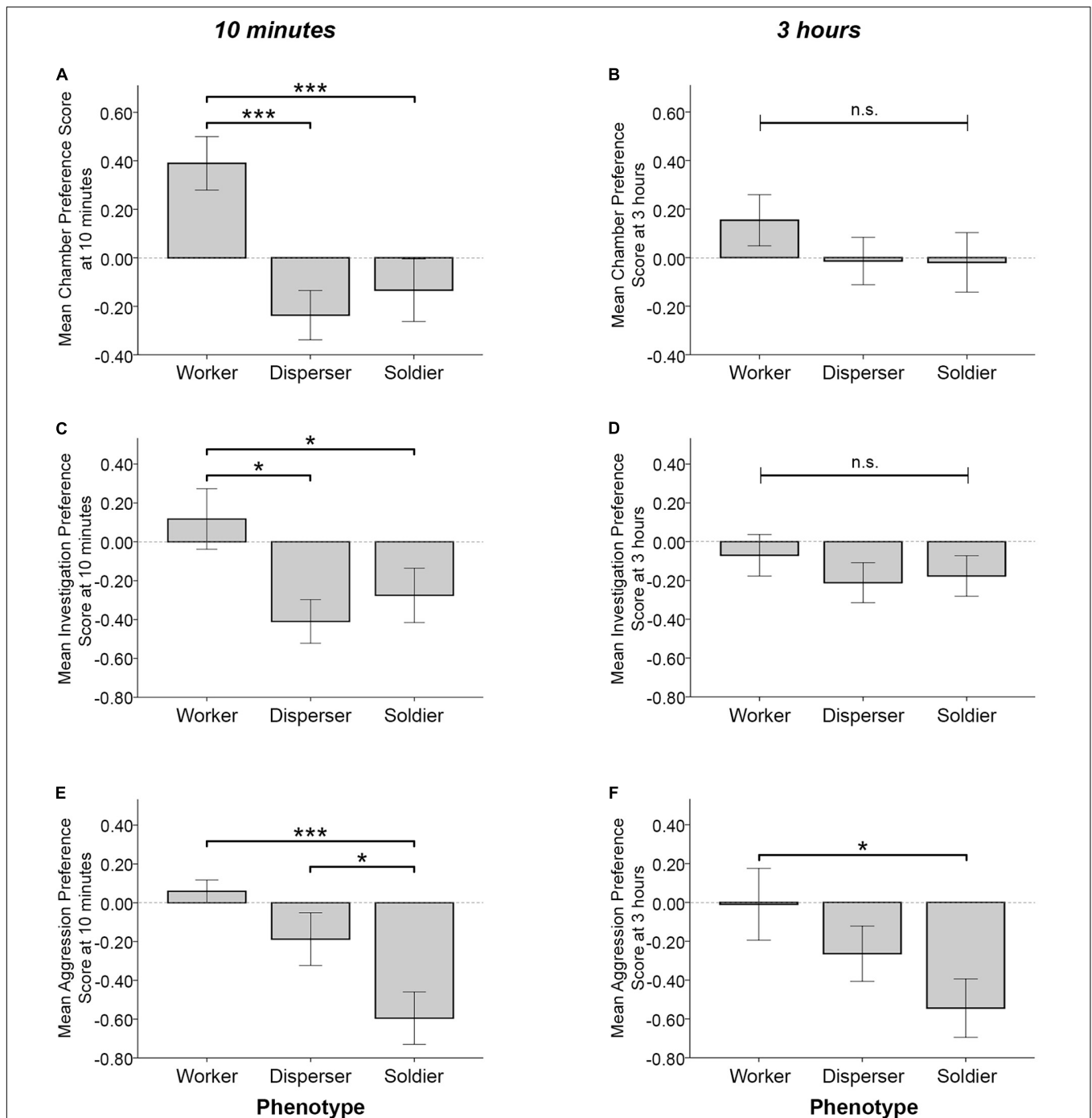
FIGURE 3 | Mean time in chamber, investigative behavior, and aggressive behavior preference scores (± SEM) for the first 10 min **(A,C,E)** and full 3 h **(B,D,F)** of the partner preference test. Workers had significantly higher chamber preference scores (positive score indicates preference for time spent in the familiar animal's chamber) than did dispersers and soldiers at the 10 min time point **(A)**, but not at 3 h **(B)**. Workers (n = 17) had significantly higher investigation preference scores (positive score indicates preference for investigating the familiar animal) than dispersers (n = 16) and soldiers (n = 20) at 10 min **(C)**, but not at 3 h **(D)**. Soldiers had significantly lower aggression preference scores (a negative score indicates preference for aggression toward the unfamiliar animal) than did workers and dispersers at the 10 min time point **(E)** and following the full 3 h test **(F)**. n.s. = not significant, *p < 0.05, ***p < 0.001 for pairwise comparisons.

p = 0.001) (**Figure 4F**). Although no significant effect of chamber type was detected for soldiers [$F(2, 17)$ = 0.461, p = 0.639, Wilks' Λ = 0.949], significant pairwise effects were found, where soldiers spent significantly more time in the familiar chamber and unfamiliar conspecific's chamber than alone in the neutral chamber (M_F = 3156.419 ± 644.195, M_N = 944.583 ± 193.839, p < 0.0005; M_U = 2503.045 ± 624.624, p = 0.017) (**Figure 4H**).
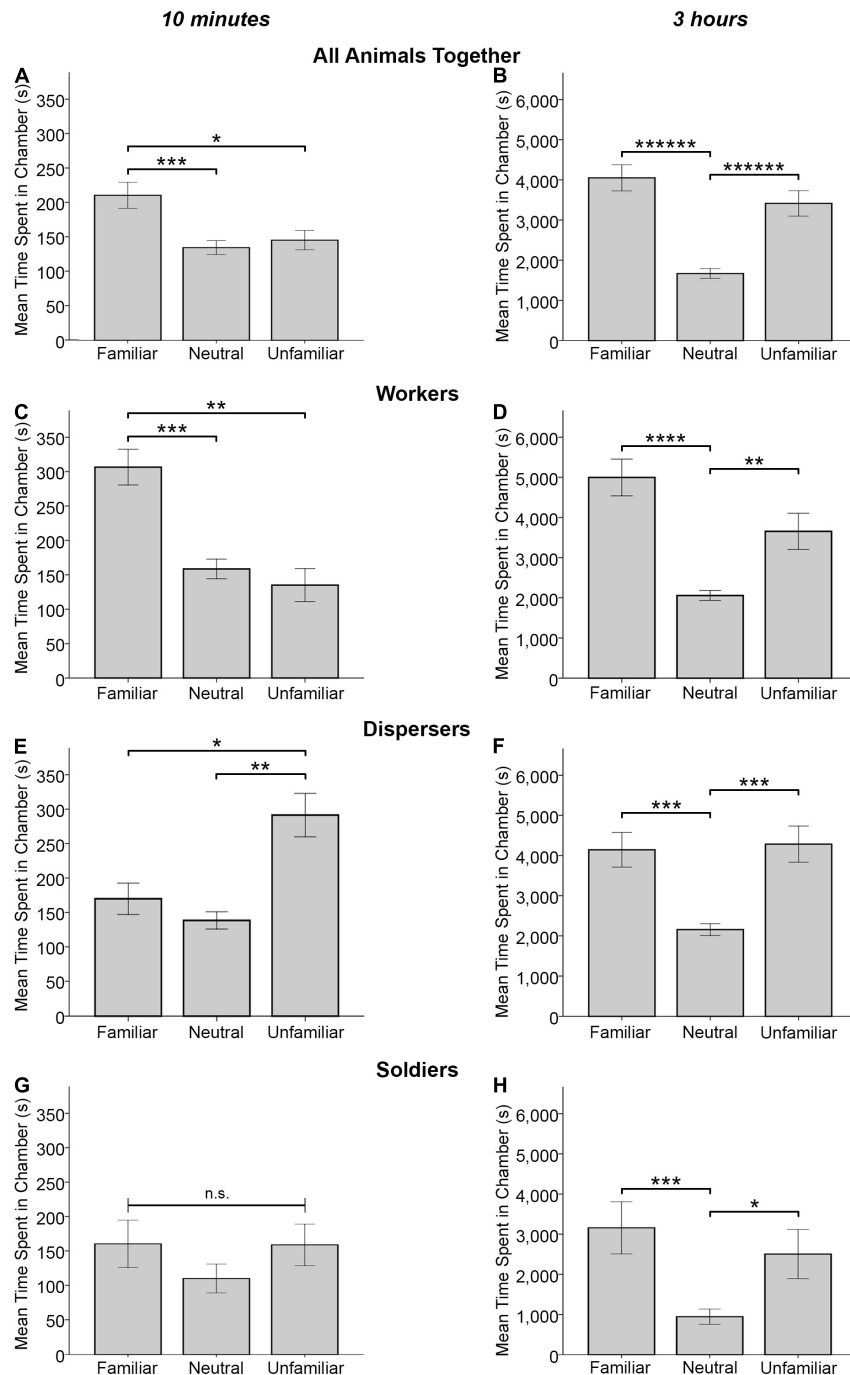
FIGURE 4 | Mean time spent in a chamber (± SEM) with either a familiar animal, unfamiliar animal, or alone in the neutral chamber by all experimental animals **(A,B)**, workers only ($n = 17$; **C,D**), dispersers only ($n = 16$; **E,F**), and soldiers only ($n = 20$; **G,H**). Data are plotted for the 10 min time point **(A,C,E,G)** and the full 3 h test **(B,D,F,H)**. All animals and all three behavioral phenotypes spent significantly more time in a chamber with another animal vs. being alone in the center chamber for the full 3 h test. At the 10 min time point, dispersers spent more time in the chamber with an unfamiliar animal vs. a familiar animal **(E)** and workers spent more time in the chamber with a familiar animal vs. an unfamiliar animal **(C**, also seen for all animals together in **A)**. n.s. = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, $******p < 0.000001$ for pairwise comparisons.

DISCUSSION

These data reveal that performance on the PPT is influenced by behavioral phenotype in non-breeding naked mole-rats.

Compared to workers, soldiers had higher preference scores for aggression toward unfamiliar conspecifics at the 10 min and 3 h comparison times (**Figure 3**). At the 10 min comparison time, workers showed a stronger chamber preference score, as well

as investigative preference score—both indicating a preference for familiar animals—compared to both dispersers and soldiers. At the 3 h time point, no phenotype differences were seen with chamber or investigative preference scores. Overall, all phenotypes spent more time in chambers with another animal vs. being alone (**Figure 4**). At the 10 min time point, workers spent more time in the chamber with the familiar animal whereas dispersers spent more time in the chamber with the unfamiliar animal (**Figure 4**). These phenotype specific patterns of time spent in each chamber were no longer present at the end of the 3 h test.

It is not necessarily surprising that soldiers had higher aggressive scores toward unfamiliar animals given they were indeed categorized as soldiers *because* they showed aggression in the out-pairing paradigm. However, these results do confirm the stability of this trait across the different testing paradigms. Importantly, in the out-pairing paradigm used for phenotyping, animals do not have the opportunity to escape the chamber and thus have reduced opportunity for social decisions. Alternatively, in the PPT, animals have three choices to make: (1) whether to be alone in the center chamber vs. with another animal, (2) whether to be with the familiar animal or the unfamiliar animal, and (3) whether to be affiliative or aggressive. The current data reveal that soldiers actively chose to be aggressive to the unfamiliar animal and they did this more than their worker/disperser siblings. Conversely, after 10 min in the PPT apparatus, worker animals spent more time in the chamber with the familiar animal and had significantly higher chamber and investigative preference scores for the familiar animal than both soldiers and dispersers. Animals classified as dispersers are somewhat intermediate in that they spent more time in the chamber with the unfamiliar animal at the 10 min time point, and had a significantly negative preference score for time in chamber and investigative behavior, indicating a preference for the unfamiliar animal but not as targets of aggression.

The stability of phenotype differences across the 3 h test varied according to behavior. For aggression, the greater preference for unfamiliar animals displayed by soldiers was significant at both 10 min and the end of the 3 h test. For investigative behavior, the greater preference for familiar animals displayed by workers was only significant at the 10 min time point; group differences were no longer significant at the end of the test. Given that naked mole-rats spend the majority of their time in the PPT doing non-social behaviors like digging (mean of total duration: 87.5%), it is possible that social investigation gets overshadowed by general activity after the initial period of investigation. From studies assessing the formation of opposite- and same-sex preferences in prairie voles (Williams et al., 1992; DeVries et al., 1997; Beery et al., 2018; Lee et al., 2019), a 3-h duration allows exploration and habituation to occur and promotes the emergence of huddling behavior toward the latter half of the study period (Beery, 2021). Although naked mole-rats do exhibit huddling behavior with colony members in the wild and laboratory (Withers and Jarvis, 1980; Sherman et al., 1992; Mooney et al., 2014), social contact in

the form of huddling was not displayed within the current study. The animals remained active throughout the entire period, never huddling with either stimulus animal. While animals were habituated to the apparatus, it is possible they will not huddle due to the presence of an unfamiliar animal (which would normally disrupt the entire colony). Similarly, the stimulus animals themselves may remain agitated due to the tethering process, preventing them from settling down. Thus, the lack of sustained preferences in naked mole-rats may reflect their agitated state in the apparatus. Furthermore, vocalizations made by the familiar or unfamiliar conspecific might also influence the focal animal's behavior. Naked mole-rats utilize antiphonal communication and their vocalizations can provide information on animal body size (Yosida et al., 2007; Yosida and Okanoya, 2009). They also produce different vocalizations in prosocial or aggressive interactions (see Barker et al., 2021a for review) and recent evidence reveals colony specific variation in vocalizations, similar to dialects (Barker et al., 2021b). It is therefore likely that vocalizations influence performance, including agitation/general activity, in the partner preference test as the focal animal gains information about familiar vs. unfamiliar and aggressive vs. prosocial individuals. Regardless, the PPT allows insight into the investigative and aggressive behavior of naked mole-rats as they relate to familiar and unfamiliar conspecifics, even if it does not capture huddling, *per se*.

The current data add to a growing body of work examining same-sex peer relationships using the PPT in rodent species with diverse social organizations. Prairie voles are socially monogamous and will cohabitate with a mate but may also form stable social groups that include extended family members (Carter and Getz, 1993). The formation of stable social groups contributes both to the selectiveness for familiar peers as well as the aggression toward unfamiliar conspecifics, especially following mating (Carter and Getz, 1993; Lee et al., 2019). While there are subtle sex differences in partner preference development, both male and female prairie voles show strong selectivity in their interactions by spending more time with familiar rather than unfamiliar same-sex conspecifics (Brusman et al., 2022). Prairie voles are also often aggressive toward unfamiliar same-sex individuals (Lee et al., 2019; Beery et al., 2021; Vahaba et al., 2021). In meadow voles, a congener species with a promiscuous mating system, animals will form social groups only during the winter season (Madison et al., 1984; Madison and McShea, 1987; Beery, 2019). Meadow voles are selective in their aggression toward strangers in that female voles will exhibit territorial behavior during summer breeding seasons but display more affiliative behavior and engage in social nesting with unfamiliar conspecifics during winter (Madison et al., 1984; Madison and McShea, 1987). In the laboratory, female meadow voles exhibit partner preference for familiar females in the PPT when housed in winter-like short photoperiods (Parker and Lee, 2003; Beery et al., 2009). Finally, in degus, communal nesting occurs in nature with closely related kin and unrelated female conspecifics coming together to form social groups (Ebensperger et al., 2004; Quirici et al., 2011; Davis et al., 2016). The social groups often change in composition across

years due to mortality and emigration, but a higher number of females in relatively stable groups confers greater fitness benefits to female degus (Ebensperger et al., 2009, 2016). In the PPT, female degus show minimal aggression toward unfamiliar same-sex conspecifics and predominantly engage in affiliative behaviors, though selective preferences are not formed based on familiarity (Insel et al., 2020). Rats and mice also do not form same-sex familiarity preferences consistent with their gregarious social structure (Beery and Shambaugh, 2021). Similar to naked mole-rats, they do not huddle with either familiar or unfamiliar conspecifics in the PPT. Use of the PPT in a comparative context has demonstrated that the test identifies species and group differences in affiliative and aggressive behavior toward familiar and unfamiliar animals, thus revealing individual differences in social decision-making, as well as capturing aspects of species-specific social organization seen in nature.

Here, we report phenotype differences in social decision-making in non-breeding, subordinate naked mole-rats. Using the PPT, we found that soldiers show a higher preference for aggression toward unfamiliar animals and a reduced preference for investigation of familiar animals compared to workers. Disperser animals were somewhat intermediate with a preference for unfamiliar animals compared to workers but reduced aggression compared to soldiers. These data are consistent with, but not confirmation of, discrete social phenotypes in the non-breeding caste of this eusocial species. Future work will need to examine the stability of these behavioral preferences across weeks and months in addition to tracking the dynamic vocal communication during dyadic/triadic interactions. Also, using the PPT to examine social decision-making in other Bathyergid species will help reveal how performance varies across closely related species with different social organization. This is important beyond the comparative PPT work done to date as it will help reveal effects due to habitat and ecology (e.g., African mole-rats are subterranean) that may be distinct from social organization, *per se*. While it is essential to study behavior in its natural context and by using ecologically relevant paradigms, using standardized laboratory paradigms across diverse species is an important complementary approach. Doing so will reveal how animals make social decisions and allow more rigorous comparison and translation across species, which will ultimately help us understand the evolution of sociality in mammals.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Toronto Animal Care Committee.

AUTHOR CONTRIBUTIONS

IT: conceptualizing the experiment, conducting and collecting all data, statistical analyses, writing and editing the manuscript, and figure creation. RM: behavior scoring, and writing and editing the manuscript. XP: behavior scoring, figure processing, and editing the manuscript. AB: conceptualizing the experiment, statistical analyses, and editing the manuscript. MH: conceptualizing the experiment, statistical analyses, and editing the manuscript. All authors contributed to the article and approved the submitted version.

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The Mating Pattern of Captive Naked Mole-Rats Is Best Described by a Monogamy Model

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Naked mole-rats form colonies with a single reproductively active female surrounded by subordinate workers. Workers perform offspring care, construction and defense of the burrow system, and food supply. Such division of labor, called "cooperative breeding," is strongly associated with the evolution of monogamous mating behavior, as seen in several mammalian lineages. This association is explained by the evolutionary theory of kin selection, according to which a subordinate adult may help to raise other's offspring if they are in full sibling relationship. In conflict with this theory, the naked mole-rat is widely considered to be polyandrous, based on reports on multiple males contributing to a colony's progeny. In order to resolve this contrast, we undertook an in-depth microsatellite-based kinship analysis on captive colonies. Four independent colonies comprising a total of 265 animals were genotyped using a panel of 73 newly established microsatellite markers. Our results show that each mole-rat colony contains a single monogamous breeder pair, which translates to a reproductive skew of 100% for both sexes. This finding, also in conjunction with previously published parental data, favors monogamy as the best-fitting model to describe naked mole-rat reproduction patterns. Polyandry or other polygamous reproduction models are disfavored and should be considered as exceptional. Overall, the empirical genetic data are in agreement with the kin selection theory.

Keywords: naked mole-rat, cooperative breeding, monogamy, polyandry, kinship analysis, microsatellite marker

INTRODUCTION

The naked mole-rat (NMR; *Heterocephalus glaber*) is a rodent species outstanding for its extraordinary longevity (maximum lifespan > 37 years) (Lee et al., 2020) cancer resistance (Tian et al., 2013; Seluanov et al., 2018), adaptation to an underground hypoxic and hypercapnic habitat (Smith et al., 2011; Fang et al., 2014; Park et al., 2021), lack of fur (Thigpen, 1940; Braude et al., 2001), and its cooperative social system (Jarvis, 1991). In NMR colonies a dominant reproductively active female, called "queen," is surrounded by subordinate workers, which take care of the offspring, defend the burrow system, and dig tunnels for foraging on plant roots and tubers

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Abbreviations: NMR, naked mole-rat.

(Jarvis, 1991; Lacey and Sherman, 1991). Such division of labor among sexually mature adults, termed "cooperative breeding," is also found in other mammals including wolfs, meerkats and beavers (Wilson, 1975; Sparkman et al., 2011; Lukas and Clutton-Brock, 2012). In these species, reproduction is significantly skewed toward one mating pair per colony, which means their breeding behavior tends to monogamy. It is assumed that the NMR queen sustains her reproductive monopoly by suppressive dominance behaviors, such as pushing and biting subordinates, during parade walks across the colony (Faulkes and Abbott, 1993; Van der Westhuizen et al., 2013). Thus, mole-rat workers usually refrain from reproductive activity as long the queen is fertile and dominant (Jarvis, 1991; Lacey and Sherman, 1991; Edwards et al., 2021).

Queens can maintain their breeding privilege for up to 17 years (Jarvis, 1991). However, extensive reshaping of colonies, which also affects the genetic composition, can occur by different mechanisms. An opportunity to take over a queen position is usually followed by social colony instability and violent fightings between high-ranking individuals (Lacey and Sherman, 1991; Van der Westhuizen et al., 2013). There is only a very small chance that animals successfully migrate to other foreign colonies (Braude, 2000) since NMRs have a fine smell- and vocalization-based kin recognition and do aggressively discriminate against foreign conspecifics (O'Riain and Jarvis, 1997; Buffenstein et al., 2022). New colonies may be formed by colony splitting, inter-colony fusion or as nascent colonies started by small groups descending from different colonies (Braude, 2000).

Cooperative breeding is tightly linked with a monogamous mating system, according to a thorough analysis of 14 independent cooperatively breeding mammal lineages (Lukas and Clutton-Brock, 2012). According to this analysis it appears that ancestors of cooperatively breeding mammals were all monogamous and that cooperative breeding without monogamy is unstable. Such a linkage is explained by the model of kin selection, according to which a subordinate adult may help to raise other's young if it has a full sibling relation to the young (Bourke, 2014). The altruistic behavior of the adult individual can function as a selective trait due to the close genetic relationship to the young sibling (50%, on average), and this is assumed to balance the cost of the adults that refrain from reproduction.

However, according to the literature, the NMR is currently viewed as polyandrous, meaning that a colony's reproduction is attributed to a single queen, but up to three breeding males (Faulkes et al., 1997; Lacey and Sherman, 1997; Buffenstein, 2005; Lukas and Clutton-Brock, 2012; Buffenstein et al., 2022). Primary evidence for this view refers to a single genetic study, on a single colony, which identified two males that contributed to colony offspring (Faulkes et al., 1997). A third male could not be excluded from paternal contribution due to limited resolution of minisatellite markers. Other studies based on behavioral observation have reinforced the classification of NMR mating behavior as polyandrous (Jarvis, 1991; Lacey and Sherman, 1991). These are, however, less conclusive since it remains unresolved whether copulation behavior leads to successful fertilization. In fact, a number of parentage studies on other species has revealed disagreements between observed mating behavior and reproduction outcome (Goossens et al., 1998; Gagneux et al., 1999; Vigilant et al., 2001).

Taken together, limited primary evidence for a small reproductive skew for males in NMR colonies leaves the question if this species poses a specific challenge to the generalized evolutionary model for cooperative breeding. In order to resolve this peculiarity, we undertook an in-depth microsatellite-based kinship analysis on four independent NMR laboratory colonies. The results indicate a high reproductive skew for both sexes, favoring monogamy as the mating model for this species, which is in agreement with the kin selection theory.

MATERIALS AND METHODS

Animals

Naked mole-rat colonies B1 and B2 were kept at the IZW in Berlin, with the approval to keep and breed NMR for research purposes by the local ethics committee of the "Landesamt für Gesundheit und Soziales," Berlin, Germany (reference no. #ZH 156; September 23, 2008). The mole-rats were housed in an artificial burrow system inside a climatized box ($2 \times 1 \times 1$ m^3 size) consisting of eight acrylic glass boxes and interconnecting tunnels. Temperature was adjusted to $25.0 \pm 2.0°C$, and humidity ranged from 40 to 70%. The chambers contained wood shavings for bedding and unbleached paper tissue as nesting material. Animals were fed with a mixed diet of vegetables, fruits *ad libitum* and cereals were provided three times per week. All newborns were marked within 12 h after birth as previously described (Jarvis, 1991; Roellig et al., 2011) and individual tissue samples were stored for later DNA extraction. For permanent marking, all pups further received a transponder microchip at an age of 3 months (7×1 mm size). Colony B1 consisted of 6 adult females, including the known queen, and 9 adult males when the monitoring started (September 2009). Over an observation period of 3 years the queen bore eleven litters with a total of 130 pups (**Table 1** and **Supplementary Figure 1**). One pup could not be sampled for analysis. Colony B2 consisted of 6 adult females, including the known queen, and 5 adult males when the monitoring started (May 2010). Over a period of 1 year the queen bore four litters, comprising 50 pups, before she died in the course of rivalry fights (**Table 1** and **Supplementary Figure 1**). Another two colonies, V1 and V2, were kept at the Vienna Zoo, Schönbrunn, Austria. Burrow systems and feeding protocols were very similar to those of colonies B1 and B2; temperature was regulated via infrared lamps, adjusting temperature to $26.0 \pm 2.0°C$. When colonies arrived at the Vienna Zoo, colony V1 consisted of six adult moles and colony V2 was already a breeding colony with 33 moles, including 11 pups from the last litter. After some successfully litters in V1 and V2, both colonies ceased breeding, at least for 2 years. At the time of sample collection (April 2012), colony V1 member size was 21 and V2 size was 39 animals (**Table 1**), and both colonies contain an unresolved mixture of adult and juvenile animals. Biopsies from the tail tips were taken from all colony members using a sterile scalpel.

TABLE 1 | Parental analysis of NMR colonies using Mendelian exclusion approaches.

Colony	Colony structure*	Informative markers	Exclusion I result	Exclusion II result
V1	5 m, 16 f	33	21 (100%) resolved animals **2 parents** 3 orphans probably co-founders	n.a.
V2	22 m, 17 f	37	38 (97.4%) resolved animals (1 ambiguous) **2 parents**	100% resolved animals **2 parents**
B1	Q, 9 m, 5 f, 129 pups	27	127/111 (98.4/86.0%)[#] resolved pups (2/18 ambiguous)[#] **1 father** 2 co-founders represent early offspring	100% resolved pups **1 father**
B2	Q, 5 m, 5 f, 50 pups	27	49 (98.0%) resolved pups (1 ambiguous) **1 father**	100% resolved pups **1 father**

Abbreviations: Q = known queen, m = adult male(s), f = adult female(s). [#]2 pups with ambiguous descent if only founder animals were considered as parents (5 alternative parental models), 18 pups with ambiguous descent if also paternal contribution of early-born male offspring was considered (46 alternative parental models). n.a. not applicable.

Ultrasound Examination

Ultrasound investigations were executed in all four colonies to investigate the reproductive conditions of the single mole-rats using a high-frequency and high-resolution ultrasonic device (Vevo 2100, VisualSonics, Inc., Toronto, ON, Canada). Reproductive activity was documented by measuring the size and condition of the reproductive organs (e.g., size, presence of corpus luteum, placental scars, sperm production) (Garcia Montero et al., 2016). General anesthesia was induced by subjecting animals inside of a small container to 5 vol.% isoflurane at an oxygen flow rate of 1.5 l/min using a Dräger Vapor 2000 system. Once the animal was asleep, anesthesia was maintained using a small mask suitable for rodents at 1.5 to 2.0 vol.% isoflurane for a maximum of 10 min. To prevent hypothermia of the moles, all procedures were performed on a heating pad (HT 200, Minitub GmbH, Tiefenbach, Germany) operating at 37°C. When fully awake, mole-rats were returned to their respective colonies.

DNA Isolation

Genomic DNA was isolated from ca. 20 mg of tissue sample using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany).

Sex Determination

Genetic sex determination based on the *DDX3Y*-psi gene locus was previously described for the NMR (Katsushima et al., 2010). We modified this concept to include the *DDX3X* gene locus as an internal reference. Degenerate PCR primers, 5'-CAG ATG GTC CAG GAG AIG CTT-3' and 5'-FAM-CCC ATA CCT TCC ATT TTC CTA A-3', were designed to target the intron 8 region of both gene loci while circumventing amplification of additional autosomal, retroposed *DDX3* gene copies. Five ng of genomic DNA was used in reactions with Taq BioMix White (Bioline), 10 pmol primers (Metabion) in a volume of 25 µl. The cycling conditions were 2 min initial denaturation at 94°C, followed by 40 cycles of 45 s denaturation at 94°C, 50 s annealing at 56°C, 60 s extension at 72°C, and a final 30 min extension

step at 72°C. Each FAM-labeled PCR product was diluted with water (typically 1:150), and 1 µl of the dilution was mixed with 10 µl optically pure formamide (Applied Biosystems) and 0.25 µl GeneScan-500 LIZ length standard (Applied Biosystems). After denaturation for 3 min at 94°C the samples were separated on POP-7 polymer on an ABI 3730xl capillary sequencer (Applied Biosystems), injecting 10 s at 1.6 kV and running at 15 kV. The electropherograms were analyzed with the GeneMapper 4.0 software (Applied Biosystems). A *DDX3Y* peak (291 bp) with a signal area greater than 0.1-fold that of *DDX3X* (237 bp) was used as an indication for male sex.

Microsatellite Markers

In total 73 novel microsatellite markers were established. This comprised three microsatellite markers developed according to a previously described enrichment protocol (Nolte et al., 2005; Leese et al., 2008), with some own modifications as described elsewhere (Molecular Ecology Resources Primer Development, Andree et al., 2010; **Supplementary Table 1**, label "Hetgla"). The method uses DNA from distantly related species for capturing of low complexity sequences in the species of interest. A bulk of 70 microsatellite markers was selected using the published *H. glaber* genome sequence (Kim et al., 2011). The genome sequence was screened for perfect sequence repetitions of unit sizes 2 and 3 bp. Microsatellites with 13–20 dinucleotide or 15–24 trinucleotide repeat units were selected as marker candidates (58,389 and 868 loci, respectively) since this length promised a balance between likely polymorphisms and a decent stutter noise during PCR amplification. PCR primers for these loci were defined using the command line version of PRIMER3 (version 2.2.3), and the primer score was used to prioritize the marker list (**Supplementary Table 1**, label "nmrsat").

Microsatellite Genotyping

PCR was done using 5 ng genomic DNA, 2.5 units of Taq polymerase (Qiagen, cat. no. 201207), 10 pmol marker-specific primers (Metabion; sequences in **Supplementary Table 1**),

one 5'-labeled with 6-carboxyfluorescein (FAM) or hexachloro-fluorescein (HEX), and 20% solution Q in a reaction volume of 25 µl. The cycling conditions were 2 min initial denaturation at 94°C, followed by 35 cycles of 45 s denaturation at 94°C, 50 s annealing at 58°C, 1 min extension at 66°C, and a final 30 min extension step at 72°C. The fluorescence-labeled PCR products were size-separated on an ABI 3730xl capillary sequencer as described for sex determination. Following automatic peak calling by GeneMapper, all electropherograms were checked for calling errors at least twice. Animals which remained orphans after a first round of kinship analysis were additionally checked for the validity of exclusive marker signals, provoking correction of two allele calls.

Kinship Analysis

Parentage was resolved at the individual level using a Mendelian test on all possible trio scenarios ("exclusion test I"), where each scenario involved the single known mother, a candidate descendant, and one of the possible fathers. For colonies which lacked family history records, each animal was tested for a possible parental role for all other animals. Iteration over trios and Mendelian testing was implemented as in-house software. Each marker was tested for X-chromosomal linkage, indicated by strict mono-allelic genotypes in males in contrast to frequent bi-allelic genotypes in females. In parallel, kinship analysis on the individual level was done using the program CERVUS (version 3.0.7) (Kalinowski et al., 2007), in order to cross-validate the findings. A second parentage test scheme ("exclusion test II") was used to resolve ambiguities after exclusion test I: given a parental model F-M (F = female parent, M = male parent) with colony-wide significance, an alternative parental model F-M', F'-M, or F'–M' (with alternative female parent F', alternative male parent M') is taken valid only if it has produced offspring that is genetically distinct from any offspring of F-M. The genotype intersection between offspring F-M and offspring F'-M' reflects the lack of resolving power in exclusion test I and quantifies the *beta* error in exclusion test II (i.e., false maintenance of parentage presumption, H_0). *Beta* likelihood was calculated from the parental genotypes, iterating the fractional intersection marker by marker. Since this *beta* calculation is based on the assumption of free marker recombination, markers with possible short-range linkage to other markers (e.g., nmrsat3763 and nmrsat3764; **Supplementary Table 1**) were excluded from this analysis.

RESULTS

Microsatellite Genotyping

We established a total of 73 microsatellite markers for the genotyping of NMRs (**Supplementary Table 1**), of which 68 markers (93%) were polymorphic in a test panel of 6 animals from three colonies. Altogether we genotyped 27 to 37 informative markers in 265 mole-rats from four captive colonies (**Table 1** and **Supplementary Figure 2**). The allele patterns indicated X-chromosomal linkage for three markers (nmrsat3637, nmrsat215074, nmrsat233031), which was confirmed by the downstream parental tests. In the progeny

genotype data we identified eight potentially novel alleles, that is, alleles that did not exist in the respective adult subpopulations. Of those alleles, six were validated as true novel alleles through repeated measurements and Sanger sequencing. The remaining two were considered as drop-outs. From this we extrapolate a germline mutation rate of $6.6 \cdot 10^{-4}$ per generation per marker. The frequency of unrecognized mutations is probably lower because mutations more likely give rise to novel alleles than converting to pre-existing alleles. Moreover, the validation rate for novel alleles indicates that the genotype mis-calling rate is lower than the rate of novel alleles, estimated to $4 \cdot 10^{-4}$.

Parentage Analysis in Captive Naked Mole-Rat Colonies

Parentage analysis at the resolution of individual offspring was done by Mendelian testing on all possible trio scenarios, also known as exclusion approach (Jones et al., 2010). In this approach, termed "exclusion test I," trios were excluded from the list of possible relations if they did not show valid Mendelian genotype patterns. In this procedure, we ignored allelic trios which involved verified spontaneous mutations (eight cases, cf. Results see section "Microsatellite Genotyping") or calling drop-outs. As a result we obtained highly resolved parentage models for all colonies (**Table 1**), including colonies V1 and V2, for which no information about the family structure was available. According to the genotype results, colony V1 contained three co-founders in addition to two parents and their 16 pups. Colony V2 was explained as a composite of two parents and their 37 pups, including one animal whose relation remained uncertain. This latter could, alternatively, have descended from two progeny individuals of the universal parents and would then be their only (surviving) pup. However, in a scenario of breeder pair switch it is very unlikely that the former breeders would have survived since breeding role takeover events are typically mediated by fatal rivalry fight in the NMR (Faulkes et al., 1997; Van der Westhuizen et al., 2013). For each of the other NMR colonies in our study, B1 and B2, we knew the identities of queen, additional founder animals and the offspring (**Table 1**). Paternity testing of the progeny against the adult founder animals gave mostly clear answers for colonies B1 (127 of 129 pups) and B2 (49 of 50 pups), indicating single universal fathers for all offspring. But, 16 additional ambiguities in the kinship model of B1 arose if we considered that grown-up progeny may have participated in breeding activity (**Table 1**). This is possible because B1 was monitored over a period of 3 years, and young NMR become sexually mature at an age of 180–225 days (Jarvis, 1991; Buffenstein, 2005; **Supplementary Text 1**). Other colonies have been monitored for a period shorter than the sexual maturation time of early offspring (colony B2), or have been analyzed in an undirected approach, considering all animals as both, parental and offspring candidates (colonies V1, V2).

So far, finding that all resolved relationships indicate one universal father per colony, and, given that ambiguous relationships always include that same universal father, we favor the kinship model with the universal father as the most parsimonious one. This is corroborated by the fact that it

does not imply mixed-sire litters. In any case, in order to quantify the relevance of ambiguous parental relationships we calculated the likelihood that a parental pair F-M produces offspring which may also be considered offspring of parental pair F-M', or vice versa (where F is the female parent, M and M' are alternative male parental candidates; **Figure 1A**). The median degree of these genotype intersections (*beta*) is 0.017 for colony B1 (maximum 0.036 in individual tests; **Figure 1B**). This small *beta* value indicates a minor loss of detection power for true additional parents if we ignore the ambiguity of intersection genotypes. That is, putative alternative paternal contributors (fathers M', M", etc.) had a very high chance to produce offspring that is genetically distinct from progeny of father M. This modified paternity test, termed "exclusion test II," was applied to all remaining ambiguities. In colony V2, the kinship model was resolved with *beta* = $1.5 \cdot 10^{-6}$, clearly indicating a single universal breeder pair for all progeny (**Table 1** and **Figure 1C**). In colony B2, the one ambiguous paternal relation was resolved in favor of a universal father with *beta* = $1.3 \cdot 10^{-3}$.

As a confirmation, the genotype-based attribution of breeders matched the results of the ultrasound-based examination. Queens identified in the Vienna colonies showed placental scars, indicative of former breeding activity, the identified breeding males were among those that had increased testis sizes (two in V1, three in V2).

Opportunities for Reproductive (Re)binding

Having observed the exclusive breeding activity of only a single male NMR per monitored colony we wondered: is it possible that the NMR actually was polyandrous (or any kind of polygamous) biologically, but there is any factor in the colony structures or settings that urged the analyzed animals into strictly bound reproductive partnerships? In other words, is there sufficient competition for reproductive partnering within our colony scenarios justifying model conclusions?

Detailed records of the age composition for two of the analyzed colonies, B1 and B2, helped to resolve this issue (**Supplementary Figure 1**). A total of 15 litters have been recorded in these colonies, while at the siring time points, between 4 and 9 adult founder males were present (average 5.3; **Supplementary Figure 1A**). We consider these the least fraction of male competitors for breeding activity. In addition, at an age of 180–225 days offspring is considered to reach sexual competence, according to literature and consistent with our own observations (Jarvis, 1991; Buffenstein, 2005; **Supplementary Text 1**). This is relevant for colony B1 which gave rise to 25 newborn males that reached the age of sexual maturation before the siring of the last litter of that colony (**Supplementary Figure 1B**). Since an incest barrier does not exist in the NMR (Reeve et al., 1990), young males may sire their solitary mothers upon reaching their age of sexual maturation, according to literature and our own observations (Clarke and Faulkes, 1999; Buffenstein, 2005; **Supplementary Text 1**). Thus, these grown-up males should also be considered as breeding competitors (on average, 9.5 per siring event in colony B1; **Supplementary Figure 1A**). In

FIGURE 1 | Probabilistic model for ambiguous parental relationships underlying the exclusion test II. A known female parent (F or F') and a candidate male parent (M or M') were evaluated for possible progeny genotypes they may generate. **(A)** Two alternative parent pairs (F-M and F'-M') produce progeny genotypes (*circles*), which may intersect (*striped region*). **(B,C)** Histograms showing the full distribution of the individually calculated progeny genotype intersections, for alternative parental animals (F' or M' in panel **A**) compared to the universally consistent breeding animals in a colony (F-M in panel **A**). The size of the intersection (*x*-axis) is scaled as fraction of genotype space F-M. **(B)** Alternative breeding males in colony B1; median intersection 0.017 (dotted vertical line). **(C)** Alternative breeding pairs (F'-M') in colony V2; median intersection 0.0000015 (dotted vertical line).

summary, the colony scenarios analyzed here contained multiple competing mating partners, both adult and newly matured, giving realistic chance for alternative mating patterns (such as polygamy, polyandry) to occur in our experimental setting. Thus, the obtained results are considered conclusive in the sense that the lacking genetic manifestation of alternative mating patterns can be attributed to the behavioral level.

DISCUSSION

The microsatellite data reported here for four captive NMR colonies reveal a single breeding pair per colony, which translates to a maximum reproductive skew of 100%. The only previous genetic study on intra-colony kinship has reported two males participating in colony reproduction (Faulkes et al., 1997). While not explicitly stated, we assume that Faulkes et al. (1997) have studied only this one colony in relevant detail. Pre-selection for high heterogeneity, as the authors state, may have favored a multi-parental result, albeit such potential bias cannot be estimated adequately. Taking together all these genetic results for the NMR, the average reproductive skew of females is 100%, that of males is 90% (one colony 50%, four others 100%). Operationally, these values let us categorize the NMR mating system as sexually monogamous, clearly distinguished from skews of about 50% which would indicate continuous competition for reproduction success. We point out, however, that monogamy in the NMR should be understood as serial monogamy, since upon death of one breeder, the surviving partner likely rebinds to a breeding successor (Lacey and Sherman, 1991; Faulkes et al., 1997; Van der Westhuizen et al., 2013) (and own observation). In addition, the high reproductive skews for the NMR fall well into the range of other cooperatively breeding species (females 88–100%, median 100%; males 76–100%, median 88%) (Lukas and Clutton-Brock, 2012), which answers the introductory question if the NMR may be an outlier to the striking evolutionary correlation between cooperative breeding and monogamous reproduction – no, given the novel results, it is in agreement.

Behavioral studies make an important contribution to detect unsolved research questions like, e.g., reproductive interaction, in the NMR. However, important for the present study, we substantially doubt that observational data can elucidate the sexual mating system in a reliable manner. Behavioral studies equate the "mating males" as "reproductive males," although breeding NMR males cannot be distinguished by morphological characteristics (e.g., body size, large external testes or protuberant penises) (Jarvis, 1991; Braude, 2000) as in other related mole-rat species (e.g., *Fukomys*) (Faulkes and Bennett, 2021). Some authors defined "copulation" as contact between the genitalia of the male and female with pelvic thrusting by the male just before and during copulation (Lacey and Sherman, 1991). However, despite its potential relevance for the precise identification of breeding males, most authors identify male breeder solely based on mating-related behaviors, such as mounting and ano-genital nuzzling (Jarvis, 1991; Lacey and Sherman, 1991; Goldman et al., 2006; Van der Westhuizen et al., 2013), which is even less reliable. It has been postulated that ano-genital nuzzling is frequently practiced by breeding NMRs in order to strengthen their bonding and to obtain chemical cues about the reproductive condition from each other (Faulkes and Abbott, 1991; Goldman et al., 2006). However, ano-genital nuzzling also occurs with and among non-breeding animals of both sexes (Jarvis, 1991; Ciszek, 2000; Goldman et al., 2006), confirmed by personal observations on colonies B1 and B2. "Mounting" is described as a behavior position in which the breeding male attempts to bring his genitals into contact with those of the queen, although

with the difference of no pelvic thrusting (Lacey and Sherman, 1991). Thus, numerous of mounting attempts occur but do not result in true copulations (Lacey and Sherman, 1991). Like ano-genital nuzzling, mounting also occurs with non-breeder males and was sometimes observed in colonies B1 and B2 when the queen was physically handicapped (e.g., otitis, suspected spinal disc herniation, or during late pregnancy). In sum, it is impossible to be certain which male is reproductive unless mating is actually witnessed (Jarvis, 1991), and this raises the concern that male contributions in NMR breeding could have been overestimated in the past, compared to our genetic evidence.

To date, no parentage studies have been undertaken and published from wild NMR colonies, presumably as a consequence of their special biology. NMR colonies typically contain 70–80 individuals, and sometimes up to 300 individuals in the wild (Brett, 1991), which significantly increases the number of possible mating pairs in kinship analysis and challenges marker depth for kinship resolution. Further, NMR colonies live in huge, subterranean burrows up to 2–3 km tunnel length (Brett, 1986). The breeding females, their mate(s) and newborn pups, representing the most important individuals for genetic paternity analysis, are usually trapped as the last members of a colony (Brett, 1991; Braude, 2000). In addition, as an incest avoidance mechanism appears to be absent in the NMR (Jarvis et al., 1994), colonies exhibit a high level of inbreeding and low levels of genetic variation within colonies (Reeve et al., 1990; Faulkes et al., 1997; Chau et al., 2018). These low levels of genetic variability and the lack of sufficient polymorphic markers previously made exclusion approaches in paternity analysis complicated to impossible (Lacey and Sherman, 1991). Addressing these challenges in the present study, we constructed a rich panel of novel microsatellite markers, which significantly extends the marker resource for the NMR (Ingram et al., 2014, 2015; Chau et al., 2018).

As the analytical strategy we chose a classical exclusion approach for several reasons: first, while alternative, probabilistic, methods rank paternity models by assigning posterior probabilities, an exclusion approach aims at the full resolution of alternative models. Second, probability priors in a probabilistic approach, namely probabilities of somatic mutations and typing errors, are usually difficult to estimate, while these may have a substantial influence on the ranking result. Third, a basic assumption of most probabilistic methods, the free flow of genetic alleles through the population, is certainly violated in NMR colony scenarios since the genotype patterns are dominated by kinship relations. The chosen exclusion approach has the advantage to cope with varying levels of relatedness. In the worst case, i.e., a high degree of relatedness, this approach requires a larger number of markers to become conclusive, but the analytical framework will not be thrown into question (Jones et al., 2010). Vulnerability of the exclusion approach against typing errors was counteracted here by a strict regime of genotype calling. Effectively, the rate of confident somatic mutations was found quite low (6.6×10^{-4} per offspring and marker), and the validation procedure for these mutations helped to narrow down estimates for typing uncertainties to the same frequency level.

Except the paternity study of Faulkes et al. (1997) no further parentage or extra-colony paternity investigations in NMR have been published. Another group, however, reported that in five wild colonies 5–8 microsatellite alleles were detected at one or more loci, which strongly suggests that these wild colonies contained individuals from more than a single breeding pair and inter-colony exchange of genetic material may occur (Ingram et al., 2015). Evidence of multiple queens in NMR colonies has been reported both in captivity and in the wild (Jarvis, 1991). These queens may simultaneously breed for some years, but the scenario usually is of shorter duration and typically leads to serious fightings, mortalities and low pup survival (Jarvis, 1991; Smith and Buffenstein, 2021). In established colonies, however, and multiple queens are a quite rare event in captive and wild NMR colonies (Braude, 1991; Jarvis, 1991). Further, based on long-term studies of 16 wild colonies of marked NMR, it has been observed that nascent colonies arise either via pairing of single mole-rats from different natal colonies or from small groups of males and females from different or same natal colonies (Braude, 2000). Inter-colony invasions, colony splitting and kidnapping have been observed among wild and captive NMR colonies, too (Lacey and Sherman, 2007; Braude et al., 2021) (S. Braude, personal communication). Independent studies on captive (O'Riain et al., 1996; Clarke and Faulkes, 1999; Ciszek, 2000) and wild NMR populations (Braude, 2000) demonstrated that outbreeding with unrelated mates is preferred in this species. Some authors therefore suggested that dispersing mole-rats would attempt to join established colonies (O'Riain et al., 1996). Although this may contribute to colony gene flow, it was demonstrated by a long-term recapture study that successful dispersal of individuals is rare and most of the nascent colonies did not appear to persist for more than 1 year (Braude, 2000). Laboratory and field studies further showed that NMR recognize colony members and aggressively discriminate against foreign conspecifics, which often results in the death of the mole-rat(s) (Lacey and Sherman, 1991; O'Riain and Jarvis, 1997) (and own observation). However, of note is one documented case in which a dispersing male successfully immigrated into an established colony and took over the breeding male position (Braude, 2000).

Some other traits have been used to infer monogamy in laboratory studies (Dewsbury, 1981), and these may serve as indicators to corroborate monogamy as a characteristic of the NMR mating system. To begin with, NMR breeding pairs are known to form stable, long-term partnerships for many years (Jarvis, 1991; Lacey and Sherman, 1991), and cooperative care for offspring was already highlighted as a social trait. In addition, subordinate individuals are physiologically suppressed by the queen and offspring exhibit a delayed onset in the sexual maturation (Jarvis, 1991; Buffenstein, 2005). In contrast to other related social mole-rat species, e.g. Fukomys, the NMR is sexually monomorphic and exhibits a balanced sex ratio on the colony level (Burda, 1990; Jarvis, 1991; Chau et al., 2018), which is prototypic for monogamous mating systems. Another, more specific characteristic is the condition of the male genitals in this species. The testes of both, breeding male and subordinates, are relatively small and intra-abdominal with only a little storage capacity for spermatozoa, a small baculum without a dorsal groove, no spines on the glans penis for vaginal anchoring (van der Horst et al., 2021), and no forming of copulatory plugs altogether indicate a low risk of sperm competition in this species (Seney et al., 2009). Fighting and aggression between NMR males is generally very rare and particularly do not occur during the queen's estrus (Jarvis, 1991; Lacey and Sherman, 1991; Clarke and Faulkes, 1998). This is in contrast to other social mole-rats, e.g., the Zambian mole-rat, where copulation often takes place irrespective of the females' reproductive cycle (Burda, 1990). In the NMR mating is found to be a rare event (Goldman et al., 2006) (and own observation) and the breeding male copulates almost during the entire estrus (2–24 h) of the queen.

To summarize, the genetic paternity results from this study together with a number of specific biological traits of the NMR consistently support the model that the preferred sexual mating system in this cooperatively breeding species is monogamy. In order to refine this model, and to resolve the underlying mechanisms for general and exceptional behavior, we would like to encourage further kinship studies on NMR colonies, particularly in the wild.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Landesamt für Gesundheit und Soziales Berlin, Germany.

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.855688/full#supplementary-material

Supplementary Data File 1 | Genotyping data of colony B1, Cervus format.

Supplementary Data File 2 | Genotyping data of colony B2, Cervus format.

Supplementary Data File 3 | Genotyping data of colony V1, Cervus format.

Supplementary Data File 4 | Genotyping data of colony V2, Cervus format.

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Socializing in an Infectious World: The Role of Parasites in Social Evolution of a Unique Rodent Family

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Transmission of parasites between hosts is facilitated by close contact of hosts. Consequently, parasites have been proposed as an important constraint to the evolution of sociality accounting for its rarity. Despite the presumed costs associated with parasitism, the majority of species of African mole-rats (Family: Bathyergidae) are social. In fact, only the extremes of sociality (i.e., solitary and singular breeding) are represented in this subterranean rodent family. But how did bathyergids overcome the costs of parasitism? Parasite burden is a function of the exposure and susceptibility of a host to parasites. In this review I explore how living in sealed burrow systems and the group defenses that can be employed by closely related group members can effectively reduce the exposure and susceptibility of social bathyergids to parasites. Evidence suggests that this can be achieved largely by investment in relatively cheap and flexible behavioral rather than physiological defense mechanisms. This also shifts the selection pressure for parasites on successful transmission between group members rather than transmission between groups. In turn, this constrains the evolution of virulence and favors socially transmitted parasites (e.g., mites and lice) further reducing the costs of parasitism for social Bathyergidae. I conclude by highlighting directions for future research to evaluate the mechanisms proposed and to consider parasites as facilitators of social evolution not only in this rodent family but also other singular breeders.

Keywords: Bathyergidae, sociality, mode of transmission, generalized transmission distance, organizational immunity, social immunity, evolution

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INTRODUCTION

Parasites (i.e., macroparasites such as ticks, fleas, lice, mites and helminths and microparasites such as viruses, bacteria and fungi) are important agents of selection. This is because they make up more than 50% of living organisms and by definition, they cause harm to their hosts (Poulin, 2007). This generates strong selective pressures which are considered one of the major evolutionary constraints that have made the evolution of sociality rare across the animal kingdom (Alexander, 1974). This is because the close proximity of individuals in a social group is assumed to facilitate the transmission of parasites between group members (Anderson and May, 1982; Rifkin et al., 2012; Patterson and Ruckstuhl, 2013).

Despite such potential evolutionary constraints, sociality has evolved repeatedly among an enigmatic group of subterranean rodents, African mole-rats of the family Bathyergidae (Faulkes et al., 1997). In fact, bathyergids exhibit a strict dichotomy between solitary species (genus

Heliophobius, *Georychus,* and *Bathyergus*) and those exhibiting cooperative breeding (genus *Heterocephalus, Fukomys,* and *Cryptomys*) with a high reproductive skew (i.e., singular breeding) where usually only a single female and a small number of males per group breed (Faulkes and Bennett, 2021; **Figure 1**). With the exception of the genus *Heterocephalus* social genera are also more speciose than solitary ones (Faulkes and Bennett, 2021; **Figure 1**). Although based on phylogenetic analyses sociality may be the ancestral state for bathyergids (Faulkes and Bennett, 2021), this is not sufficient to account for the prevalence of this extreme form of sociality in this rodent family that has only been reported for about 3% of all bird and mammal species (Lukas and Clutton-Brock, 2012a). Similarly, only about 5% of mammals exhibit monogamy but this mating system is prevalent among social mole-rats and some of their close relatives although the degree of genetic monogamy differs between species and localities (Lukas and Clutton-Brock, 2012a, 2013; Faulkes and Bennett, 2021). Monogamy cannot only limit the exposure to parasites, but has also been identified as a precursor for the evolution of cooperative breeding in singular breeding societies (Cremer et al., 2007; Lukas and Clutton-Brock, 2012b). For Bathyergidae ecological constraints to dispersal and the unpredictability of rainfall as well as encounters with food sources in habitats with high variance in rainfall have traditionally been proposed to be the driving force behind the evolution of sociality (Jarvis et al., 1994; Faulkes et al., 1997; Faulkes and Bennett, 2021). Large environmental variability, particularly with regards to rainfall has generally been proposed to be a key predictor of the evolution of cooperatively breeding bird and mammal species (Jetz and Rubenstein, 2011; Lukas and Clutton-Brock, 2017; Firman et al., 2020). However, this hypothesis has been criticized as singular breeding species also occur in relatively begin habitats (Shen et al., 2017). It would also not account for the occurrence of several cooperatively breeding bathyergid species in more mesic habitats while plural breeding or loosely social species are entirely absent from the Bathyergidae (Bennett and Faulkes, 2000; Šumbera et al., 2012; Patzenhauerová et al., 2013). While no other animal taxon exhibits this dichotomy of social systems, singular breeding is commonly found in social insects. Parasites have been implicated as important driver of the evolution of sociality in eusocial insects (Biedermann and Rohlfs, 2017; Cremer et al., 2018). To date, the possible role of parasites for other singular breeding species has not been investigated. In the remainder of this article, I will explore the role that parasites may have played in the evolution of sociality and singular breeding of bathyergids.

PARASITE TRANSMISSION AND THE SUBTERRANEAN NICHE

Infection with parasites is a function of the exposure and susceptibility of individuals to parasites (Poulin, 2007). From a parasite's perspective this requires a two-step process: encountering a host and invading it (Schmid-Hempel, 2021). Exploiting the subterranean niche may have aided in the evolution of sociality by reducing the exposure to parasites. It has been noted repeatedly that subterranean rodents have

a significantly impoverished macroparasite species richness compared to similar sized terrestrial species while microparasite infection has rarely been studied (Scharff et al., 1996; Bartel and Gardner, 2000; Rossin and Malizia, 2002; Hubálek et al., 2005; Rossin et al., 2010; Viljoen et al., 2011b; Lutermann and Bennett, 2012; Cutrera et al., 2014; Lutermann et al., 2015, 2019; Archer et al., 2017; Fagir et al., 2021). However, since the majority of the other subterranean rodent families comprises mostly of solitary species and none are singular breeders (Jarvis and Bennett, 1993), a reduction of parasite exposure in the subterranean niche alone cannot account for the prevalence of singular breeding species among Bathyergidae. Regardless, bathyergids differ from most other subterranean families by living in sealed burrow systems, rarely venturing above ground and only some of the solitary species include above ground vegetation in their diet (Stein, 2000). At the same time, the buffered environmental conditions in the burrows of bathyergids with stable, warm temperatures and high humidity (Buffenstein, 2000; Šumbera, 2019) also provide ideal microhabitats for parasites such as fungi and arthropods that experience favorable conditions year-round (Marshall, 1981). Nevertheless, the subterranean niche may have been an ideal starting point for social evolution in bathyergids due to the constraints it puts on contact rates between hosts.

In a recent paper Schmid-Hempel (2021) proposed that from a parasite's perspective the opportunity for transmission is the crucial difference between infecting solitary compared to a social host. This applies regardless of the vast range of group size and social organization in many social species. Several meta-analyses have concluded that group size *per se* affects parasite transmission or disease risk (Côté and Poulin, 1995; Rifkin et al., 2012; Patterson and Ruckstuhl, 2013). However, using group size as a proxy for sociality is a convenient but oversimplified approach that entirely ignores the many facets of group living. Instead Schmid-Hempel (2021) posits to use four key elements of sociality that affect what he calls "generalized transmission distances": the temporal, spatial, genetic and ecological proximity of hosts that incorporate many dimensions of social organization. Group living may be temporary, e.g., for breeding purposes or perennial. In the case of bathyergids species, rainfall is a key determinant of group stability for social species but also affects breeding opportunities for both solitary and social species and foraging activity which may determine the encounter probability with parasites (**Figure 2A**; Spinks et al., 1999, 2000; Young et al., 2010; Torrents-Ticó et al., 2018). Transmission can occur within as well as between groups representing different spatial scales (**Figure 2A**). The patterns of genetic relatedness can vary widely between host species but relatedness is particularly high in cooperative breeders where breeding is limited to a small number of individuals and most group members are offspring of the breeders (i.e., singular breeders) as is the case in all social mole-rats (Bishop et al., 2004; Burland et al., 2004; Patzenhauerová et al., 2013; Ingram et al., 2015). If transmission of a parasite is determined by the genetic make-up of the host, closely related hosts in close spatial proximity for extended periods of time, such as in social Bathyergidae, could greatly facilitate the spread of a parasite (Schmid-Hempel, 2021). Lastly, their social
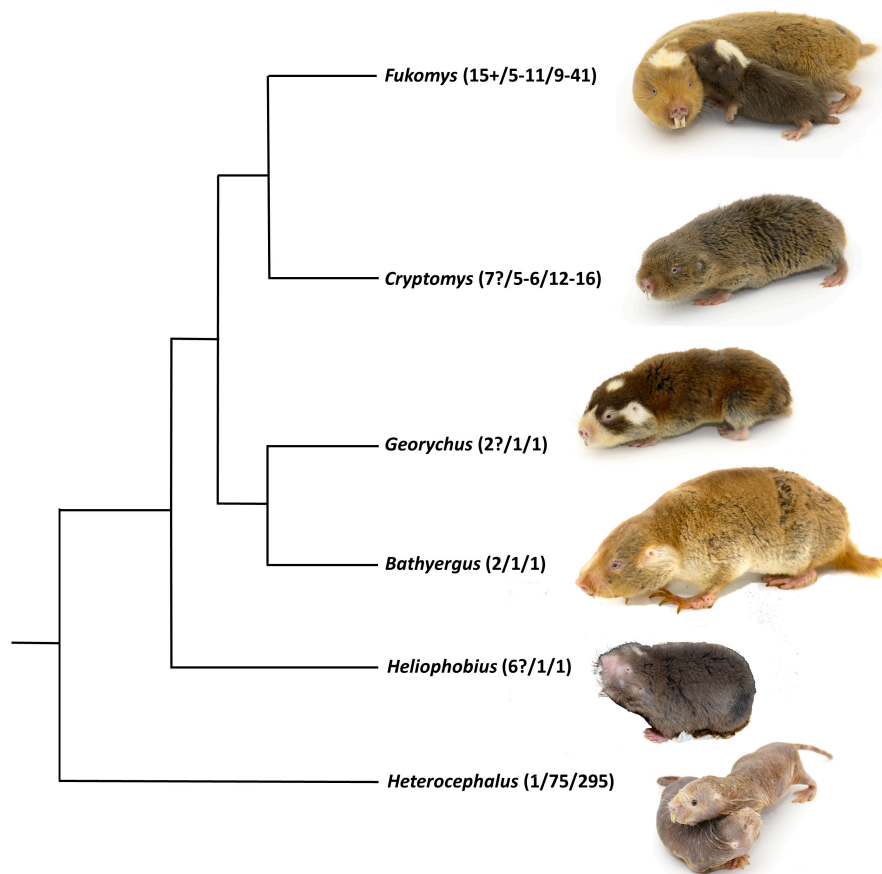
organization and cooperation allows social species to extend their ecological niche (Schmid-Hempel, 2021). Consequently, in social bathyergids the exploitation of arid habitats with highly dispersed food sources is thought to be enabled by group-living (Bennett and Faulkes, 2000; Faulkes and Bennett, 2021). However, it also extends to the structure and dimensions of their burrow systems with larger systems potentially exposing individuals to more parasites.

Incorporating these four elements into a "generalized transmission distance" suggests fundamental difference in the transmission dynamics between solitary and social hosts that parasites face (Schmid-Hempel, 2021; **Figure 2A**). As illustrated in **Figure 2A** parasites experience a unimodal, intermediate transmission distance across a host population when parasitizing solitary hosts (Schmid-Hempel, 2021). In contrast, distances within groups are short, and this is where most transmission occurs (**Figure 2A**). Conversely, between group transmissions distances are far and consequently transmission frequencies are low resulting in a bimodal distribution of transmission distances (**Figure 2A**). These differences lead to fundamentally different selection pressures in the host-parasite systems of solitary and social hosts. For subterranean species, the transmission distances between groups are further exaggerated due to

the dispersal constraints posed by the challenges of below-ground dispersal (**Figure 2C**). The antagonistic relationship between parasites and their hosts suggests that the fitness implications of these differences in transmission distances and how these may have shaped the evolution of sociality in bathyergids should be considered separately (Schmid-Hempel, 2021). Accordingly, I will address these separately for hosts and parasites in the following.

THE HOST PERSPECTIVE

Parasite infection depends on the exposure (i.e., encounter) as well as susceptibility (i.e., invasion and proliferation) of an individual. The highly skewed distribution of parasites (also called overdispersed) across host populations suggests that these two parameters can differ widely among host individuals (Poulin, 2007).

Individual Defenses

Hosts generally employ three strategies to reduce either exposure or susceptibility to parasites, i.e., by avoiding encounters with parasites, suppressing parasite proliferation and/or minimizing
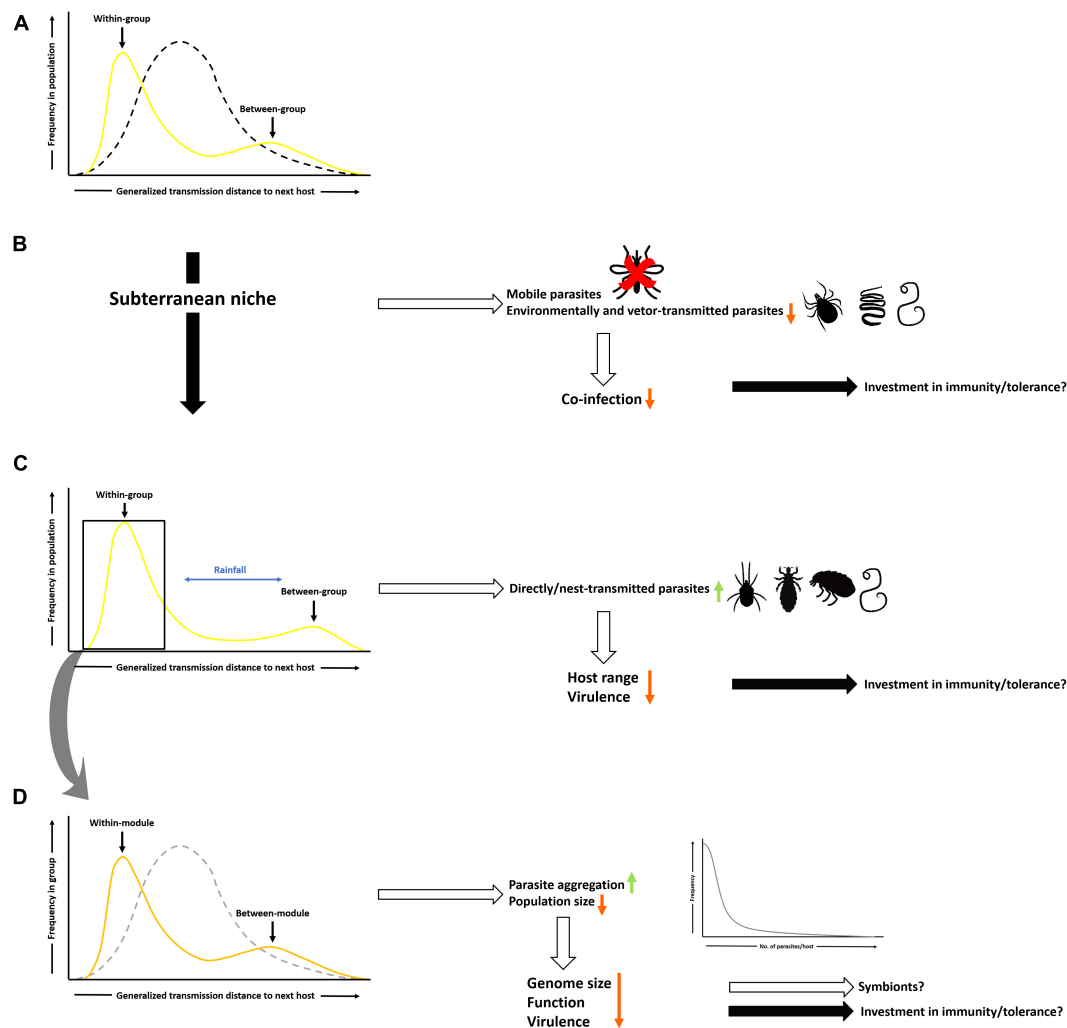
FIGURE 2 | Compositive distance space [i.e., generalized transmission distance (GTD) derived from spatiotemporal, genetic and ecological distance between hosts, a theoretical measure of transmission distance] and its predicted effects on parasite exposure and selection pressures on parasites and bathyergid hosts. This measure incorporates characteristics of the social organization and group size of a particular species. **(A)** GTD is unimodal for solitary hosts (black, dashed line) but bimodal for social hosts (yellow line) due to short GTDs between group members (within-group) but long GTDs between groups. **(B)** Living in a subterranean environment excludes mobile parasites and reduces exposure to environmentally-transmitted or vector-borne parasites (e.g., ticks, helminths). For parasites (open arrows) this reduces their degree of co-infection with variable host investment (solid arrows) in physiological defenses (i.e., resistance vs. tolerance). **(C)** In a subterranean environment GTDs for within and between-group transmission vary with rainfall and soil properties but are generally larger for the latter compared to terrestrial species. These conditions favor directly transmitted parasites (e.g., mites), but pose constraints on their host range and virulence with uncertain consequences for host investment in physiological defenses. **(D)** Behavioral responses of hosts (i.e., organizational immunity) can further generate differential patterns of GTDs depending on their presence (orange line) or absence (gray, dotted line). The former causes further parasite aggregation (curve on the bottom right) and reductions in population size. For parasites this can result in reductions in genome size and associated function as well as further decreases of virulence and potentially evolution of symbiotic relationships with unknown consequences for physiological defenses employed by hosts.

parasite damage (Råberg et al., 2009; Medzhitov et al., 2012; Hart and Hart, 2018). While parasite avoidance is largely limited to behavioral modifications, preventing parasite proliferation and reducing damage also requires physiological responses that are more costly (Viney et al., 2005; Boots et al., 2009; Medzhitov et al., 2012).

Avoiding Parasite Encounters

The most efficient and at the same time probably least costly anti-parasite strategy an individual can employ is the avoidance

of parasites by actively reducing encounter probabilities (Hart and Hart, 2018). This includes the avoidance of areas with high exposure but also of infected conspecifics including when choosing mates (Hart and Hart, 2018). However, subterranean species may be limited in the choice of habitats they explore or in their mate choice due to the costs of digging (Vleck, 1979; Šumbera, 2019). This could have been one factor contributing to the differences in microhabitat choice between solitary and social bathyergids with the former often using more easily workable soil types (Romanach, 2005; Lövy et al., 2012). In addition,

evidence suggests that solitary species more frequently venture above ground for breeding dispersal which may also permit more flexibility in mate choice (Stein, 2000). While in social Bathyergidae individuals may act aggressively toward unfamiliar conspecifics, active discrimination based on infection status has rarely been tested (Lutermann et al., 2022). Within a burrow system individuals can reduce their exposure to any parasites present by using several sleeping chambers and/or the use of distinct chambers for various purposes (Roper et al., 2002). The most obvious may be a toilet chamber that will limit the deposit of urine and feces, that could be the source of directly transmitted helminths or pathogens, spatially. Using these chambers only for brief periods would furthermore reduce the parasite exposure. Similarly, the use of food chambers reduces the chances of consuming contaminated food. Such a dedicated chambers are already apparent in the burrow systems of solitary bathyergids (Thomas et al., 2009, 2012; Šumbera et al., 2012).

Grooming is known to be an effective means of removing ectoparasites (Mooring et al., 2004; Hart and Hart, 2018). In addition, the incorporation of plant materials containing volatiles inhibiting the growth and survival of ectoparasites and bacteria in nesting material can reduce exposure (Hemmes et al., 2002). However, it is not known in how far this is employed by bathyergids and possible medicinal properties of the of the plants they use have rarely been explored.

Suppressing Parasite Proliferation (Resistance)

Once parasite infection has occurred a host can employ several mechanisms to suppress its proliferation. Self-medication in the form of the consumption of plant material or substances with medicinal properties can be an effective behavioral strategy to limit the amplification of parasites once an infection has occurred (Hart and Hart, 2018). This anti-parasite behavior can be observed in a wide range of animal species (Neco et al., 2019). It is unknown whether bathyergids also practice this behavior. However, the physical constraints of the subterranean environment make it unlikely that they are selectively seeking such plants. Nevertheless, the "geophyte farming" described for some Bathyergidae may include bulbs and tubers with medicinal properties that are kept for such a purpose (Jarvis and Sale, 1971; Lovegrove and Knight-Eloff, 1988; Jarvis et al., 1998). In addition, the inclusion of bulbs and tubers with highly toxic secondary plant component reported for several bathyergids (Bennett and Faulkes, 2000) may also assist in suppressing the proliferation of pathogens. These plants often also have a high fiber content and require the aid of symbiotic microorganisms in the cecum to break down the cellulose (Buffenstein, 2000). It has been reported that such microorganisms can also help their host to combat parasites in some species (Kreisinger et al., 2015; Peachey et al., 2017; Leung et al., 2018). It remains to be seen whether they play a similar role in bathyergids although this has been suggested for at least one species (Debebe et al., 2017; Braude et al., 2021). Both, direct competition for resources with invading parasites but also the production of chemicals in response to infection have been reported (Kreisinger et al., 2015; Leung et al., 2018). In the case of mole-rats such toxins may act directly in the host's body or could be taken up through the coprophagy practiced by these animals.

Currently, however, no empirical data is available for bathyergids to support this hypothesis.

In adaption to the high temperatures and humidity in the sealed burrow systems mole-rats have lowered metabolic rates and less dense pelage to prevent the build-up of heat and facilitate heat dissipation (Bennett and Faulkes, 2000; Buffenstein, 2000; Šumbera, 2019). In addition, they may temporarily become hypothermic in response to hypoxia although this response appears to be restricted to social bathyergids (Cheng et al., 2021). Lowered body temperatures could also slow down the proliferation of some pathogens and parasites. If mole-rats would actively downregulate their body temperatures in response to infection, similar to the response to hypoxic conditions, they could thus contain infection. Although it is unclear whether body temperature was downregulated actively, resting metabolic rate of Natal mole-rats (*Cryptomys hottentotus hottentotus*) infested with cestodes (*Raillientina* sp.) was reduced providing indirect support for such a mechanism. However, this could only be sustained at a low to medium cestode abundance while it was similar to that of uninfested animals in those carrying large burdens of cestodes. Furthermore, mole-rats are known to regulate the temperature in their intestines to provide ideal conditions for their symbionts suggesting that they are able to make selective adjustments in body temperature (Buffenstein, 2000).

A suite of behavioral (i.e., sickness behaviors) and physiological changes in the host in response to microparasite infections form part of the acute phase response that can be observed across a wide range of taxa (Hart, 1988; Adelman and Martin, 2009). Sickness behaviors include reductions in overall activity, food intake, and libido but also increases in body temperature causing a fever are part of this phenomenon. It allows the host to conserve energy that can be channeled into immune responses but also deprives the parasite of essential nutrients for growth and replication (Adelman and Martin, 2009). Fever and sickness behaviors have also been recorded for several mole-rat species (Urison et al., 1993; Viljoen et al., 2011a; Lutermann et al., 2012) and likely play a role in combating infections with microparasites in bathyergids as well.

The immune system of mammals is complex and comprised of two arms, innate and adaptive immunity, and is probably the most important line of defense against parasites once transmission has occurred (Adelman, 2010). Innate immune responses are considered relatively cheap and broadly target foreign molecules. Conversely, adaptive immune responses are antigen-specific and induced by exposure to foreign antigens (Lochmiller and Deerenberg, 2000; Klasing, 2004). Triggering this arm of the immune system is energetically costly and requires trade-offs with other demands (e.g., growth, reproduction) an individual might experience and hence, affect individual fitness (Lochmiller and Deerenberg, 2000; Lee, 2006; Martin et al., 2008; Martin, 2009). In concert with the genetic make-up of an animal as well as co-infections with other parasites the costs of immune responses are likely to be the reason why immune responses vary considerably within and between individuals (Viney et al., 2005; Altizer et al., 2006; Råberg et al., 2009; Bordes et al., 2012). However, this investment can pay off when it confers immunity to re-infection with the same parasite species. In mammals it

may furthermore be transmitted to offspring *via* the placenta or *via* antibodies in the milk produced by mothers (Boulinier and Staszewski, 2008; Roth et al., 2018). Apart from extensive studies in naked mole-rats (*Heterocephalus glaber*) the immune function of bathyergids has received limited attention (Lutermann and Bennett, 2008; Lutermann et al., 2012; Lin and Buffenstein, 2021). Innate immune responses of *H. glaber* to parasites are potentially modified as they lack natural killer cells. At the same time, their macrophages exhibit a higher production of pro-inflammatory cytokines and they possess a type of neutrophils that can produce several antimicrobials at large quantities properties that are not known from other rodents (Lin and Buffenstein, 2021). It is unknown whether similar modification are also present in other bathyergid species but the composition of blood cells in highveld mole-rats (*Cryptomys hottentotus pretoriae*) was modified by infection with parasites (Lutermann et al., 2012).

Minimizing Parasite Damage (i.e., Tolerance)

The costs associated with resistance as well as the possible tissue damage involved (i.e., immunopathologies) may be the reason why some individuals or species may opt for tolerance rather than resistance (Viney et al., 2005; Råberg et al., 2009; Schulenburg et al., 2009; Best et al., 2012; Medzhitov et al., 2012). Although well-established in plant hosts, this strategy has only recently garnered attention in animals and studies of tolerance in animals are limited (Baucom and de Roode, 2011; Medzhitov et al., 2012; Budischak and Cressler, 2018). The host can attempt to minimize the costs of tissue damage from either the parasite or the immune system using a variety of mechanisms (Medzhitov et al., 2012). These include cellular stress responses triggered by stress-response systems dedicated to particular stressors such as high levels of reactive oxygen species (ROS) or hypoxia (Medzhitov et al., 2012). ROS have been studied in all three social genera, most extensively in *H. glaber*, but not in relation to parasites (Mendonça et al., 2020; Braude et al., 2021; Buffenstein et al., 2022; Jacobs et al., 2021a,b). Like other subterranean rodents Bathyergidae are adapted to the hypoxic conditions in their burrow systems and this also includes range of physiological adaptations to reduce oxidative stress (Schülke et al., 2012; Ivy et al., 2020; Logan et al., 2020). Adaptations to the subterranean niche may hence, have equipped bathyergids with a physiological "tool kit" that could also prove useful in parasite tolerance and may make them particularly tolerant to parasite infections. Tolerance to infection with helminths has been shown in *C. h. pretoriae* where males chronically infested with *Mathevotaenia* sp. show neither reductions in body mass nor changes in androgen levels (Lutermann et al., 2012, 2022). However, simulation of a secondary bacterial infection resulted in significant reductions of testosterone levels for helminth-infested but not healthy males suggesting lower tolerance to invasion of a second parasite (Lutermann et al., 2012).

Hosts are unlikely to pursue only a single strategy but employ a mixture of all of these, potentially in a parasite-specific manner (Best et al., 2008; Boots et al., 2009; Råberg et al., 2009) as all are clearly beneficial. The use of the various strategies may also change temporally depending on other demands (e.g.,

reproduction) and resource availability (Altizer et al., 2006; Budischak and Cressler, 2018).

Group Defenses

Cremer and colleagues suggested that singular breeding social insects (i.e., eusocial Hymenoptera and termites) achieve parasite protection at a group-level through the sum of defenses employed by group members that affect the exposure and susceptibility of social hosts to parasites and called this "social immunity" (Cremer et al., 2007, 2018). Similar to individual host defenses, social immunity can be distinguished into avoidance, resistance and tolerance. In addition to a behavioral and physiological component it has an organizational components that is unique to social organisms (Cremer et al., 2018). While aspects of social immunity have been the focus of many eusocial insects (Cremer et al., 2007, 2018; Stroeymeyt et al., 2014; Schmid-Hempel, 2021), its applicability to social vertebrate species has not been explored (Van Meyel et al., 2018).

Avoiding Parasite Encounters

Behavioral strategies play an important role in parasite avoidance at the colony level. Exploiting soil types that pose greater constraints to underground dispersal may already act as an effective defense against between-group parasite transmission (**Figure 2B**). In addition, Bathyergidae are known to be xenophobic (Riain et al., 1997; Spinks et al., 1998; Clarke and Faulkes, 1999; Ganem and Bennett, 2004; Bappert et al., 2012). Although this could be partially attributable to the risk of reproductive competition in social species, an alternative, and not mutually exclusive, function may be to reduce the risk of parasite transmission by intruding conspecifics (Freeland, 1976, 1979). This hypothesis has not yet been addressed for Bathyergidae, but does not appear to always apply (Lutermann et al., 2022).

In addition to self-grooming, allo-grooming, the grooming of conspecifics, can further reduce the risk of parasite transmission. Since the rate of allo-grooming should increase with group size this could be an effective way to reduce the risk of parasitism in social species including social bathyergids. It could account for the negative correlation between ectoparasite burdens and colony size found in several social bathyergids (Viljoen et al., 2011b; Lutermann et al., 2013; Archer et al., 2016). In eusocial insects allo-grooming also significantly affected the outcome of interspecific competition between co-infecting parasites (Milutinović et al., 2020). The possible role of allo-grooming for parasite control has not been explored in Bathyergidae.

Resistance and Tolerance

While avoidance, resistance and tolerance are clearly beneficial to both solitary and social host individuals, social species may have more resources to invest (Ezenwa et al., 2016). Resource availability can constrain a host's ability to use either resistance or tolerance as a strategy to combat parasite infection (Budischak and Cressler, 2018). However, if increased access to resources is one of the benefits of group-living, social species should have additional means to invest into parasite defenses (Ezenwa et al., 2016). For example, in Natal mole-rats energy stores in the form of fat increased with colony size (Lutermann et al., 2013). This

is likely to translate into more energy being available for parasite defenses and although this has not been explicitly tested for any bathyergid, the negative correlations between colony size and parasite burden reported for several *Cryptomys* species suggests that this might be the case (Viljoen et al., 2011b; Lutermann et al., 2013). The additional resources available can either be used for stronger immune responses but can also increase the tolerance of social hosts to parasite infection as shown in other singular breeding mammals (Almberg et al., 2015).

Exposure to infected conspecifics can result in the activation of the immune system of uninfected individuals in anticipation of an infection (Kavaliers and Colwell, 1992, 1994). Hence, encounters with infected intruders as well as colony mates could prime individuals in social Bathyergidae and in turn lower their susceptibility to parasites. Furthermore, coprophagy between individuals or from adult offspring to newly recruited offspring may allow for the transmission of both immune-stimulating substances as well as a diverse symbiont community that could provide additional parasite defenses (Ezenwa et al., 2016) similar to what has been reported for some social insects (Leclaire et al., 2014; Powell et al., 2014; Cremer et al., 2018). These hypotheses have not been addressed for bathyergids but deserve attention in the future.

Organizational Immunity

Parasite transmission is often linked to contact rates between individuals (May and Anderson, 1978; Altizer et al., 2003). This is illustrated by several network analyses showing that microbial fauna reflects contact networks of inter-individual contacts (Drewe, 2010; Blyton et al., 2014; VanderWaal et al., 2014). In addition, this may be facilitated by the close relatedness in singular breeding species (Cremer et al., 2007, 2018; Schmid-Hempel, 2021). First proposed for social insects, the organizational immunity hypothesis posits that division of labor as well as behavioral flexibility that modulates contact rates between group members in response to infection should constrain parasite spread within a colony (Naug and Camazine, 2002; Cremer et al., 2007; Stroeymeyt et al., 2014).

The temporal or spatial separation of group members can reduce parasite transmission by reducing contacts (i.e., exposure), both direct or indirect, between group members (Evans et al., 2020; Lucatelli et al., 2021). The use of different sections of the burrow system at different times could be a means of temporal separation. A number of studies has addressed activity patterns in Bathyergidae although mostly focusing on activity rhythms when in isolation (Oosthuizen and Bennett, 2022). Nevertheless, in laboratory conditions activity patterns differed between individuals for several social bathyergids (Oosthuizen et al., 2003; Hart et al., 2004; De Vries et al., 2008; Schielke et al., 2012; van Jaarsveld et al., 2019). Similarly, they differed between group members in the field (Šklíba et al., 2012, 2014, 2016; Šumbera et al., 2012; Lövy et al., 2013; Francioli et al., 2020; Finn et al., 2022). Several of the latter studies also observed distinct differences in activity patterns between breeding and non-breeding colony members with the former exhibiting less activity which could lead to differential exposure and susceptibility to parasites for colony members.

The greater complexity of burrow systems of social compared to solitary bathyergids is probably not simply a results of greater foraging efficiency or a larger number of foragers (Lovegrove, 1988; Spinks et al., 1999; Le Comber et al., 2002; Le Comber, 2006; Sichilima et al., 2008; Šumbera, 2019). Burrows containing several nesting and/or toileting areas allow colony members to rest or defecate separately, both in time and space, and this can impede within-group transmission *via* reduced exposure. In fact, several studies have reported the differential use of burrow systems by colony members (Lacey and Sherman, 1991; Šumbera et al., 2012; Lövy et al., 2013; Šklíba et al., 2016; Francioli et al., 2020) which is likely to affect parasite exposure. At the same time, singular breeding and high relatedness among colony members in social Bathyergidae also means that losing non-breeders due to parasite infection is less costly while these individuals still accrue inclusive fitness benefits (Cremer et al., 2018; Schmid-Hempel, 2021). To date, the removal of infected group members, either of their own volition or forcibly by other group members, as observed in social insects, has not been reported for bathyergids.

In addition to the reproductive division of labor, the division of labor of other tasks and/or specialization on particular tasks observed in eusocial insects can reduce the spread of parasites among colony members, particularly if those tasks are carried out in different parts of the nest or burrow (Cremer et al., 2007, 2018; Stroeymeyt et al., 2014). In more flexible eusocial insect species, usually those with smaller group sizes, task specialization changes with time and environmental conditions and may vary based on age, sex or infection status of the individual concerned (Tofts and Franks, 1992; Cremer et al., 2007, 2018; Stroeymeyt et al., 2014). Although task specialization has been suggested for naked mole-rats and several *Fukomys* species in early studies (Jarvis, 1981; Bennett and Jarvis, 1988; Lovegrove, 1988; Gaylard et al., 1998; Wallace and Bennett, 1998) this was not confirmed in later ones (Lacey and Sherman, 1991; Thorley et al., 2018; Siegmann et al., 2021). However, several studies have reported that an individual's age, sex and/or breeding status correlates with its contributions to cooperative tasks in these bathyergid species (Thorley et al., 2018; Zöttl et al., 2018; Siegmann et al., 2021). No such division of labor could be identified in *Cryptomys* species, but few studies have addressed this in this genus (Bennett, 1989; Moolman et al., 1998). In eusocial insects age-related cooperative behavior also determines exposure to parasites (Stroeymeyt et al., 2014; Cremer et al., 2018). However, additional division of labor may also be constraint by group size and more likely in species achieving larger group sized (e.g., naked or Damaraland mole-rats). In accordance with this hypothesis, age-related cooperative behavior has also been identified in eusocial bathyergids but links to parasite transmission have not been explored. Similarly, age effects on parasite burden have not explicitly been addressed although effects of breeding status in some species may be an indication of this as breeders are presumably the oldest individuals in a group.

The task-specific division of labor can also affect the microbiome of individuals, either because they differ in their exposure to microorganisms or because exchange of microorganisms occurs predominantly between individuals carrying out the same task (Münger et al., 2018;

Sinotte et al., 2020). In eusocial insects these differences are assumed to also increase the efficiency with which certain tasks are carried out and increase the extraction of nutrient that also fuel immune responses (Iorizzo et al., 2020; Sclocco and Teseo, 2020). Although the number of studies investigating the effects of the division of labor on the microbiome remains limited, the available information suggests distinct microbiomes for breeders and non-breeders as well as different worker castes in eusocial insects (Sclocco and Teseo, 2020; Sinotte et al., 2020). These differences can also be linked to differences in metabolomics including more stimulated immune systems in foraging workers (Quque et al., 2021). Currently, no information is available in how far this may also apply to social Bathyergidae.

The above information suggests that social bathyergids have various additional avenues of parasite defense, many of which do not rely on costly mechanism. Behavioral mechanisms also allow for a great deal of flexibility and may only be employed after a parasite infection has occurred, further reducing their costs.

THE PARASITE PERSPECTIVE

The fitness of parasites can be partitioned into two components the success within and that between hosts (Schmid-Hempel, 2021). The former requires the successful invasion and multiplication, growth or acquisition of resources to eventually produce transmission stages or offspring. Conversely, the latter is determined by the successful transmission of this parasite propagules to new hosts. In the following I will consider the two fitness determining processes separately.

Within-Host Success

There are three host traits that determine the within host-success of a parasite: the immune response of the host, host predictability and the presence of other parasites or symbionts that may compete for host resources (Schmid-Hempel, 2021).

Host Immune Responses

As outlined above, hosts can employ one of two immune strategies in response to an infection; resistance or tolerance (Råberg et al., 2009; Baucom and de Roode, 2011; Medzhitov et al., 2012). While the former can effectively reduce parasite survival and proliferation the latter does not. At the same time, host tolerance extends the period of propagule production and hence, is likely beneficial for the success of parasites (Budischak and Cressler, 2018).

Overall, host individuals in bathyergid groups may differ in their suitability as hosts for parasites invading a social group. Indeed, asymmetric distribution of parasites between breeders and non-breeders or between the sexes has been reported for some parasite species of social bathyergids (Ross-Gillespie et al., 2007; Viljoen et al., 2011a; Archer et al., 2016, 2017; Fagir et al., 2021). However, this was not the case for other social Bathyergidae (Fagir et al., 2021). Similarly, such differences may be age-based (Silk and Fefferman, 2021).

Social rank can affect immune responses as shown in recent meta-analyses (Habig and Archie, 2015; Habig et al., 2018).

Although patterns varied widely among the studies included, there was a general trend for dominant individuals to carry higher parasite burdens. The authors proposed that this was linked to the greater investments by dominants to achieve and maintain their status (Habig and Archie, 2015; Habig et al., 2018). However, this pattern was not apparent in cooperatively breeding species. For singular breeders such as social bathyergids the kin structure of the group would likely preclude such competition for dominance that is usually linked to reproductive activity. While studies exploring this relationship are limited for bathyergids, Natal mole-rats breeders exhibited stronger fever responses to a simulated infection and had significantly larger spleens, an organ important for the storage and circulation of immune cells (Lutermann and Bennett, 2008; Viljoen et al., 2011a). This may be due to energy savings of breeders that partake to a lesser extend in energetically costly cooperative tasks.

Host Predictability

Hosts with larger body sizes, in better condition, that are more abundant and/or long-lived are a more predictable resource from a parasite perspective and this benefits its persistence and proliferation (Combes, 2001). With few exceptions (i.e., Giant mole-rats, *Fukomys mechowi*), social bathyergids are smaller in body size than solitary species however, they are more abundant due to living in groups (Bennett and Faulkes, 2000). At the same time, individuals are likely to be in better body condition in social species and fat stores increased with group size for Natal mole-rats (Lutermann et al., 2013). While longevity does not increase with sociality *per se*, it does in singular breeding species where it is mostly observed for reproductive individuals (Lucas and Keller, 2020; Downing et al., 2021; Kennedy et al., 2021; Korb and Heinze, 2021; Vágási et al., 2021). While there is currently no information on longevity for the genus *Cryptomys*, members of the other two social genera (i.e., *Heterocephalus* and *Fukomys*) are well-known for their long life-spans, particularly in breeders (Dammann and Burda, 2006; Dammann et al., 2011; Schmidt et al., 2013; Lewis and Buffenstein, 2016). This suggests that social Bathyergidae may be particularly predictable and localized hosts from a parasite's perspective.

Co-infections

Infections with a single parasite are the exception rather than the rule in nature (Behnke et al., 2001; Cox, 2001), but is suggested to be more common for social species (Altizer et al., 2003; Schmid-Hempel, 2021). Since parasites rely entirely on their hosts for resources this can cause competition between co-infecting parasites for limiting host resources (Pedersen and Fenton, 2007). Such competition can reduce an individual parasite's success but can also facilitate it (Knowles et al., 2013; Pedersen and Antonovics, 2013; Fagir et al., 2015; Hoffmann et al., 2016; Mabbott, 2018). In addition to this direct interaction between co-infecting parasite species, they can also interact indirectly *via* the host's immune system and infection with one parasite may enhance or reduce the successful invasion and/or proliferation of another parasite (Pedersen and Fenton, 2007). Furthermore, the dependence on beneficial microorganisms bathyergids require to break down

their unpalatable herbivorous diet (Bennett and Faulkes, 2000; Buffenstein, 2000) makes competition for resources between parasites and endosymbionts similarly likely.

Between-Host Success

Parasites are required to complete three steps to achieve between-host fitness gains starting with leaving the current host (Schmid-Hempel, 2021). While this step likely does not differ fundamentally between solitary and social hosts, the subsequent transmission steps (i.e., encountering and infecting) to a new hosts are characterized by substantial differences in general transmission distance (Schmid-Hempel, 2021; **Figure 2**).

EVOLUTIONARY CONSEQUENCES FOR HOSTS AND PARASITES

By definition host-parasite relationships are antagonistic with each side attempting to increase their fitness at the expense of the other (Poulin, 2007). In the resulting evolutionary arms race parasites depend entirely on their hosts for their survival while this does not apply in a similar fashion to hosts (Dawkins and Krebs, 1979). Furthermore, the number of parasites is generally larger and their generation time usually shorter than that of their hosts suggesting that selection will mostly act on parameters of the host-parasite relationship that provide the greatest fitness returns for parasites (Poulin, 2007; Schmid-Hempel, 2021). In the case of social bathyergids this would be the within-group transmission which is likely to occur more frequently than that between colonies (Archer et al., 2016; **Figure 2C**). Consequently, selection of defense mechanisms reducing within-colony transmission should be under strong selection pressure in social bathyergids (Hawley et al., 2021; Schmid-Hempel, 2021). At the same time, parasites of social bathyergids should experience stronger selection pressures than their hosts, mainly on mechanisms aiming to increase the transmission rate for parasites. In the following I will address the resulting selection scenarios for hosts and parasites separately.

Selection on Hosts

As behavioral measures can substantially affect parasite transmission and are highly flexible at comparatively low costs, selection pressures from parasites should act strongly on behavior (Ezenwa et al., 2016; Hawley et al., 2021). The presumed higher transmission in larger groups selects for smaller group sizes (Altizer et al., 2003; Rifkin et al., 2012; Patterson and Ruckstuhl, 2013). Under such circumstances selection can instead favor preferential social interactions with certain individuals that can ultimately lead to modularity to reduces parasites transmission (Freeland, 1976, 1979; Griffin and Nunn, 2012; Nunn et al., 2015). However, evidence for this is weak possibly due to the opposing selection pressures associated with the benefits of group-living including lower predation pressure, increased foraging efficiency, transfer of protective microbes and information that may counter pressures on reductions of group size (Ezenwa et al., 2016; Townsend et al., 2020; Hawley et al., 2021). In social bathyergids the ecological constraints posed by the subterranean

niche already generate between-group modularity (**Figure 2C**). In addition to the benefits of improved foraging efficiency in groups, ecological and behavioral factors have likely played an important role in bathyergids in tipping the balance in favor of group-living (Faulkes and Bennett, 2021). Ecological constraints to dispersal likely have also relaxed selection pressures on avoiding encounters with parasites through infected intruders. This could also account for the lack of avoidance of odors from infected males observed in highveld mole-rats and the readiness of females of several bathyergid species to engage in copulations with unfamiliar males (Lutermann et al., 2022).

Modularity can occur between as well as within groups (e.g., organizational immunity) and the selection pressure exerted by parasites is likely to contribute to modularity at a colony level (**Figure 2D**). The predominance of within-group transmission in social bathyergids suggests that selection for organizational immunity, including the reproductive division of labor, should be a major evolutionary trajectory in this family. Avoidance and hygienic behaviors can be observed across the full spectrum of sociality (Meunier, 2015; Van Meyel et al., 2018). This often leads to asymmetries in inter-individual contact rates which can differ widely across a range of social systems from solitary to eusocial (Sah et al., 2018). Such modularity between less connected sub-groups is common among social species and increases with group size across species (Griffin and Nunn, 2012; Sah et al., 2018; Evans et al., 2021). At the same time, parasite-mediated selection should favor greater investment in hygienic behaviors including allo-grooming, burrow hygiene or the removal/isolation of infected group members, but reduce the incidence of social behaviors that can increase parasite transmission such as agonistic behaviors (Hawley et al., 2021). This is more likely to evolve in kin groups where inclusive fitness benefits reduce competition for resources. However, the evolution of full organizational immunity, including the reproductive division of labor, is likely constrained in species with multiple breeders due to the lack of inclusive fitness benefits as a result of lower relatedness. In contrast, in closely related hosts, parasites can favor the evolution of singular breeding and organizational immunity as has been proposed for eusocial insects (Naug and Camazine, 2002; Biedermann and Rohlfs, 2017; Cremer et al., 2018). Similarly, parasites could have generated an important selection pressure for the monogamous ancestors of social mole-rats to invest into organizational immunity in response to parasitism due to the high dispersal barriers for offspring (**Figure 2D**). Group sizes differ substantially between social bathyergids and social insects with the latter having group sizes that may exceed those of mole-rats by three orders of magnitude. This is relevant as implementing and maintaining organizational immunity may be constrained by group size. However, it has recently been shown for singular breeding ambrosia beetles (*Xyleborinus saxesenii*) living in small groups, that offspring delay breeding dispersal in the presence of microparasite infection (Nuotclà et al., 2019). The additional investment in hygienic behaviors by philopatric offspring significantly reduced the detrimental effects of parasite infection and hence, lowered the costs of sociality. If similar effects can be accrued by non-breeding group members in social bathyergids this could also account for the philopatry in

bathyergid species where dispersal constraints are relaxed (e.g., *F. mechowii* or *F. anselli*). At the same time, such benefits would be an additional incentive for offspring in species with high dispersal constraints (e.g., *H. glaber*) to forgo investment into reproduction and hence, parasites may also have contributed to the evolution of singular breeding and reproductive suppression in Bathyergidae. The division of labor for other tasks has been questioned for social bathyergids (Lacey and Sherman, 1991; Thorley et al., 2018; Siegmann et al., 2021). Recent mathematical models suggest significant fitness benefits for such division of labor (Udiani and Fefferman, 2020). However, this only applied in the presence of parasites when age-based division of labor reaped more benefits than fixed task specialization. Consequently, some forms of organizational immunity may be more flexible in bathyergids.

While both resistance and tolerance are strategies available to solitary as well as social species and although parasite burden is frequently assumed to be larger in social species (Altizer et al., 2003; Rifkin et al., 2012; Patterson and Ruckstuhl, 2013), from the arguments outlined above it is not clear that social species should exhibit a greater investment in immune defenses than solitary species (Schmid-Hempel, 2021 and references therein, **Figure 2**). Comparative studies in social bees and cooperatively breeding birds suggest a greater investment in immune defenses in the latter (Stow et al., 2007; Spottiswoode, 2008). However, such increased investment in immunity is energetically costly and should more likely be favored in singular breeding species as breeders can benefit from the contributions of related non-breeders (e.g., greater access to food, less investment in foraging or parental care) that in turn increase their inclusive fitness in this way. Conversely, tolerance may carry fitness costs to the host as it does not affect the parasite burden (Medzhitov et al., 2012; Budischak and Cressler, 2018). Once again, the benefits of group-living in the form of greater access to resources can, however, alleviate these costs as has been shown for other singular breeding species (Almberg et al., 2015). Similarly, theoretical work suggests that high burdens of parasites with low virulence should select for hosts to employ tolerance rather than resistance as a defense strategy (Bonds et al., 2005). Thus, organizational immunity can provide effective protection against parasites in singular breeders while additional investment in immune response may not be required or even be lower in singular breeders (**Figure 2**).

Due to the reproductive division of labor the effective population size of singular breeding species is smaller than in communally breeding or solitary species and this can cause a loss of genetic diversity (Hoban et al., 2020). For social insects this results in reduced negative or purifying selection across the genome (Imrit et al., 2020). Similarly, there is evidence for a link between effective population size and selection of genes involved in parasite recognition and immune activation in Bathyergidae. Kundu and Faulkes (2004) report evidence for purifying selection of exon 3 of the *MHC II BLA-DQα1* gene, coding for the transmembrane protein not directly involved with parasite recognition, from solitary (*Heliophobius argenteocinereus*) to social bathyergids (*Cryptomys hottentotus hottentotus*) that was less pronounced in the two species with the largest reproductive skew (*F. damarensis* and *H. glaber*) and the smallest effective

population sizes. At the same time, evidence for positive selection on exon 2, coding for the antigen recognition site, was stronger in *C. h. hottentus* compared to the solitary as well as the other social species (*F. damarensis* and *H. glaber*; Kundu and Faulkes, 2004). Furthermore, high mortalities observed in laboratory colonies of *H. glaber* as a result of artificial viral infections suggest that immune responses may be weaker in the species with the lowest effective population size (Ross-Gillespie et al., 2007; Artwohl et al., 2009). Conversely, investment in immune priming *via* social cues from infested conspecifics should be strongly selected for in social bathyergids to reduce within-group transmission (Schmid-Hempel, 2021).

These examples illustrate that despite the costs of parasitism, selection against group-living should be reduced for singular breeding Bathyergidae. At the same time, selection on behaviors reducing parasite transmission, particularly within colonies such as organizational immunity, should be stronger than selection on physiological immunity, particularly if they provide protection from a range of parasite taxa (Hawley et al., 2021). The kin structure of social bathyergids is a key element affecting the inclusive fitness benefits of such anti-parasite strategies and similar advantageous effects cannot be expected for plural breeders.

Selection on Parasites

Social behavior of hosts can have profound effects on the population structure and evolution of virulence of parasites (i.e., parasite-induced harm to the host) (Ezenwa et al., 2016; Hawley et al., 2021). The genetic diversity and effective population size of a parasite increases in gregarious species with large groups that exhibit only weak modularity (**Figure 2D**). Such species also tend to sustain a larger diversity of parasite species (Côté and Poulin, 1995; Rifkin et al., 2012; Patterson and Ruckstuhl, 2013). However, in species with high modularity, such as in social bathyergids, parasite transmission is greatly impeded and consequently the genetic population structure of parasites should be much more distinct and the effective population size smaller (Hawley et al., 2021; **Figure 2D**). In host species that exhibit organizational immunity these effects are further exacerbated as it constraints parasite transmission within groups (Cremer et al., 2018). This should also affect patterns of parasite aggregation (**Figure 2D**). Parasite populations are often characterized by a skewed distribution, also called overdispersion, with a small number of host individuals sustaining the majority of parasites (Woolhouse et al., 1997; Wilson et al., 2002). While such skews may be absent in host populations with frequent contact or close proximity between host individuals, it should be pronounced in hosts where inter-individual contact rates are low (Hawley et al., 2021). These effects are illustrated for bathyergid parasites by observations that group membership is a good predictor of parasite infection (Viljoen et al., 2011a; Lutermann et al., 2013; Archer et al., 2016). Evidence for similar effects at a colony level due to organizational immunity are less clear, but differences in parasite burden between breeders and non-breeders have been reported for some social bathyergids (Viljoen et al., 2011b; Lutermann et al., 2013; Archer et al., 2016). Overdispersion of parasites among hosts results in the

aggregation of parasites on certain host individuals that in turn leads to increased competition between these parasites for host resources and greater variance in reproductive success of competing parasite individuals (Poulin, 2007; Hawley et al., 2021).

These effects of host behavior on parasite population structure and aggregation also affect selection on parasite virulence (Hawley et al., 2021; Schmid-Hempel, 2021). Reductions in host connectivity or increased modularity reduce transmission rates, conditions also favored by low between-group contacts and organizational immunity, select for greater parasite virulence (Ebert, 1998; **Figures 2C,D**). However, in host populations with distinct modularity high virulence effectively results in a depletion of hosts. This would negatively affect parasite fitness as observed in two incidences of viral infections of naked mole-rats in captivity (Ross-Gillespie et al., 2007; Artwohl et al., 2009). These fitness implications would exert selection pressures for the evolution of low virulence on parasites (Hawley et al., 2021), a scenario also applicable to social bathyergids. The fragmentation of parasite populations due to organizational immunity could furthermore lead to reductions in parasite genome size and possibly loss of functional abilities (**Figure 2D**) as observed in lineages that become commensals in eusocial insects (Leggett et al., 2013; Conlon et al., 2021). In highly modular social hosts dispersal constraints would also result in closely related parasite individuals exploiting hosts. Due to the inherent inclusive fitness benefits of such a scenario selection should favor decreased transmission rates and lowered virulence to reduce kin competition among such parasites (Hawley et al., 2021 and references therein). Thus, high modularity generated by low between-group contact rates (**Figure 2C**) as well as behavioral barriers to within-group transmission (**Figure 2D**) should lower the optima for both transmission and virulence for parasites exploiting hosts such as social Bathyergidae. At the same time, this can be expected to result in lower co-infection rates, further relaxing selection for virulence in co-infecting parasites (Hawley et al., 2021). In addition, the long life expectancy observed in social bathyergids and the related increased opportunities for transmission should lower selection pressures on parasite virulence (Bonds et al., 2005).

If social interactions of hosts are biased in favor of kin, parasites transmitted between such hosts exploit hosts with a similar genetic make-up. In singular breeding species with large group sizes (e.g., naked mole-rats) this does not only resemble the conditions experienced during the serial passage of parasites through the same host (Ebert, 1998), but it is also similar to those encountered by parasites in monocultures which facilitate transmission between hosts (Baer and Schmid-Hempel, 1999; Altermatt and Ebert, 2008; Ekroth et al., 2019). Ultimately, the high degree of relatedness between hosts, as observed within groups of social bathyergids, can favor the evolution of host specialization (**Figure 2C**) as successful transmission and proliferation in alternative hosts is no longer required (Kassen, 2002; Bono et al., 2017). In its extreme form such specialization can not only increase parasite fitness but can be a route for the evolution of benign symbionts (Hughes et al., 2008; Biedermann and Rohlfs, 2017; **Figure 2D**). Similarly, host predictability

should facilitate host specialization in order to increase parasite fitness (Combes, 2001). This possibility has received limited attention in the literature to date (Hawley et al., 2021).

Selection pressures experienced by parasites will also differ with the immune strategy employed by the host; since host resistance reduces parasite fitness this strategy should result in selection for increased virulence in parasites. Empirical evidence of the benefits of sociality in the form of greater access to resources suggest that social species do not necessarily exhibit more investment in immunity thus relaxing selection for virulence in parasites (Almberg et al., 2015; Ezenwa and Snider, 2016). Both strategies might be equally employed by host individuals regardless of their social system and from an evolutionary perspective the mean and variance across a host population rather than individual differences are relevant for selection (Schmid-Hempel, 2021).

Based on the arguments laid out above, parasites of social mole-rats should experience population bottlenecks that reduce their species and genetic diversity while selecting for reductions in virulence, but possibly high transmission and host specialization (**Figure 2**). In fact, for bathyergids there is some evidence for such evolutionary forces having acted on mesostigmatid mites of the genus *Androlaelaps*. Although they are not necessarily restricted to social bathyergids, the majority of the species parasitizing Bathyergidae have not been reported for any other host family (Lutermann et al., 2019). Overall, it appears that selection pressures exerted by behavioral strategies employed by social bathyergids should be stronger than those posed by immune strategies as the latter are less likely to differ from those encountered in solitary hosts or those with multiple breeders.

Interplay Between Sociality, Parasite Mode of Transmission and Life Cycle

The forces of selection acting on both hosts and parasites are also dependent on the mode of transmission, mobility and the type of life-cycle of a parasite with the former likely evolving in response to host defenses that also affect parasite virulence (Poulin, 2007; Antonovics et al., 2017; Hawley et al., 2021; Schmid-Hempel, 2021). The mode of transmission (i.e., method used by parasite) can be either vertical or horizontal. Horizontal transmission can further be distinguished into direct transmission *via* physical contact, airborne (i.e., micrcoparasites), or indirect, i.e., environmentally (e.g., contaminated food, soil or water) or vector-borne (Antonovics et al., 2017). For many parasites more than one mode of transmission may be used and the relative importance of each mode in a particular host-parasite system will be important for their evolution (Antonovics et al., 2017). Generally, parasites with a vertical mode of transmission decrease in virulence because only surviving offspring that reproduces can further transmit the parasite while those with horizontal mode of transmission increase in virulence (Ebert, 2013; Antonovics et al., 2017). However, the kin structure of social bathyergids makes distinctions between these two routes challenging. On the one hand, although vertical transmission is easily possible, the high reproductive skew in mole-rat societies

means that the majority of parent-offspring transmissions will not be successful for parasites with obligate vertical transmission as these offspring will never breed and are thus unable to transmit the parasite to their progeny. At the same time, within a colony, transmission will mostly happen between colony members due to their close physical and genetic proximity (see details above). Although this is technically horizontal transmission, the distinction from vertical transmission is largely formal. In contrast, between-colony transmission is clearly horizontal but occurs much less frequently. Due, to low between-group contact rates, lack of a clear distinction between vertical and horizontal mode and the limited number of group members in the case of social bathyergid hosts, the evolution of virulence of parasites using a horizontal mode should be constrained for parasites (Hawley et al., 2021). This would be further exacerbated by organizational immunity that leads to modularity and hence, further constraints to transmission within bathyergid groups. Similarly, the monogamous mating strategy of social Bathyergidae is an effective measure against directly transmitted parasites during sexual contacts (Antonovics et al., 2017).

While horizontally transmitted parasites requiring direct contact between hosts, particularly virulent ones, should constrain the evolution of sociality the dilution effects provided by group members favor selection of sociality in the presence of mobile parasites that may also act as vectors for microparasites (Hart and Hart, 2018; Hawley et al., 2021). However, the subterranean environment effectively eliminates vector-borne microparasites relying on mobile vectors (e.g., mosquitoes) for bathyergids (**Figure 2B**). Consequently, selection on defenses against these types of parasites should be low in social bathyergids. While no flying vectors are known for Bathyergidae, a number of relatively mobile vectors such as fleas have been reported for several bathyergids (Lutermann et al., 2019; Fagir et al., 2021). Fleas can either be directly transmitted between hosts or *via* shared space use such as nests (Krasnov, 2008). Intriguingly, roughly half the flea species observed for bathyergids to date are host generalists and vectors of zoonotic pathogens while the other half appear to be host specialists that only exploit social bathyergids (**Table 1**). If opportunities for between-group transmission are limited, even for relatively mobile parasites, such specialization should be favored by selection. This should also remove opportunities for such vectors to contract microparasites. Thus, it may not be surprising that screenings for *Bartonella* spp., that are most likely vectored by fleas, have been negative for social common and Damaraland mole-rats but not solitary *B. suillus* (van Sandwyk, 2007). However, solitary *G. capensis* were also negative for these microparasites. Only two tick species, less mobile vectors with environmental transmission, have been reported for solitary Bathyergidae and both species appear to be host generalists parasitizing a range of small mammals (Horak et al., 2018; Lutermann et al., 2019; **Table 2**). Microparasites are the least studied parasites for Bathyergidae and natural infections have only been assessed for one fungal (*Emmonsia parva*) and two bacterial taxa (*Bacillus cereus* and *Mycoplasma* spp.). Of these *Mycoplasma* spp. could be vector-borne while the other two are environmentally transmitted *via* contamination of the

TABLE 1 | Overview of insect parasite species, their mode of transmission and host range reported for the family Bathyergidae to date.

Taxon/species	Transmission/host range	Host	References
Siphonaptera	**Direct/nest**		
Ctenophtalmus ansorgei	Generalist	*Fukomys bocagei*?	Segerman, 1995
Ctenophtalmus edwardsi	Generalist	*Cryptomys hottentotus**	Segerman, 1995
Cryptopsylla ingrami	Specialist (species-specific?)	*Cryptomys h. hottentotus*	Segerman, 1995; Archer et al., 2014
Dinopsyllus ingens	Specialist (species-specific?)	*Bathyergus suillus*	de Graaff, 1964
Dinopsyllus zuluensis	Specialist (species-specific?)	*Cryptomys h. natalensis*	Segerman, 1995
Procaviopsylla creusae	Specialist[a]	*Cryptomys hottentotus**	Segerman, 1995
Xenopsylla georychi	Specialist (species-specific?)	*Fukomys bocagei*	Segerman, 1995
Xenopsylla philoxera[a]	Generalist	*Cryptomys hottentotus**	Segerman, 1995
		Cryptomys h. mahali	Fagir et al., 2021
Xenopsylla piriei	Generalist	*Cryptomys hottentotus**	Segerman, 1995
Anoplura	**Direct/nest**		
Eulinognathus hilli	Specialist (genus-specific?)	*Cryptomys h. hottentotus*	Archer et al., 2014
		Cryptomys h. natalensis	Ledger, 1980
Eulinognathus lawrensis	Specialist (species-specific?)	*Bathyergus suillus*	Ledger, 1980
Eulinognathus sp.	?	*Fukomys damarensis*	Lutermann et al., 2015
Linognathus sp.	?	*Cryptomys h. pretoriae*	Viljoen et al., 2011b

*Old host record lacking geographic information, host could be Cryptomys or Fukomys sp.
[a] Possible misidentification or accidental host as species-specific for Procavia capensis.
?Relationship unknown or uncertain.

soil (Hubálek et al., 2005; Retief et al., 2017, 2021). All of these microparasites appear to be host generalists. However, while prevalences were generally greater for social compared to one solitary hosts, this did not apply to *B. suillus* which had prevalences exceeding those of social species (*C. h. hottentotus*, *F. damarensis*) for both bacteria.

The most speciose ectoparasite taxon infecting Bathyergidae is represented by mites with at least 18 species (**Table 2**). For trombiculid mites (i.e., chiggers, genera: *Austracarus*, *Euschöngastia*, *Gahrliepia*, and *Schoutedenichia*) only the first instars are generalist parasites while all other stages are soil dwelling and consequently transmission is environmental (Shatrov and Kudryashova, 2006). Thus, it is not surprising that these parasites have been reported from a range of social bathyergids but only one solitary species as the larger burrow system of the former may increase their exposure to these mites.

TABLE 2 | Overview of acari parasite species, their mode of transmission and host range reported for the family Bathyergidae to date.

Taxon/species	Transmission/ host range	Host	References
Ixodidae	**Environmental**		
Ixodes alluaudi	Generalist	*Bathyergus suillus*	de Graaff, 1964
		Georychus capensis	
Haemaphysalis leachi/elliptica	Generalist	*Bathyergus suillus*	de Graaff, 1964
Acarinae	**Direct/nest**		
Myonyssoides capensis	Specialist (species-specific?)	*Cryptomys h, hottentotus*	Zumpt, 1961
Androlaelaps capensis	Specialist (family-specific)	*Bathyergus suillus*	Zumpt, 1961; de Graaff, 1981; Viljoen et al., 2011b; Archer et al., 2014; Lutermann et al., 2015, 2019
		Georychus capensis	
		Cryptomys h. hottentotus	
		Cryptomys h. mahali	
		Cryptomys h. pretoriae	
		Fukomys damarensis	
		Fukomys darlingi	
Androlaelaps cryptomius	Specialist (*Bathyergus*, *Georychus*)	*Bathyergus janetta*	Zumpt, 1961; Lutermann et al., 2019
		Georychus capensis	
Androlaelaps eloffi	Specialist (species-specific?)	*Cryptomys h. mahali*	Zumpt, 1961
Androlaelaps georychi	Specialist (species-specific?)	*Georychus capensis*	Zumpt, 1961
Androlaelaps marshalli	Generalist	*Cryptomys hottentotus**	Zumpt, 1961; Viljoen et al., 2011b
		Cryptomys h. pretoriae	
Androlaelaps scapularis	Specialist (family-specific)	*Bathyergus janetta*	Zumpt, 1961; Till, 1963; Viljoen et al., 2011b; Archer et al., 2014; Lutermann et al., 2015, 2019
		Bathyergus suillus	
		Cryptomys h. hottentotus	
		Cryptomys h. mahali	
		Cryptomys h. natalensis	
		Cryptomys h. pretoriae	
		Fukomys damarensis	
Androlaelaps tauffliebi	Specialist (*Bathyergus*, *Fukomys*)	*Fukomys mechowi*	Till, 1963; Lutermann et al., 2015, 2019

(Continued)

TABLE 2 | (Continued)

Taxon/species	Transmission/ host range	Host	References
		Fukomys damarensis	
		Bathyergus janetta	
Laelaps liberiensis	Generalist	*Cryptomys h. mahali*	Fagir et al., 2021
Ornithonyssus bacoti	Generalist	*Cryptomys hottentotus**	Zumpt, 1961
		Bathyergus suillus	
Bathyergolichus bathyergians	Specialist (species-specific?)	*Bathyergus suillus*	Zumpt, 1961; Lutermann et al., 2019
Bathyergolichus zumpti	Specialist (species-specific?)	*Georychus capensis*	Zumpt, 1961; Lutermann et al., 2019
Radfordia ensifera	Specialist (species-specific?)	*Cryptomys h. hottentotus*	Archer et al., 2014
Radfordia rotundata	Specialist (species-specific?)	*Cryptomys h. natalensis*	Zumpt, 1961
Radfordia sp.		*Fukomys damarensis*	Lutermann et al., 2015
Trombiculidae			
Austracarus polydiscum	Generalist	*Cryptomys hottentotus**	Zumpt, 1961
Euschöngastia bottegi	Generalist?	*Heterocephalus glaber*	de Graaff, 1964
Gahrliepia nana	Generalist?	*Cryptomys hottentotus**	Zumpt, 1961
Schoutedenichia crocidurae	Generalist?	*Cryptomys hottentotus**	Zumpt, 1961
Unidentified trombiclid	Generalist?	*Cryptomys h. hottentotus*	Archer et al., 2014; Lutermann et al., 2015, 2019
		Fukomys damarensis	
		Georychus capensis	

Old host record lacking geographic information, host could be Cryptomys or Fukomys sp.
?Relationship unknown or uncertain.

The remaining 14 mite species reported for bathyergids rely predominantly on direct transmission and are often the most prevalent and abundant parasites reported for a host species (Viljoen et al., 2011b; Archer et al., 2014; Lutermann et al., 2015, 2019; Fagir et al., 2021). Only three of these species are known host generalists (*Androlaelaps marshalli*, *Laelaps liberiensis* and *Orntihonyssus bacoti*) while the remaining 11 species appear to be host specialists for Bathyergidae either at the family, genus or species level (**Table 2**; Lutermann et al., 2019). This suggests a potentially close co-evolutionary history between these mites and bathyergids that should help to shed light on the selection pressures experienced by both actors. Blood-sucking lice (Anoplura) are the most sedentary ectoparasite taxon of bathyergids and require direct contact between hosts for transmission (Kim, 2006). This is also why most louse species have a narrow host range, often using a single host species, which

also appears to apply to the two species identified in Bathyergidae with one potentially specific to the host genus (*Eulinognathus hilli*) and another to the species (*E. lawrensis*) (Zumpt, 1966; Lutermann et al., 2019; **Table 1**). Thus, the patterns of host specialization across the ectoparasite communities described for bathyergids to date appears to support the hypothesis for a link between parasite transmission mode, host social behavior and parasite specialization. As laid out above this specialization should also include lowered virulence in these parasites and consequently, stronger selection pressures on behavioral rather than physiological immunity for social bathyergids.

The picture arising from the helminth community described for Bathyergidae is somewhat less clear. This is partially due to the lower taxonomic resolution with most parasites only being identified to genus level (Lutermann et al., 2019; **Table 3**). However, cestodes have complex life cycles that require an intermediate host (often an arthropod) in their life cycle (Georgiev et al., 2006). Hence, they need to adapt to both an invertebrate and vertebrate which makes host specialization much less likely as host encounters tend to depend mostly on stochastic processes (Antonovics et al., 2017). This could not only account for the limited number of cestode species observed in Bathyergidae to date, but would also make the evolution of specific behavioral or physiological responses less likely (Antonovics et al., 2017). At the same time, basic hygienic behaviors (e.g., grooming to reduce intermediate hosts such as mites or fleas) and organizational immunity in the form of dedicated toileting areas can be effective means to reduce the transmission of cestode propagules. Nematodes have a more diverse range of transmission modes including direct, environmental or transmission *via* an intermediate host, but this is often not well established for a species (Anderson, 2000). However, many of the genera observed to infect Bathyergidae are host generalists with a wide geographic distribution (Anderson, 2000; **Table 3**). Possible exceptions are four nematode species (*Ortleppsrongylus bathyergid*, *Mammalakis macrospiculum*, *M. zambiensis,* and *Paralibyostrongylus bathyergid*) that have to date only been found in bathyergids with three of them retrieved from *B. suillus* (Lutermann et al., 2019; **Table 3**). Helminths are famous for their ability to manipulate the immune responses of their hosts and may strongly interact with symbiotic microorganism (Maizels et al., 2004). Hence, they often do not trigger strong immune responses from their hosts, at the same time, they require host survival for successful proliferation. Consequently, they may favor tolerance responses in hosts.

Rather than an individual parasites species the composition of parasite communities exploiting a host and the virulence of common parasites will determine selection on particular social behaviors as well as the extent to which such behaviors reduces the associated fitness costs (Hawley et al., 2021). This in turn will shape the evolutionary trajectories for the parasites involved. The majority of macroparasites exploiting Bathyergidae seem to fall into one of two broad categories: directly transmitted host specialists (at species, genus or family level) and indirectly (environmentally or *via* intermediate hosts) transmitted host generalists with the former occurring at the highest prevalences (Viljoen et al., 2011b; Lutermann et al., 2013, 2019;

Archer et al., 2014, 2017; Fagir et al., 2021). Consequently, selection on the bathyergid host should be strongest on behavioral (e.g., hygienic behaviors, organizational immunity) instead of physiological control strategies while for those parasites selection will act to favor benign parasites with direct transmission.

FUTURE DIRECTIONS AND CONCLUSION

Exploiting the subterranean niche has posed a number of challenges for Bathyergidae such as hypoxia, hypercapnia and foraging and dispersal constraints. However, the latter constraints likely have also limited the exposure and transmission to parasites which in turn should have made major contributions to the evolutionary steps of members of this family toward sociality and singular breeding. Living in sealed burrow system has reduced the exposure of bathyergids to mobile, environmentally and vector-transmitted parasites (**Figure 2B**). The resulting reductions in the prevalence, abundance, co-infection rate and parasite species diversity and hence, costs of parasitism should also have lowered the threshold for group-living. At the same time, the constant physical conditions and low generalized transmission distance within groups should favor directly transmitted arthropod parasites of bathyergids (**Figure 2B**). While social behaviors such as allo-grooming are effective in controlling such parasites the finite host size should induce evolution toward host specialization and lowered virulence in these parasites (**Figure 2C**). This can be facilitated further by organizational immunity including the reproductive division of labor observed in all social Bathyergidae (**Figure 2D**). This scenario also suggests that selection on behavioral defense strategies has been much stronger for Bathyergidae than that experienced on physiological defenses. Hence, venturing below ground and exploring soils that are difficult to work and regions of unpredictable rainfall have likely contributed to the evolution of extreme reproductive skew observed in naked and Damaraland mole-rats (*Fukomys damarensis*) that is unique among vertebrates with a social structure that bears resemblance to what can be found in eusocial insects. This is also illustrated by the stark contrast of social systems in bats that are not only extremely mobile and can occur in very large groups, but also harbor one of the most diverse assemblages of parasites among vertebrates (Luis et al., 2015; Han et al., 2016; Webber and Willis, 2016). However, although there might be some kin structure in bats, they do not exhibit singular breeding.

There are a number of hypotheses that require testing and research directions that can help to consolidate or revise the framework laid out here that I will outline below. Firstly, I anticipate that across the range of bathyergid species overall parasite burden (e.g., prevalence, abundance and/or diversity) should decrease from solitary to social species with increasing reproductive skew (i.e., increasing group size), but also with progressively more challenging soil properties and less predictable rainfall patterns. At a species level, similar patterns should be apparent between different geographic localities experiencing different climatic and soil conditions that affect

TABLE 3 | Overview of helminth parasite species, their mode of transmission or life-cycle and host range reported for the family Bathyergidae to date.

Taxon/species	Transmission/life cycle	Host range	Host	References
Nematoda				
Hexametra sp.	Complex	Generalist	*Fukomys anselli*	Lutermann et al., 2018
Capillaria sp.	Complex	Generalist	*Fukomys mechowi*	Scharff et al., 1997
Heligmonina sp.	Direct/environmental	Generalist	*Cryptomys h. pretoriae*	Viljoen et al., 2011b
Neoheligmonella sp.	Direct/environmental	Generalist	*Cryptomys h. hottentotus*	Archer et al., 2017
Ortleppstrongylus bathyergi	?	Specialist (species-specific?)	*Bathyergus suillus*	de Graaff, 1964; Lutermann and Bennett, 2012
Mammalakis macrospiculum	?	Specialist (family-specific?)	*Bathyergus suillus*	de Graaff, 1964; Lutermann and Bennett, 2012
			Cryptomys h. hottentotus	Archer et al., 2017
Mammalakis zambiensis	?	Specialist (species-specific?)	*Fukomys anselli*	Junker et al., 2017
Protospirura muricola	Complex	Generalist	*Heliophobius argenteocinereus*	Scharff et al., 1997; Tenora et al., 2003; Lutermann et al., 2018
			Fukomys anselli	
			Fukomys kafuensis	
			Fukomys mechowi	
Protospirura numidica	Complex	Generalist	*Fukomys anselli*	Lutermann et al., 2018
Protospirura sp.	Complex	Generalist	*Fukomys anselli*	Viljoen et al., 2011b; Lutermann et al., 2018
			Cryptomys h. pretoriae	
Ascarops africana	Complex	Generalist?	*Cryptomys h. natalensis*	Lutermann et al., 2013
Paralibyostrongylus bathyergi		Specialist (species-specific?)	*Bathyergus suillus*	Lutermann and Bennett, 2012
Trichostrongylus sp.	Direct/environmental	Generalist	*Bathyergus suillus*	de Graaff, 1964
Trichuris sp.	Direct/environmental	Generalist	*Bathyergus suillus*	Lutermann and Bennett, 2012; Archer et al., 2017; Lutermann et al., 2019
			Georychus capensis	
			Cryptomys h. hottentotus	
Cestoda	**Complex**			
Inermicapsifer arvicanthidis		Generalist	*Heliophobius argenteocinereus*	Tenora et al., 2003
Inermicapsifer madagascariensis		Generalist	*Cryptomys h. nimrodi*	de Graaff, 1981; Scharff et al., 1997
			Fukomys kafuensis	
			Fukomys mechowi	
Inermicapsifer sp.		Generalist?	*Fukomys anselli*	Lutermann et al., 2018
Mathevotaenia sp.		Generalist?	*Cryptomys h. pretoriae*	Viljoen et al., 2011b
Raillietina sp.		Generalist?	*Cryptomys h. natalensis*	Lutermann et al., 2013
Rodentolepis cf. *microstoma*		Generalist	*Fukomys anselli*	Lutermann et al., 2018
Rodentolepis sp.		Generalist?	*Bathyergus suillus*	Lutermann and Bennett, 2012
Taenia sp.		Generalist?	*Bathyergus suillus*	Lutermann and Bennett, 2012
Echinococcus sp.		Generalist?	*Georychus capensis*	de Graaff, 1964

?Relationship unknown or uncertain.

between-colony contact rates. More specifically, the community composition of parasites can be expected to shift from more to less mobile parasites and reductions in vector-borne as well as environmentally-submitted parasites with increasing dispersal constraints (e.g., soil hardness, rainfall patterns) for the host. Specifically, low prevalences, abundance and species diversity of mobile and vector-borne parasites can be expected for social hosts in more arid localities or during periods of low rainfall and larger group sizes. At the same time, directly transmitted parasites such as relatively benign parasites, e.g. mites, could increase in species diversity. This is due to the genetic bottlenecks, they would experience but possibly also increases in prevalence

and abundance due to hosts employing tolerance rather than resistance as defense strategy in larger groups.

Conversely, evidence for elements of organizational immunity should increase with increasing dispersal constraints and increasing group sizes both within and between bathyergid species. This is already evident in the degree of physiological suppression of reproduction of non-breeders (Faulkes and Bennett, 2021), but other aspects of organizational immunity need to be explored as well including but not limited to spatial and temporal segregation between colony members. The subterranean environment exploited by Bathyergidae hampers to some extend studies of behavioral mechanisms although

technology can provide some remedy (Šklíba et al., 2016; Finn et al., 2022). Conversely, and despite their limitations, laboratory settings can allow for detailed behavioral observations. Such comparisons should be conducted at three levels: between bathyergid species that differ in group sizes (see **Figure 1**), across a range of colony sizes for each social species, as well as individual colonies that grow in size as they mature. At each of these levels the risk of social transmission and hence, the need for organizational immunity will change with group size and dispersal constraints. Importantly, a lack of evidence for organizational immunity beyond the reproductive division of labor does not inevitably disprove the role of parasites or the significance of organizational immunity as the presence of parasites may be required to induce such flexible behavioral responses (Nuotclà et al., 2019). Laboratory settings allow for experimental manipulations of parasite burdens and will thus be a good test of this hypothesis. Since the effectiveness of behavioral responses also depends on the mode of transmission such manipulations should be conducted with different parasite taxa.

Behavioral patterns such as contact rates can also be measured indirectly. Individually marked parasites can be used to track their transmission between group members and shed light on contact rates (Zohdy et al., 2012). The larger effective population size and shorter generation time of parasites also allow insights into host movements based on the genetic population structure of their parasites as well as co-speciation patterns between hosts and parasites (Hugot, 2006; Nieberding and Olivieri, 2007). Hence, studies of the population structure but also genome size of bathyergid parasites can provide insights into the interaction between these hosts and their parasites. Given their species diversity mites may prove particularly useful in this regard and mite species exploiting a large number of bathyergid species, such as *Androlaelaps scapularis* and *Androlaelaps capensis*, would allow for direct comparisons of population structure across a number of bathyergid species.

Virulence of parasite species can be tested by artificial infestation of individuals. For many of the bathyergid parasites this will, however, firstly require a better understanding of their life cycles. Nevertheless, infections with directly transmitted parasites (e.g., mites) should already be feasible and not only allow to assess their level of virulence but also the physiological defenses of their hosts. This can be carried out in isolated individuals and those in groups to evaluate the contributions of individual and social factors. By varying the resource availability (e.g., access to food) during such experimental manipulations one will furthermore be able to determine the role of resources and contributions of immunity vs. tolerance to host responses (Budischak and Cressler, 2018). Using different numbers or species of parasites (i.e., co-infection) can furthermore shed light on both the competitive ability of particular parasites and the resulting costs for hosts.

The assessments of the microbiome should be extended to a range of bathyergid species. Importantly, rather than simply providing inventories of the species composition they should be linked to group and individual characteristics that constitute once again a reflection of between and within-group contacts and possibly organizational immunity. Their potential role in mediating resistance or tolerance benefits can be evaluated by linking them to (natural or experimental) infection patterns. Lastly, the use of theoretical models using the biological parameters provided by such studies can allow the testing of assumptions and further specification of conditions that facilitated social evolution in bathyergids, similar to studies conducted for social insects (e.g., Udiani and Fefferman, 2020).

The scenario presented here is based on incomplete information as exhaustive and long-term parasite assessments (including those for microparasites) are lacking for many bathyergid species, most notably naked mole-rats. Also, more extensive behavioral observations are necessary and immunity and tolerance needs to be assessed in a greater range of species. Nevertheless, the scenario presented integrates knowledge on host and parasite biology to shed light on the relationships between bathyergids and their parasites and is the first to explore the role of the latter for social evolution in Bathyergidae. This illustrates how subterranean living and social structure can reduce the exposure to parasites and thus, substantially lower the costs associated with living in groups while the benefits of sociality such as better resource acquisition are apparent. In turn, bathyergid parasites have likely experienced substantial constraints to the evolution of virulence as well as experienced genetic bottlenecks that made them more benign. This will help to further shed light on social evolution in this unique family but also the role of social behaviors for parasite evolution.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Zambian Mole-Rats: 33 Years on the Scene and What We Still Do Not Know and How We Could Learn It

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This article surveys more than three decades of research on Zambian mole-rats (genus *Fukomys*, Bathyergidae), pointing out some unanswered questions and untested hypotheses and suggesting approaches to address them. These research proposals range from sensory ecology topics, the main research field, covering different (even not yet identified) senses, orientation in time and space, communication, studies on aging, population dynamics, and the survival strategies of mole-rats during yearly floodings in the Kafue Flats. Discussion includes cryptozoological investigation into the existence of strange mole-rat species in some Zambian localities as reported by local communities, the study of mole-rats in assumed contact (hybrid?) zones of special interest, (cyto)genetic studies of hybrids of selected species, and a non-invasive study of population and family structure and dynamics with help of endoscopes. In each case, there is a rationale, reasoning, hypothesis, and suggested methical approach.

Keywords: *Fukomys*, sensory ecology, behavioral ecology, ecophysiology, spatial orientation, Zambia

INTRODUCTION

Thirty-seven years ago (1985), in Lusaka, Zambia, I first held a mole-rat, now known as *Fukomys anselli*, in my hands. In 1986, I was the first to bring these animals out of Africa, to Germany, where I succeeded in breeding them. This began my 33-year-long career, involving (mainly laboratory) multi- and interdisciplinary research on mole-rats. In 1990, my student assistant from the University of Zambia, and later Ph.D. student at the University of Duisburg-Essen, friend and colleague, Mathias Kawalika, moved from Lusaka to Ndola and became acquainted with the local giant mole-rats, now known as *Fukomys mechowii*. These guinea-pig-sized animals live in larger families, the biology of which was virtually unknown to scholars at that time, though they were familiar to local people. Dr. Kawalika sent me several live animals. The Zambian mole-rats were kept and studied at my home and in institutions where I worked, including J. W. Goethe-University (Zoological Institute, Faculty of Biology and Senckenberg Institute of Anatomy, Faculty of Medicine) in Frankfurt am Main, and at the University Duisburg-Essen (Department of General Zoology, Faculty of Biology) in Essen.

Both species were investigated mainly in the laboratory for diverse aspects of sensory biology, anatomy, physiology, systematics and taxonomy, and behavioral biology. The research was later extended to field studies by former students and colleagues. Existing knowledge on the Zambian mole-rats was recently summarized in Begall et al. (2021) and Caspar et al. (2021a).

Active research on mole-rats had ended with my retirement in 2018, but the baton has been handed down to my students. Because of mainly financial and manpower-related reasons as

well as lack of time, several research ideas were never realized, and these hypotheses have never been published. Being convinced that they do not deserve to be forgotten, I would like to share these ideas here in the hope that they might trigger interest and inspire the next generation of young researchers.

SENSORY ECOLOGY

Sensory ecology explores "how the animals acquire information and respond to it" (Dusenbery, 1992). Bathyergid mole-rats (as well as other subterranean mammals) are excellent animal models to investigate these questions. They live in a monotonous, uniform, simple sensory environment, deprived of most cues and signals available to animals aboveground, yet they must also orient in space and time, forage, find partners, communicate, and be warned of danger. On one hand, we can expect degeneration of senses because of lack of use, we must also expect specializations and specific adaptations to compensate for handicaps. My interest in the biology of subterranean mammals in general and in their senses, in particular, was drawn in 1979 by a seminal review article by Eviatar (Eibi) Nevo (1979). In the meantime, this field has become a goldmine of research for many students and scholars (reviewed in, e.g., Burda et al., 1990a; Burda, 2006, 2021; Begall et al., 2007a,b; Buffenstein et al., 2021, and numerous papers cited therein and in this article). I would like to point out here some persisting questions.

Hearing

Subterranean mammals rely to a great extent on audition for communication and to be alerted to danger. The only hitherto published reports on burrow acoustics revealed that in the tunnels of blind mole-rats (*Spalax*), airborne sounds of 440 Hz propagated best whereas lower and higher frequencies were effectively attenuated (Heth et al., 1986) and that in mole-rat (*Fukomys*) tunnels, low-frequency sounds (200–800 Hz) are not only least attenuated but also their amplitude may be amplified like in a stethoscope (up to twice over 1 m) (Lange et al., 2007). Morpho-functional analyses classified the ear of subterranean mammals as a low-sensitivity and low-frequency device (i.e., Burda et al., 1992, 1989; Burda, 1990; Müller et al., 1989, 1992; Mason, 2001; Schleich and Busch, 2004; Lange and Burda, 2005; Mason et al., 2016; Pleštilová et al., 2016, 2021). Concordantly, hearing (at least in the studied bathyergid, spalacid, and geomyid species) is characterized by low sensitivity and a restricted frequency range of best hearing tuned to low frequencies (0.5–4 kHz) (Müller and Burda, 1989; Heffner and Heffner, 1990, 1992, 1993; Kössl et al., 1996; Brückmann and Burda, 1997; Gerhardt et al., 2018; Barker et al., 2021; Caspar et al., 2021b). Some authors considered the restricted hearing in subterranean mammals vestigial and degenerate due to under-stimulation (Heffner and Heffner, 1990, 1992, 1993). In contrast to this view, moe-rats have a rich (mostly low-frequency) vocal repertoire (see paragraph below) and progressive structural specializations of the middle and inner ear. Thus, other authors have considered these hearing characteristics adaptive, in that hearing sensitivity has decreased during the evolution of subterranean mammals to

avoid over-stimulation of the ear in their natural environment (Burda, 2006, 2021; Lange et al., 2007).

We may assume that the local increase in amplitude occurs at the expense of decreasing the amplitude somewhere else and that there may be a specific "stethoscope region" at a certain distance from the sound source, where the amplification effect is particularly pronounced. Given the lowest hearing threshold in *Fukomys anselli*, 7 dB SPL (Brückmann and Burda, 1997), we may expect that the measured stethoscope effect would amplify the signal, lowering the threshold to 0.5 dB SPL. This would mean that in their natural environment, mole-rats display best hearing sensitivity in a range that is comparable to that of most other mammals, including humans. I do not expect that contact communication calls need to be adapted (either in their intensity or even in their frequency) to the tunnel acoustics. Neither do I expect that hearing is primarily adapted to vocal communication. I expect that hearing is adapted to the tunnel acoustics – i.e., hearing of distant (primarily substrate-borne) acoustic cues provides a warning of danger. Vocalization then must match the given hearing range.

Testing the hearing sensitivity of subterranean rodents in a laboratory in the open field may, thus, not correctly reflect their sensory capacities in natural tunnels where certain signals may be enhanced. In analogy with hearing research on dolphins, which is performed in their natural milieu, in water, the hearing parameters of mole-rats should be studied in their natural acoustic environment, in tunnels.

Vocalization

It is generally expected that vocal-repertoire size correlates positively with group size (e.g., McComb and Semple, 2005). It is thus not surprising that with an average colony size of 78 individuals (Sherman et al., 1992) and 12–18 different adult vocalizations (Pepper et al., 1991; Barker et al., 2021, respectively), the naked mole-rat has been long considered to be the rodent with the largest vocal repertoire. (Note that mechanical sounds and juvenile vocalizations are not considered in this survey.) Later studies showed that many *Fukomys* species with much smaller mean family sizes (7–12 individuals) have a similarly rich adult repertoire compared to that of the naked mole-rat: 16 adult vocalizations in *F. mechowii* (Bednářová et al., 2013), 13 in *F. anselli*, (Credner et al., 1997); 11 in *Fukomys darlingi* (Dvořáková et al., 2016); 10 in *Fukomys micklemi* (Vanden Hole et al., 2014), while eight sounds were described in solitary *Heliophobius argenteocinereus* (Knotková et al., 2009). Thus, the richness of the vocal repertoire in African mole-rats may be influenced not only by group size but also by social complexity and stability (cf. Burda et al., 2000).

Importantly, however, to assess the real richness of the vocalization repertoire we have to study it in all potential social interactions (i.e., also during courtship and during the entire period of care for pups), which is not easy since these animals do not breed in captivity. We may expect that vocalization in solitary animals may be more complex and richer than is known. To give an analogy: let us monitor the vocalization of a lonesome human. Unless she/he talks to herself/himself (soliloquy), we would conclude that the vocal repertoire of this human is much

more restricted than the vocal repertoire of humans living (and being monitored) in a group. Moreover, in captivity, we will never be able to simulate all possible interactions and different situations, which may occur in nature. To illustrate the issue: an animal may have a specific call if it encounters a snake but until we have simulated such a situation in the laboratory we will never learn about it. Animals in natural tunnels may elicit sounds that we never hear in the open field.

The other question of interest is whether the pups vocalize in the ultrasonic range like pups of muroid rodents (cf. e.g., Okanoya and Screven, 2018). In many epigenetic rodent species, pups are raised similarly to subterranean rodents in closed underground nests, i.e., in a similar acoustic environment. Also, the morphological substrate for vocalization can be expected to be similar in all rodent pups. The problem is, however, that mothers in mole-rats most probably would not hear and react to ultrasonic calls. Nevertheless, it is possible to produce signals/cues even if we cannot perceive them. Just think of producing smell signals carrying information about our sex, stress, emotions, and diseases, which we cannot perceive but a dog can. Such ultrasonic "signals" do not play a role in communication anymore but they might represent a kind of "atavisms." However, that ultrasonic vocalization was not found in pups of *Heterocephalus glaber* (Pepper et al., 1991; Barker et al., 2021). Studies that recorded vocalizations in *Fukomys* (see above) were limited by the technical equipment used, which did register sounds above 20 kHz.

To summarize, the reason we have not recorded the entire vocalization in solitary mole-rats is because they have no opportunity to reveal it in captivity. Moreover, to make conclusions about the role of vocalization in communication, we need to conduct playback experiments. Studies of vocalization in the ultrasonic range in pups would be of interest. Morpho-functional comparative studies of the vocal apparatus of subterranean rodents enabling them vocalization in the low-frequency range are still missing.

Olfaction

The sense of smell of mole-rats has been studied with respect to olfactory discrimination of familiar and unfamiliar individuals, conspecifics (Heth et al., 2002a, 2004), and root kairomones (Heth et al., 2002b; Lange et al., 2005). We expect that the underground environment, which lacks air-currents, is not particularly odorant-rich, and that odorants are predictable. This assumption, however, contradicts the conclusions of a study on the diversity of the study of olfactory receptor subgenome within a single family of olfactory genes (OR7), suggesting that mole-rats can recognize a broad range of odorants (Stathopoulos et al., 2014). Behavioral tests of smell sensitivity and the discrimination capacities of mole-rats are needed to learn what are the specific and convergent olfactory capabilities distinguishing subterranean rodents from their epigeic counterparts.

The idea of "blind" foraging in herbivorous underground dwellers (Lovegrove and Wissel, 1988) has been challenged by showing that subterranean rodents of several species use olfaction to discriminate between soils in which plants had or had not been growing (Heth et al., 2002b; Lange et al., 2005).

These laboratory studies should be, however, conducted under (semi)natural conditions in the field to learn which geophytes are present, under which conditions, at which distance, and how precisely they can be targeted in the field.

The randomness versus directionality of food searching in mole-rats could be tested *in situ* using small beds of diverse plants (carrots and potatoes). These can be established (as controls not vegetated squares will be watered) at given distances from an established burrow system in a random pattern. This potential study could assess the probability that and how long it takes, for the mole-rats to discover and exploit these beds.

Burda et al. (1999a) have shown that subterranean rodents generally start to eat carrots offered to them from the lower end of the root while rodents that are not specialized root eaters display no preference for a particular root end, or they eat from the upper end. Although the adaptive meaning and evolutionary origin of this convergent feeding behavior are understandable, the proximate (sensory) basis enabling distinction between the two root poles remains a challenge for future research. The study demonstrated that subterranean rodents can very finely distinguish between different parts of plant organs and exhibit thus unanticipated sensory abilities. A follow-up study of perceptual mechanisms and cues enabling them to identify the polarity of a root may certainly prove to be of great interest, not only for ecologists and sensory biologists but also for botanists and applied sciences such as pest control.

Vision

The eyes of the African mole-rats are relatively small; however, the optical apparatus, including the retina, contain all the structures of a typical mammalian eye (Peichl et al., 2004). The retina is normally developed and possesses all characteristic layers. The photoreceptor layer is dominated by rods but contains an unusually high proportion of cones (approximately 10%), which solely express short-wavelength-sensitive (SWS) cone photopigment (Peichl et al., 2004). The main problem with the interpretation of the anatomical, physiological, and behavioral findings regarding the visual apparatus of subterranean mammals and the open question is, why the eye in *Fukomys* is superficial and only quantitatively reduced but qualitatively normally developed, while in some other subterranean mammals, like the blind mole rat, *Spalax*, it is subcutaneous and structurally degenerated? Why *Spalax* possess a long wave-sensitive-opsin but no SWS-opsin, whereas the situation in *Fukomys* is the opposite? Although adaptive explanations as well as non-adaptive ones (the side effects of different adaptations) have been suggested [reviewed in Burda (2021)], they always considered each group (Bathyergidae and Spalacidae) separately and not from the point of view of the otherwise apparent convergence.

Distant Thermoperception

Distant thermoperception in dark underground burrows where the ambient temperature is buffered and uniform would be an advantageous sensory ability to be alerted to conspecifics, intruders, open burrows, and even plant storage organs. A variety of infrared radiation (IR)-detecting receptors are present in animals (e.g., crotalid and boid snakes, vampire

bats, and some insects) which aid them in foraging or otherwise enhance their fitness [reviewed in Campbell et al. (2002)]. My former colleague at the University Duisburg-Essen, botanist Prof. Hardy Pfanz, drew my attention to the fact that underground plant organs, roots, bulbs, tubers, etc. act as IR-light conductors (Sun et al., 2005). One can indeed wonder why such a sensory ability has not evolved (or has not yet been discovered) in subterranean mammals?

Decades ago, I have observed that Ansell's mole-rats were able to detect a warm object (a cup) placed in the cage beside their paths and that they approached it and explored it. The observations were only anecdotal but motivated us (Sabine Begall and me) to design a two-choice test-apparatus and experimental protocol 20 years ago. Students performed pilot experiments to test this ability under controlled laboratory conditions. The results were quite promising and we suggested that the specific nose (rhinarium) of mole-rats might be the seat of putative IR-detectors. Our application for research funding was not successful and the project has been abandoned and subsequently forgotten. The idea of distant thermoperception and IR-detection by mole-rats should be recaptured. It is noteworthy that recently distant thermoperception was recorded also in domestic dogs (Bálint et al., 2020). The respective authors further hypothesized that the dog rhinarium is particularly sensitive to radiating heat thanks to its coldness. It may be of interest and significance in the context of distant thermoperception that the nose area in mole-rats is permanently colder than other body areas (Šumbera et al., 2007).

Spatial Orientation (Dead Reckoning and Magnetoreception)

Simple observations of captive mole-rats moving in their home cages and simple maze experiments reveal very good spatial memory, which seems to be based on proprioception and dead reckoning. Navigation over longer distances in the field is probably assisted by magnetoreception. Maze experiments with mole-rats may be complicated by the fact that the animals seem to learn the maze after one or few trials and the "errors" in the following passes represent explorative trips. Mazes should be also longer and/or more complex than those used for experiments with laboratory rodents. Mole-rats are relatively good swimmers (Hickman, 1978) and a water maze may be an option for an experimental design to study the role of magnetoreception in navigation (cf. Phillips et al., 2013).

Experiments in classical and modified mazes and circular arenas have heuristic potential to study navigation strategies (always considering both: dead reckoning and magnetoreception). Care has to be taken, as in all such experiments generally, that we consider and exclude the possibility of leaving olfactory tracks, providing unconscious cues for piloting (e.g., in that the experimentator waits for the animal at the goal/outlet), magnetic disturbances in buildings (particularly at the floor where iron wires, electric and water installation may be laid).

Experiments like those described by Etienne et al. (1993) should be applied also to mole-rats. Experiments should be done with mirrored mazes to find out whether mole-rats use external (allothetic) cues (e.g., magnetic compass direction) or internal (idiothetic) cues as directional reference. Apart from tube perspex mazes also circular arenas with one or more outlets are suitable for navigation studies in mole-rats. The tube maze may connect the home cage (nesting chamber) with a food chamber or nest-material provisioning chamber (see Section "Conditioning").

Captive mole-rats prefer to run along the walls of their home cages in a clockwise direction. Having observed this phenomenon, we found it so conspicuous that wanted to use it as a behavioral assay and hypothesized that the moving pattern may be affected by changing the magnetic field. When the animals were put into a circular arena, they started to collect tissue papers from their home cage litter given as substrate into the experimental arena to provide familiar odors and thus alleviate the stress from a new environment and the nest-building assay was born (Burda et al., 1990b). The mole-rats gathered strips of paper and still circled along the arena wall in a clockwise direction. This phenomenon deserves to be studied systematically (and also in the southern hemisphere). On a similar note, the late Graham C. Hickman once suggested monitoring the direction of the spiral coiling of the vertical ventilation burrows ("chimneys") of subterranean mammals in the southern and northern hemispheres.

The nest-building assay, i.e., recording in which compass direction the rodents build their nests in a circular arena, is a simple experiment and its heuristic potential is not yet exhausted [reviewed in Burda et al. (2020)]. It remains also unclear whether the nest-direction in mole-rats is species- or population-specific (cf. Oliveriusová et al., 2012) or whether it may be entrained (Deutschlander et al., 2003).

Studies of spatial orientation of mole-rats in the field may be inspired by experiments by Kimchi and Terkel (2003a,b), Kimchi et al. (2004) on blind mole rats). Experiments by Eloff (1951) with interrupted or blocked runways should be recapitulated in a systematic way, always noticing the compass direction of the affected tunnel. Homing ability of captured and translocated mole-rats should be studied. Compass direction and straightness of tunnels in newly established burrow systems (e.g., after translocation of a founder pair of animals) should be monitored. The hypothesis of the magnetic field as a heading indicator to keep the course of burrowing (Malewski et al., 2018) should be tested also in localities under high voltage power lines where mole-rats might also occur (cf. Burda et al., 2009).

Chronobiology

Chronobiology (orientation and structuring behavior in time) in African mole-rats has been the subject of several studies in the laboratory and the field. A diversity of cues has been found to function as *zeitgebers*: especially light (cf. e.g., Oosthuizen et al., 2003), although its biological role underground is not clear, and temperature (Šklíba et al., 2014), although at least in the nest 50 cm and deeper under the surface ambient temperature is buffered and constant (Burda et al., 2007). Besides, that small predictable daily fluctuation of the magnetic field may serve as *zeitgeber* (cf. Wiltschko and Wiltschko, 1995). Such

small variations are masked by electromagnetic disturbances in standard laboratories and animal rooms.

My former colleague at the University Duisburg-Essen, geologist Professor Ulrich Schreiber, drew my attention to another potential and thus far unstudied *zeitgeber:* periodic circadian oscillation (rise and fall) of the levels of groundwater, the so-called earth tide, which is analogous to sea tides, and also caused by the combined effects of the gravitational forces exerted by the moon and the sun, and the rotation of the earth (Sugisaki, 1981; Sato, 2006). The rising groundwater pushes soil gases up and their pressure in the atmosphere of the underground burrows thus predictably changes during the day. It remains unknown, however, whether mole-rats can detect changes in those gases (carbon dioxide, helium, argon, etc.).

A white head spot (bles) is typical of *F. anselli*, *Fukomys kafuensis*, *Fukomys damarensis*, *F. micklemi*, and other *Fukomys* species from southern and western Zambia (southwards of 15th latitude) (cf., Burda et al., 1999b; Van Daele et al., 2004) but is missing in most *F. mechowii* and in the examined, *Fukomys amatus*, and *Fukomys whytei*, *F. vandewoestijneae*, and Kasama mole-rats (cf., Macholan et al., 1998; Kawalika et al., 2001; Van Daele et al., 2004, 2013; Burda et al., 2005). Even in species where the headspot is present it is variable in size and shape. The adaptive significance of the bles in many *Cryptomys* and *Fukomys* species remains obscure but it has been suggested that being an unpigmented part of the skin it may facilitate penetration of light to the pineal organ and thus be involved in photoperception and control of photoperiodicity (Lovegrove et al., 1993). However, chronobiological data for *Cryptomys* and *Fukomys* are very ambivalent, and large polymorphisms in the size and shape of the white head spot, even in its very presence, weaken the idea of its functional significance. Nevertheless, having briefly examined the large collection of specimens of mole-rats in the Natural History Museum of Zimbabwe in Bulawayo in 1993 (then under the auspices of Fenton P. D. "Woody" Cotterill), I noticed that there may be a general latitudinal gradient in the size of the bles, decreasing and disappearing from the south toward the equator, reflecting thus also changes in annual photoperiodicity and seasonality of breeding (**Figure 1**). The bles in museum specimens can be planimetrically measured and the hypothesis of the latitudinal gradient should (and relatively easily could) be statistically tested.

Nociception

The naked mole-rat became famous for its virtual insensitivity to acid, capsaicin, and histamine. This feature was observed also in some other studied bathyergid species. The phylogenetic distribution of this somatosensory property among bathyergids is inconsistent and the *Fukomys* species outside Southern Africa have not been examined [reviewed in Lewin et al. (2021)]. I have observed that captive Ansell's mole-rats learned to accept and eat chili peppers (Habanero) without problems. I suggest that offering mole-rats chili peppers as food complement and observing their behavioral reaction would represent a simple, cheap, non-invasive screening test for the presence or absence of capsaicin insensitivity among species instead of injecting the substances.

"Pumping"

Bednářová et al. (2013) describes the "chest thumping" in *Fukomys mechowii*. The animals hit rhythmically the floor of the perspex tube in an artificial tunnel system. They produced thus audible vibrations with the highest frequency of the signal at 1.63 kHz and the loudest frequency of the audible part at about 0.45 kHz. The sequences contained from three to four thumps and were separated by breaks of 0.6 s. The mole-rats produced this sound in the closed space of the tube. The sounds were recorded mainly in two situations: during aggressive encounters (males) and feeding (females) – the female entered the tube and started to produce seismic signals after the food was inserted into the terrarium. The function of these signals in giant mole-rats is still unclear. Similar behavior has been, however, reported as so-called "pumping" also in other mole-rat species: in *Cryptomys hottentotus* (Eloff, 1958; Poduschka, 1978) and in *F. damarensis* (Bennett and Jarvis, 1988). The authors discussed pumping in the context of warning and antipredator behavior, although no clear conclusion could be made.

I have observed pumping in captive *F. anselli* and *F. kafuensis*, in both sexes, but relatively rarely and almost exclusively if the animal was in a tube. It did not matter how long the tube was and whether it was open on one or both ends or closed or whether the animal was in the tube alone or not. Only very seldom have I observed this behavior also in the "open field," in an animal put into a bucket. Behavior could be described as pumping, not thumping or drumming, and was not audible. It remains to be clarified whether pumping represents signaling or sensing. In both cases, one can imagine that pumping in a tunnel produces slight air currents, which can be perceived by the family members or will be echoed if the tunnel is plugged in front of the animal. Alternatively, the meaning of pumping is not to exhale (and produce air currents) but to inhale the air (and analyze it). Indeed, this was also the impression of Eloff (1958) who writes: "the abdomen is flattened as if the animal inspires: then the spinal column is curved, bent ventrally and this action is repeated several times." He further suggested that a break in the tunnel might be detected in this way, through the displacement of a volume of air. The fact that cigarette smoke in the room provoked (though not reproducibly) animals to engage in pumping is interesting and could be of possible relevance.

Conditioning

The testing of sensory capabilities by behavioral methods is based on operant conditioning. Positive reinforcement in mole-rats is not an easy task: they do not drink free water, and reinforcement by food has also several constraints (the animals have very limited body fat reserves and when forced to starve, they very quickly lose water). One possibility would be, rather than feed them at a given place, to provide food pieces that they would repeatedly retrieve into their "storage room." I have observed, however, that mole-rats are tireless nest builders and use every opportunity to gather and retrieve nest materials (e.g., strips of tissue paper). This material is, contrary to food pieces, odorless, which is its further advantage. It can be used as a reward at the goal of a maze or in

FIGURE 1 | Stuffed specimens of *Fukomys* mole-rats showing the variability in the size of the headspot (bles) in the collections of the Natural History Museum of Zimbabwe in Bulawayo. **(Left)** Mole-rats (incl. *F. mechowii*) from Northern Zambia, **(Right)** mole-rats (incl. *F. damarensis*) from Southern Africa.

association with sensory signals, which would announce that it is available.

AGING

Mole-rats are famous for their longevity and slow aging (cf. Braude et al., 2021; Buffenstein and Craft, 2021). The studies on this aspect of life history would profit if we could study it in the field, without the necessity of long-term capture-recapture studies. For this purpose, we would need a biological marker of age, which could be easily obtained from animals in the field by biopsies without the necessity to sacrifice them. Only recently, an epigenetic age estimator (clock) was developed for the naked mole-rat, enabling us to assess (from skin biopsies) whether epigenome undergoes age-related changes (Horvath et al., 2022).

The Corti organ of the inner ear represents a perfect aging biomarker, accurately quantifiable – unfortunately, the animals have to be sacrificed and preparation of the organ of Corti by the technique of the so-called surface specimens is not a routine method. The number of hair cells is given at birth and in the course of life, the hair cells only die and leave visible scars behind which can be exactly counted (**Figure 2**). Since mole-rats under natural conditions do not experience any noise overstimulation and are not exposed to ototoxic drugs, all the missing hair cells are expected to be due to physiological aging (cf. also Burda, 1978; Úlehlová et al., 1984).

ECOPHYSIOLOGY

Living under hypoxic and hypercapnic conditions is a frequently mentioned stress that subterranean mammals have to cope

with (cf. Nevo, 1979, 1999; Braude et al., 2021; Park et al., 2021). There are also many studies providing data on the atmosphere and microclimate (temperature and humidity) in the burrows (cf. Burda et al., 2007; Holtze et al., 2018). I would, however, like to point out that all the measurements were done in unobstructed burrows and empty nest chambers, i.e., without burrow inhabitants being present in close vicinity of the measuring probes.

Microclimate and atmosphere in an occupied nest chamber, i.e., with inhabitants being present, is surely a different one. A digging mole-rat having only a little air volume between its
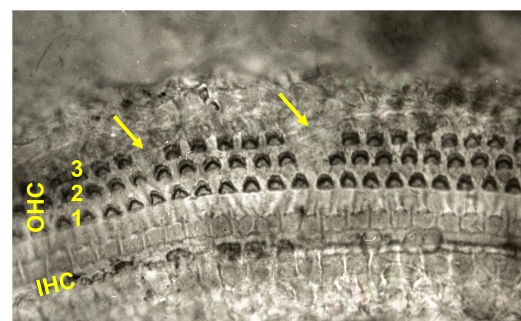
FIGURE 2 | Total surface specimen of the organ of Corti of a shrew (*Sorex araneus*). The geometrical pattern consists of three rows (1–3) of cuticular plates of outer hair cells (OHC) separated by a row of inner pillar cells from a single row of cuticular plates of inner hair cells (IHC, not in focus). Altogether 4 OHC are missing on account of the physiological involution of the auditory neuroepithelium (arrows). Ehrlich's hematoxylin × 375. See Burda (1978) and Úlehlová et al. (1984) for further illustrations and description of the phenomenon.

snout and the end of a tunnel in front of it, is also likely exposed to much higher hypoxic and hypercapnic stress than an animal moving in a free superficial runway tunnel.

SPECIATION, GENETICS

Contact Zones

Contact (hybrid?) zones between *Fukomys* species in Zambia (and elsewhere) require systematic studies. In our laboratory, *F. anselli* (2*n* = 68) and *F. kafuensis* (2*n* = 58) successfully breed producing reproductive offspring (2*n* = 63). The mechanism of chromosome hybridization has not been studied.

Distribution of Giant Mole-Rats: A Case for Cryptozoology?

My former student assistant at the University of Zambia, later Ph.D. student, colleague, and friend, Mathias Kawalika (1962-2006), wrote the following paragraphs in his Ph.D. thesis (Kawalika, 2004):

"*It remains unclear whether there are hybrid zones between neighboring Fukomys species or not, it remains unclear whether the areas of distribution (of diverse Fukomys species) overlap at some places and whether some of the species occur sympatrically. Indeed, even the map of distribution of the giant mole-rat published by Ansell (1978) (Figure 59), i.e., a species of mole-rats which can be distinguished from other species by its size, needs revision. Ansell himself admits that the record from Malawi is questionable. Also, no giant mole-rats were captured during recent rather intensive mole-rat collecting in the Southern, Western, and North-Western Province (i.e., the strip between the Zambezi and the Kafue rivers) (Van Daele et al., 2004).*

However, it should be of interest to note that in Kabwe as well as in Kasama I have been assured by local hunters that there live both, small and giant mole-rats sympatrically. The locals distinguish both species even by name: kakoko and mfuko, respectively. They claimed that even a "super-giant mole-rat" occurs in the area. Similarly, in Chongwe, two types of mole-rats (giant and common) were reported. In Chibale, the sympatric occurrence of C. amatus and C. mechowi could be proved (Scharff, 1998, and own unpublished observations). In Ndola (Chichele) I have captured medium-sized mole-rats which also do not grow in captivity (Burda, personal communication), yet subsequent karyological study revealed a karyotype typical for C. mechowi. It should be noted that in this locality, occurrence of two distinct forms was indicated also by parameters of the burrow system. Thus, I have found at one site (in early June 2000) within an area of 50 m × 25 m, 225 mounds and tunnels having 75–110 mm in diameter, whereas just about 100 m from this sector there were tunnels which ranged from 50 to 75 mm in diameter and there were only about 60 mounds within the same size area. There is no doubt that further field studies are needed to verify or falsify the claims of local hunters and to answer the points raised in this section.

Interviewing of several hunters (in Kasama area) leads to the assumption that there are four types of mole-rats in the area: (a) common (small) mole-rats; (b) giant mole-rats; (c) multi-colored giant mole-rats; (d) white super giant mole-rats. The super giant mole-rats are about the size of small puppies which puts them above 800 g. This was evident from one animal already prepared and cooked in a pot. (Interestingly super-giant mole-rats are recorded also from the Kabwe area.) The hunter (in Bwembya village) revealed that the animal had a white coat which is usually the case for all the super giant animals he has ever caught. The common moles have been described by all hunters as always gray without the white head spot. The giant mole-rats are of two types, the normally colored (as from Ndola) and the multi-colored which usually have a white belly and brown upper colored body. The giant mole-rats (types b, c, and d) can coexist in the same location whereas they cannot and never share their habitat with those of common mole-rats (type a). This was evident also from the size of the burrows inspected. The common mole-rats were more confined to lower altitudes with gray sandy soils whereas the giant were confined to brown soils. The closest distance observed between the habitat of the giant mole-rats and that of the common ones was 1 km and both localities were separated by the escarpment. Local hunters clarified that the super giant mole-rat was less common in comparison to the giant mole-rat. They capture it only occasionally.*"

The indigenous knowledge of local mole-rats should not be underestimated, though it has to be viewed critically in some aspects (Burda and Kawalika, 1993). The reports of Mathias Kawalika surely deserve to be taken seriously and to be checked.

Sex Ratio

The subterranean habitat is considered structurally simple and microclimatically stable (Nevo, 1999), thus subterranean mammals may be suitable models for the study of sex-ratio adjustment. There seems to be inconsistency between laboratory and field data as far as the sex ratio is concerned.

The sex ratio among wild captured young (<1 year) giant mole-rats (*F. mechowii*) was male-biased (1.22) but among adults (>1 year), the proportion of males decreased (0.96) (Kawalika and Burda, 2007). Taking all wild-captured animals together, the overall sex ratio was equal (1.07). Sampling was roughly equally distributed throughout space and time as well as with respect to capture methods so there was probably no bias due to the sampling method. Data on captive families are inconsistent with the field data due to a strong female-biased neonate sex ratio of 0.54 among laboratory-born animals (Scharff et al., 1999).

F. anselli shows a female-biased neonate sex ratio of 0.85 (Begall and Burda, 1998) and even 0.44–0.73 in wild captured animals (Kawalika and Burda, 2007; Sichilima et al., 2011, respectively).

The following hypotheses explain the above findings and should be considered and tested:

(1) The skew toward neonate females is an artifact of captive breeding and does not reflect the natural situation. A reverse (i.e., male-biased) sex ratio should be expected among neonates in the field to explain a higher proportion of males among young wild-captured mole-rats.

(2) With growing older, males disappear from the population. There are indications of a higher dispersal rate of adult males in *F. mechowii* (Kawalika and Burda, 2007). A male-biased higher dispersal rate has been reported

also in *F. damarensis* (Hazell et al., 2000). It should be, however, noted that a male-biased dispersal rate is deduced from the observed higher aboveground activity of males. A hypothesis should be tested that while males tend to disperse aboveground, females disperse rather underground (cf. Šumbera et al., 2012; Torrents-Ticó et al., 2018; Mynhardt et al., 2021). On the other hand, there is no indication of higher intrinsic mortality in captive male mole-rats (Dammann and Burda, 2007). It is noteworthy that female-female inter-familial encounters (at least in captivity) are more aggressive and lethal (Burda, 1989).

(3) There is a sex-linked age-polyethism, expressed in the higher activity and/or trapability of subadult males. With increasing age, males may become more cautious or less active. However, previous observations on captive animals do not suggest any marked difference in this aspect (own observation, Burda, 1990; Zöttl et al., 2016).

Finding answers to the questions of why sex ratios in newborn captivity-bred giant mole-rats deviate so markedly from equality, may prove to be significant for gaining insights into the ecology of mole-rats in particular, and for understanding phenomena affecting sex-ratios in mammals in general.

The following exciting hypothesis could explain the phenomenon and should be tested: In *F. anselli* and *F. mechowii*, two types of females occur: homogametic females: XX and heterogametic females: XY*. Due to the early loss of YY* zygotes, XY* females would produce twice as many phenotypical females as males. XY* females in the S. American field mice (*Akodon*) have been described already 35 years ago. It was shown that in *Akodon*, XY* females persist at high frequencies (up to 30%) and are better breeders than XX (Bianchi, 2002). An unusual sex determination system has been found also in an unrelated subterranean rodent: the mole-lemming (Just et al., 2002). Existence of heterogametic (XY*) females at different frequencies in the populations and our captive breeding stock and higher mortality of younger males (observed also in breeding colonies) could explain all the observed phenomena.

SEXUAL DIMORPHISM AND AGE STRUCTURE

The question of why there is a marked sexual dimorphism (at least between breeding males and their oldest sons on one side and females on the other side) in *Fukomys* mole-rats has no clear answer. The mole-rats are monogamous, and because of isolation, family stability, and incest avoidance (Burda, 1995) there is seemingly no significant competition for mating which would act as a sexual selection pressure. Nevertheless, I have observed (Burda, 1995, 1999, own observations) that incest avoidance between mothers and their sons is not as strong as between siblings and between the father and his daughters. This means that there is a competition between the breeding male and his male offspring and thus also between the brothers. This competition may be the motor driving the body size increase in males. An even more significant index signaling the body strength

may be, however, the breadth of the (upper) incisors. It should be noted that teeth are testosterone target organs used for signaling dominance status in diverse mammals (baboon or musk deer being familiar examples). I have observed that old breeding males which may be no longer the largest ones in the family still had the broadest incisors. Unfortunately, I have never measured this trait systematically to test the hypothesis. The males themselves may assess this strength index during their typical sparring (playful) fights which have a character of mouth (teeth) wrestling: the animals lock their incisors together and sway from side to side. The males do not bite each other but – in case of serious fights between non-familiar males – they try to break the incisors of the rival. Eventually, the defeated male shows an appeasement posture (Burda, 1989). The fight between two females has a different character: a female does not respect the appeasement postures (if any), does not try to fight mouth-to-mouth but bites the retreating animal (Burda, 1989).

Caspar et al. (2021c) have quantified sexual size dimorphism and have measured also the width of the upper incisors in *F. anselli*. While the authors have found differences between both sexes, they found no significant differences between breeders and non-breeders. Their study sample consisted of animals of known age and most specimens originated from different families. This approach is not suited to test my hypothesis that the breeding (dominant) male in a family ("king") can be recognized based on the width of its upper incisors and that the wrestling with locked incisors between males serves to compare the strength and to establish the dominance. The males normally measure their strength in this way with other family members (i.e., within their respective families), not with strangers from different families. Moreover, the study by Caspar et al. (2021c) involved captive animals with non-breeders being on average 58 months old. This situation is most probably not natural. We should expect that in nature, the mean lifespan (coexistence within a family) of a non-breeder is "only" 31 months (Burda et al., 2000). This means that a 58-month-old non-breeder in nature would not be any more competitive with its father but would have been expelled, or "voluntarily" have abandoned its respective family or have died of a different cause before reaching that age.

Based on my observations (Burda, 1989, 1990, 1995, 1999) and the above assumptions we can expect that older male non-breeders (sons) from an established (longer lasting) family will be larger and will have wider upper incisors than the younger males. If they invaded a fledging (smaller) family with a younger "king," they would be able to defeat him and mate with the "queen," which is always ready "to have an affair" (Burda, 1995; Bappert et al., 2012). In a family with an older "king," they will surrender but will be adopted into the family. They might mate also with a non-breeding female as there will be no incest avoidance (Burda, 1989, 1990, 1995). All existing observations about the dispersal and genetic structure of mole-rat families in nature (Patzenhauerová et al., 2013; Torrents-Ticó et al., 2018; Mynhardt et al., 2021) are consistent with conclusions derived from our observations on captive animals (Burda, 1989, 1990, 1995; Bappert et al., 2012).

To avoid any misunderstanding: sporadic copulation in *Fukomys* mole-rats does by far not guarantee fertilization (Burda, 1990, 1995; Willingstorfer et al., 1998). Moreover, the replacement of the "king" by his son can be expected only when the "king" is already old and weak. At that time, however, the mother ("queen") is most probably also old and her reproductive potential is reduced (Willingstorfer et al., 1998). Hence, a family bond between mother and son has only a low chance of arising and thriving, and consequently, a much lower chance of being discovered and identified by researchers. On the other hand, taking over a "kingship" by an unfamiliar male invader may frequently escape our attention. This case may affect smaller fledging families with a younger breeding male invaded by an older, bigger male. If this happens, the family has still only a few members (offspring of the founder) and after some time they disappear or are in a minority and the invader could be considered by researchers to be the original founder and "king" of the family who has been probably cuckolded (offspring of the first king would be considered to be the result of extra-pair copulations of the "queen").

STUDY OF THE STRUCTURE, DYNAMICS, AND RHYTHMICITY OF MOLE-RAT FAMILIES IN THE FIELD

Most of the information on life histories (reproductive biology, aging, etc.) and the activity of mole-rats have been gained in the laboratory. Thus far, information on wild living mole-rats (and subterranean mammals in general) could only be obtained only using invasive and time consuming methods, requiring long-term projects – capture-marking-recapture methods and telemetry. Because of the apparent time- and manpower-demands as well as technical problems, only a few studies have been undertaken, limited to only a few burrow systems in limited areas.

With Mathias Kawalika, we prepared a new original and innovative method, which would be simple, elegant, and non-invasive and would enable us to survey and monitor many families of mole-rats in their respective nest chambers *in situ* in different localities over a time span determined only by possibilities of the researchers to revisit the study places. Unfortunately, in 2006 Mathias passed away due to malaria and the project was never realized.

The principle of the projected method is the following (**Figure 3**):

(a) The nest chamber will be localized (see below).

(b) A chimney will be drilled by a soil driller from above into the ceiling of the nest chamber. The mole-rats are not able to plug a hole in the ceiling of the chamber and are not disturbed by cold and red light. We assumed that the mole-rats would not permanently abandon a nest chamber in the building in which they invested a lot, i.e., they would return after the disturbance due to drilling has ceased.

(c) An endoscope will be introduced, meaning the animals can be counted and video-monitored. Thanks to their white head spots (blesses) they (at least *F. anselli*) can be recognized individually.

(d) Continuous monitoring of the nest chamber throughout the whole day and in regular time intervals will provide data about the circadian and circannual rhythmicity of individual animals and family dynamics (breeding etc.).

(e) An endoscope will be introduced, and the animals will be counted and video-monitored. In this way, we get information on family size and also the population size in a given area, family (and population) dynamics over a given time period (breeding data, recruitment of new family members, losses), daily and seasonal activity patterns (note that mole-rats in many species can be individually recognized based on the size and form of the head spot).

(f) Temperature, humidity, oxygen, carbon dioxide, and methane concentrations can be measured *in situ* in occupied nest chambers. These data are thus far generally missing.

Nest Chamber Localization

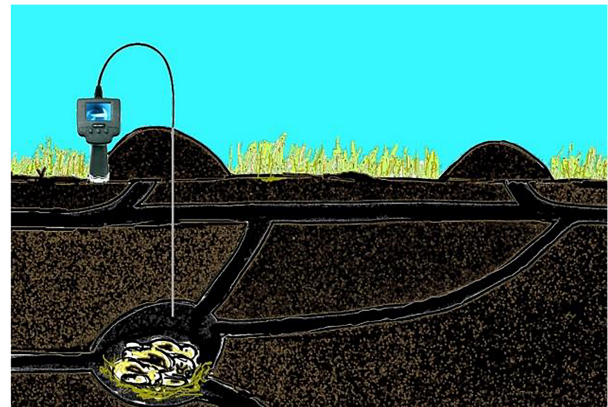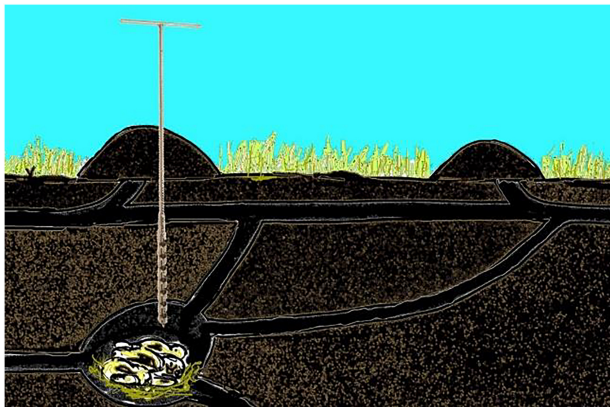There are several possibilities to localize the nest, which may be combined.

FIGURE 3 | Illustration of the proposed method of nest chamber drilling **(Left)** and endoscope monitoring **(Right)**.

(a) An experienced local mole-rat hunter will localize the nest chamber. (Mathias Kawalika succeeded to recruit a very experienced mole-rat hunter, Mr. Kawesa from Ndola, who was able to locate nest chambers of mole-rat families.) Should localization of the nest chamber by a hunter fail for whatever reason in some habitats, we might use further complementary approaches to narrow down the nest location.

(b) The telemetry method (an animal is captured at the periphery of the burrow system and collared in order to show us the way to the nest).

(c) Metal search detector can be used in sandy Kalahari soils and involves inserting bait strips of cotton tissues partly into burrows. Metal rings are sewn into these strips. Mole-rats pull down the strips and transport them as nest materials into the nest chamber. The nest is then located by a metal detector.

(d) Sound localization using sensitive spy microphones (note that mole-rats are very vocal animals) (see chapter vocalization above).

(e) Employment of a ground penetrating radar ("georadar") has become a realistic option in recent years.

SURVIVAL STRATEGIES DURING FLOODS

Mathias Kawalika noticed that in the Kafue Flats and specifically in the Lochinvar National Park, mole-rats were present in areas that have been regularly seasonally inundated by floods. The question arises of how the mole-rats survive flood events. Local people claimed that mole-rats occupy termite mounds during floods and that animals from different colonies gather peacefully in one place. We did not have the opportunity to test these claims.

CONCLUSION

The biology of African mole-rats was virtually unknown 50 years ago (cf. Kingdon, 1974). In the last few decades, the number of published papers on mole-rats has grown enormously. Even if they have become the best known wild (and certainly the best known African) rodents in many aspects of biology, there are still many enigmas and mysteries, which will no doubt keep many other scientists busy for their whole academic careers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no permit is required for this kind of study which is a theoretical one and is based on published peer-reviewed studies.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Self-Domestication Underground? Testing for Social and Morphological Correlates of Animal Personality in Cooperatively-Breeding Ansell's Mole-Rats (*Fukomys anselli*)

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Ansell's mole-rats (*Fukomys anselli*) are sexually dimorphic subterranean rodents that live in families consisting of a single breeding pair and their late-dispersing non-breeding offspring. Most individuals exhibit a conspicuous white head patch, which results from integumental depigmentation. Alongside other morphological, physiological, and social characteristics, skin depigmentation in these social rodents mirrors traits that presumably evolved as byproducts from selection against aggression in domestic animals, making them a potential candidate species for a self-domesticated wild mammal. Here we explored whether the expression of the white head patch, sexual dimorphism, and reproductive division of labor are reflected by different personalities in Ansell's mole-rats. We tested locomotory activity and risk-taking as well as aggression and affiliative behavior in 51 individuals originating from nine captive families in various experimental set-ups. In line with the concept of animal personality, we recovered consistent individual responses over time. While sex had no influence on any tested variable, reproductive status was found to affect risk-taking behavior but not other personality dimensions. Discriminant function analysis revealed that family members clustered more closely together than expected by chance, suggesting that group affiliation rather than sex or social status determines behavioral profiles in this species. Finally, we failed to recover any consistent correlation between head patch expression and behavior, which conflicts with predictions of the self-domestication hypothesis. We argue that many domestication-like traits in Ansell's mole-rat and its congeners evolved in the framework of subterranean adaptation and call for a cautious application of the self-domestication concept to wild mammals.

Keywords: behavioral type, burrowing rodent, neural crest, reproductive skew, sociality

INTRODUCTION

Ansell's mole-rat (*Fukomys anselli*) is a social bathyergid rodent from central Zambia that permanently lives underground in self-excavated tunnel systems (Begall et al., 2021a) extending up to several hundred meters in length (Šklíba et al., 2012). It displays a suit of morphological as well as sensory adaptations to subterranean life, including a cylindrical body shape, reduction of body appendages, and strongly procumbent incisors employed for digging (Begall et al., 2021a). Its eyes

are profoundly reduced in size severely constraining visual perception of shapes, size, and movement (Němec et al., 2004). However, Ansell's mole-rats are able to distinguish between darkness and light (Wegner et al., 2006), and their eyes appear to be involved in sensing the Earth's magnetic field (Marhold et al., 1997; Caspar et al., 2020). Hearing is confined to low frequencies (Müller and Burda, 1989; Gerhardt et al., 2017), and their somatosensory system is supposedly well developed, as is the case in other subterranean mammals (Park et al., 2007). Odors (such as anogenital scent or urine) help mole-rats to distinguish familiar from unfamiliar conspecifics and to identify family members (Heth et al., 2004; Leedale et al., 2021).

Ansell's mole-rats, like all its congeners in the genus *Fukomys* as well as the distantly related naked mole-rat (*Heterocephalus glaber*), are cooperative breeders that live in groups with high reproductive skew. In Ansell's mole-rats, families typically comprise about ten individuals, but groups may contain up to 25 animals (Begall et al., 2021a). There is only one monogamous reproductive pair in each family (i.e., breeders, at times also called king and queen), and the offspring (non-breeders or helpers) may remain in their natal family for an extended period of time (Patzenhauerová et al., 2013). The non-reproductive mole-rats contribute to the survival of their younger siblings, for instance, by excavating and maintaining tunnels as well as by retrieving pups that stray away from the communal nest (Begall et al., 2021a). In free living *Fukomys* mole-rats, breeders are more likely to range in the vicinity (<10 m) of the nest and they also spend more time within the nest compared to non-breeders that may be found at distances of >90 m away from the nest chamber (Lövy et al., 2013; Šklíba et al., 2016). In line with that, breeders of the congeneric Damaraland mole-rat (*Fukomys damarensis*) have been shown to be less active than non-breeders in the wild (Francioli et al., 2020). However, no significant differences in the locomotor activity between Ansell's mole-rat breeders and non-breeders could be recovered in the laboratory (Schielke et al., 2012). Due to the reproductive division of labor and strong philopatry of offspring, Ansell's mole-rats and their congeners have been considered to be eusocial mammals by some authors (Burda et al., 2000; Burland et al., 2002). There is, however, a debate about the applicability of this term, which was originally introduced to describe the lifestyle of social insects such as ants and termites, to mole-rats of the genus *Fukomys*. It has recently been shown that in wild Damaraland mole-rats, the presence of non-reproductive helpers in a burrow system only weakly increases the fitness of the breeding female (Thorley et al., 2021). Furthermore, *Fukomys* helpers fundamentally differ from workers in many social insects in not showing evidence for task specialization (*F. anselli* – Šklíba et al., 2016; *F. damarensis* – Thorley et al., 2018); a trait that is also lacking in naked mole-rats (Siegmann et al., 2021). Zöttl et al. (2016) showed that the individual cooperative investment of *Fukomys* helpers increases with age and appears to be a consequence of age-related polyethism as is also the case in other cooperatively breeding mammals, such as meerkats (*Suricata suricatta*). There were only small differences in the investment of cooperative behavior between the sexes, and if present it was biased towards females. For instance, females provided significantly more alloparental care (a behavior that was very rarely observed overall) and tended to invest more time into nest building and digging than males (within their first year of life – Zöttl et al., 2016). At least in the Damaraland mole-rat, males tend to disperse earlier and more frequently than the more philopatric females (Zöttl et al., 2016; Torrents-Ticó et al., 2018). Males are also more likely to invade family groups to challenge established breeders (Mynhardt et al., 2021). The greater body mass, relative skull dimensions and broader incisors of males might have evolved in the context of such intrasexual conflicts (Caspar et al., 2021b).

Ansell's mole rats (as well as their congeners) display remarkable longevity, reaching a maximum age of about 22.2 years (Dammann et al., 2011; Begall et al., 2021a). The average (and maximum) lifespan of breeders in captivity is about two times that of non-breeders, resulting in a bimodal aging pattern for the species (Dammann and Burda, 2006). In the congeneric giant mole-rat (*Fukomys mechowii*) it was recently recovered that cortisol levels in hair are significantly higher in adult non-breeders living together with their parents compared to those of breeders (Begall et al., 2021b). This finding may indicate higher stress levels in non-breeders and could explain the earlier onset of senescence.

Among the many unusual traits of the Ansell's mole-rat and other *Fukomys* species are some that mirror characteristics of domesticated mammals. These include markedly low stress hormone levels (Ganem and Bennett, 2004), increased tolerance and lowered aggression toward conspecifics (restricted to group members in captivity, see Begall et al., 2021b; compare Ganem and Bennett, 2004), localized depigmentation of the integument (see below), and brains that are smaller than the average for similar-sized rodents (Kruska and Steffen, 2009). In fact, the combined presence of these traits could be interpreted as evidence for so-called self-domestication in *Fukomys*.

The concept of self-domestication has originally been invoked to explain various derived phenotypic as well as behavioral characters that differentiate modern humans (*Homo sapiens*) from their ancestors and other primates (reviewed by Hare (2017); see also Theofanopoulou et al. (2017)). However, it has also been adopted for non-human primates, such as bonobos (*Pan paniscus* – Hare et al., 2012) and marmosets (*Callithrix jacchus* – Ghazanfar et al., 2020), with calls to extend it further to other mammalian groups. Self-domestication entails the emergence of specific behavioral and morphological traits in natural populations which are otherwise characteristic for those domesticated by humans (Hare, 2017). The main feature of lineages considered to be self-domesticated is increased social tolerance and reduced aggression (Hare, 2017), importantly mediated by low levels of circulating stress hormones (compare Albert et al., 2009; Wilkins et al., 2014). This reduction in stress hormone levels is hypothesized to chiefly relate to hypofunction of the adrenal glands, which develop from neural crest cells in the mammalian embryo. Due to the action of pleiotropic genes regulating the differentiation and migration of the embryonic neural crest cells, selection against aggression would not only affect the adrenal glands but further give rise to alterations in other neural crest-derived structures, causing localized depigmentation of the integument (although this is not

found in humans), as well as a reduction in brain, jaw, and tooth size (Wilkins et al., 2014). The collective of these various altered traits is commonly denoted as the "domestication syndrome" which is supposed to be found in both self-domesticated and human-domesticated lineages (Trut, 1999; Wilkins et al., 2014; but see Lord et al. (2020) for a rebuttal).

Whether the term "self-domestication" is actually fitting for what it aims to describe and how conclusive the available evidence for the hypothesis is, continues to be hotly debated (Sánchez-Villagra and van Schaik, 2019; Losey, 2021). In fact, the only traits constituting the "domestication syndrome" which are universally expressed in domesticated mammal lineages are increased tameness and integumental depigmentation, calling its scope into question (Sánchez-Villagra and van Schaik, 2019).

Among non-synanthrope wild rodents (compare Geiger et al., 2018 for a relevant study on a commensal population of house mice), *Fukomys* is indeed striking in fitting the self-domestication concept in several crucial aspects. Apart from the aforementioned traits, *Fukomys* group members provide allomaternal care for the altricial offspring of the family and communicate with each other through an elaborate repertoire of social vocalizations, which surpasses that of most other rodents in complexity (Bednářová et al., 2013). Although not typical of domesticated lineages, these traits have also been hypothesized to characterize alleged self-domesticated species of primates, including humans and marmosets (Ghazanfar et al., 2020).

The most conspicuous domestication-like trait in Ansell's mole-rats is the white dorsal head spot, which results from local depigmentation of the integument. In most *Fukomys* species, individuals of both sexes display such a white bles ("occipital patch" – De Graaff, 1964; "blaze" – Burda, 1989). In some species, it might taper caudally along the spine (e.g., *F. damarensis*, *Fukomys micklemi*; De Graaff, 1964; pers. obs.) while it is usually constrained to the occipital region of the head in others (e.g., *F. anselli*, *Fukomys foxi* – Williams et al., 1983; pers. obs). Yet in other taxa, it is missing altogether or is only found in few individuals of a given population (*F. mechowii* and *Fukomys vandewoestijneae* – Caspar et al., 2021a; some species/populations of the *F. whytei* species group – Faulkes et al., 2017). In all species that express it, the size and shape of the bles varies considerably (e.g., De Graaff, 1964; Williams et al., 1983). Depigmentation in other parts of the integument might occur (particularly on the medial ventrum and the mandibular area) but is never as consistently present as the head patch (pers. obs.). Apart from efforts to assess its potential value for taxonomy, the *Fukomys* head patch has received little research attention, and both the proximate and ultimate causes for its expression remain enigmatic.

Since partial depigmentation of the integument is often championed as being a hallmark indicator of (self-)domestication (Sánchez-Villagra and van Schaik, 2019), a nuanced examination of its evolutionary ties to social behavior in *Fukomys* appears warranted. To support the notion of self-domestication, a correlation between head patch expression and other traits considered to derive from selection against aggression should be demonstrable at the individual level (Ghazanfar et al., 2020).

In this study, we aim to explore whether individual personalities are present in Ansell's mole-rats and how they are linked to sex, social status, and the expression of the white dorsal head patch. Over the past decades, numerous works have demonstrated different temporally stable personalities or behavioral syndromes in populations of vertebrates as well as invertebrates (Gosling, 2001; Sih et al., 2004; Bell et al., 2009; Kralj-Fišer and Schütt, 2014), and it would come as a surprise if Ansell's mole-rat would not comply to this concept. The current literature on animal personality considers five major temperament trait categories or personality dimensions: aggressiveness, boldness, exploration, activity, and sociability (Sih et al., 2004; Réale et al., 2007; Beckmann and Biro, 2013). Personality studies on rodents so far included species such as domestic guinea-pigs (*Cavia porcellus* – Zipser et al., 2013), bank voles (*Myodes glareolus* – Šěchová et al., 2014), ground squirrels (*Urocitellus beldingi* – Dosmann et al., 2015), and as a representative of the group of subterranean rodents, the Talas tuco-tuco (*Ctenomys talarum* – Fanjul and Zenuto, 2020). Individuals that show consistent behavior over time and across contexts for two or more behavioral traits are considered to have a personality. However, in some studies only one of these criteria was met (and also just for some behaviors that have been studied) but the concept of animal personality was considered to apply to the species anyway (Zipser et al., 2013; Fanjul and Zenuto, 2020).

Based on the notable sexual dimorphism in body size and weaponry (Caspar et al., 2021b), we expected to find personality differences between the sexes in Ansell's mole-rats, particularly in feistiness and aggressiveness. Furthermore, we hypothesized that reproductive and non-reproductive individuals represent different behavioral types concerning boldness and/or activity based on previous observations in the wild as well as captivity (Šklíba et al., 2016; Zöttl et al., 2016; Houslay et al., 2020). Since genetic studies have shown that behavioral types are to some extent heritable (van Oers et al., 2005; van Oers and Mueller, 2010), we also expected that individuals of the same family would cluster closer together than chance would suggest. Finally, the self-domestication hypothesis proclaims that more intense integumental depigmentation is indicative of a stronger disruption of neural crest cell migration, predicting that head patch size in mole-rats would correlate with increased docility and social tolerance (Ghazanfar et al., 2020) – at least if this trait is assumed to represent an evolutionary byproduct of selection against aggression. To our best knowledge, this is the first attempt to test the applicability of the self-domestication concept in a non-domesticated rodent species by studying the potential coupling of individual-level personality and morphology.

MATERIALS AND METHODS

Subjects

A total of 51 Ansell's mole-rats (*F. anselli*; karyotype: $2n = 68$) from nine families have been studied. This species attains sexual maturity at approximately 18 months (Bappert et al., 2012). We therefore classified animals with an age of at least 548 days at the time of the first experimental trial as adults, and

categorized them either as "reproductive" or "non-reproductive" based on social status. Animals that were less than 548 days old at the first trial were classified as "immatures." The mean age ± SD of reproductive animals (6 males and 6 females) was 3,103±1,279 days (range: 1,915–6,135). Non-reproductive adults (15 males and 5 females) were on average 965±328 (595–1,894) days old. Immature juveniles (11 males and 8 females) had a mean age of 349±173 days (37–531).

All mole-rats were born in captivity at the University of Duisburg-Essen and genealogically derive from founder individuals captured in the vicinity of Lusaka, Zambia, which is the type locality of the species (Begall et al., 2021a). The founder individuals (approximately 150) came to Europe in the course of several expeditions undertaken between the mid 1980s and early 2010s. The animals were kept in glass terraria ranging in size (W × L × H) from 45 cm × 70 cm × 40 cm to 60 cm × 140 cm × 40 cm, depending on family size. The terraria were littered with sawdust and enriched with flower pots serving as nest chambers, and plastic or wooden tubes. Hay and paper tissue strips were regularly provided as nesting material. Room temperature was kept constant at 24 ± 1°C with a relative humidity of 40–50%. Light conditions were regulated with an artificial light-dark cycle (12 L: 12 D, lights on at 8:00 a.m.). Given that Ansell's mole-rat families behave highly xenophobic in captivity (Begall et al., 2021a), they are kept in isolation from each other.

Experimental Behavioral Assays and Quantification of Head Patch Size

Different dimensions of animal personality were quantified in five experimental set-ups.

Open-Field Test

We used an open-field assay to assess activity and exploration. Each test lasted 5 min and was video-recorded. The animal was placed in the middle of a uniformly illuminated custom-made open-field set-up (80 cm × 80 cm, 8 × 8 = 64 squares with an edge length of 10 cm each), the floor and walls (height: 29 cm) of which were made of non-transparent PVC. We used ANY-maze software (version 5.3, Stoelting group) to measure the covered distance (and speed) of the individuals exploring the open field. Furthermore, we measured the time the animals spent in the center of the field (inner 4 × 4 = 16 squares).

Bubble Wrap Test

To assess risk-taking behavior, we used a custom-made set-up of two cubic boxes (plexiglass, edge length = 20 cm) that were connected by a 40 cm long tunnel (plexiglass, width: 15 cm, height: 10 cm). The tunnel floor was lined with several layers of commercial bubble wrap, covered in plastic foil (width: 15 cm, length: 20 cm, height: 1 cm). Since the bubble wrap covered the tunnel floor from wall to wall, the animals had to walk over it in order to cross the tunnel. One of the boxes (left/right) served as a starting chamber, the identity of which was pseudorandomized. A metal grid closed the entrance to the tunnel, which was opened approximately 10 s after the animal was put into the starting chamber. We measured the latency from opening the starting

chamber to the animal first touching the bubble wrap with at least one paw; furthermore, we measured the total time to the completion of the task (from opening the starting chamber until the subject stood with all four paws on the foil). If the animal did not complete the task within 5 min, it was returned to its respective home terrarium. The maximum time of 300 s was noted in this case. We conducted a control test (same set-up without bubble wrap) to investigate whether the bubble wrap was indeed treated as a relevant obstacle by the mole-rats.

Aggressive Encounter

For the aggressive encounter test, we used the same set-up as for the bubble wrap test (but omitting the bubble wrap lining) with an unfamiliar same-sex individual of a different family being put into one of the boxes. A metal grid prevented direct contact between both individuals. The focal animal was put into the starting chamber and after approximately 10 s the metal grid was opened, allowing the focal animal to explore the tunnel leading to the box with the unfamiliar conspecific. We measured the time the focal animal spent near the metal grid separating it from its conspecific, the time the subject lingered at the metal grid when the unfamiliar animal was simultaneously present (close contact), and the number of times the focal animal got in contact with the metal grid. During this assay, we regularly observed behaviors unambiguously indicating aggressiveness or fierce arousal like biting into the grid, intensive sniffing, urinating at the grid, and hopping with the hind feet. Each trial lasted 5 min. The close contact time was measured as an absolute value, but it should be replaced by relative values in future studies.

Affiliative Encounter

We used the same set-up and measured the same variables as for the aggressive encounter assay, but this time a familiar individual (same sex as the focus animal) of the same family was put into box 2. Behaviors observed during the affiliative encounter set-up were friendly (accompanied by contact calls) or neutral but never aggressive.

Handling

Finally, we scored the animals' evasiveness and docility during handling on a scale of 0–3. While staying in their home terraria, the mole-rats were gently lifted up manually by the experimenter (LB) grasping the skin near the tail-base. The animals were held for approximately 10 s. A maximum of one point was given for each of the following three behaviors, yielding the highest possible score of 3 if all three were observed: Tries to escape handling by elopement; attempts to bite; emits threat vocalizations.

Behavioral Consistency

Behavioral consistency across different contexts was tested for the aggressive and affiliative encounter situations, because for both contexts the same structural set-up has been used and the same variables were measured.

After each trial all materials were cleaned with a mild detergent and dried with paper towels.

We tested each animal twice in the respective assays to check for response consistency over time. At least 4 weeks had to pass between the first and second trial. For each animal, a maximum of one test per day was performed. Not all mole-rats in our sample could be tested in all scheduled assays/trials because some of them were unexpectedly moved to another animal facility and thus unavailable for retests.

The relative size of the white head patch was quantified from photographs of the individual animals. Mole-rats were photographed in containers outside of their home terraria. Each photo was taken with the camera positioned perpendicular to the respective animal, while its head was held outstretched and in parallel to the ground. We used ImageJ (Schneider et al., 2012) to measure the total area of the patch, subsequently divided by the squared inter-aural distance, to arrive at an individual estimate of patch size relative to head dimensions. For each individual, absolute patch size and interaural distance were quantified based on a single photo. The experimenter conducting the behavioral assays (LB) was blind to the aim of the study concerning patch size-related analyses.

Data Analysis

All statistical analyses were performed in R Studio (R Core Team, 2021). Data and model residuals were tested for normal distribution by means of the Shapiro–Wilk test. In case of a detected normal distribution, parametric tests have been used, otherwise the data were transformed by applying $\log(x + 1)$ or a square root function. For pairwise comparisons [e.g., results of trial 1 versus results of trial 2, total time to cross the tunnel with bubble wrap versus without (control) pairwise t-tests or paired Wilcoxon-tests have been employed].

Based on the five experimental set-ups, a total of 11 behaviors (plus one control test) were recorded across two test series (trial 1/trial 2). For each of the eleven behavioral variables, the mean of the two trials obtained for each individual was calculated and analyzed using principal component analysis (PCA), employing the *pr.comp()*-function of the R package *MASS* (Venables and Ripley, 2002). Since only complete individual data-sets could be used, the sample size fed into the PCA was confined to 44 individuals. Principal components (PCs) with eigenvalues >1.0 were retained for interpretation and a subsequent discriminant function analysis. Following Martin and Réale (2008), variables with factor loadings >0.4 were considered to contribute greatly to the respective component.

We used the individual PC scores generated by the PCA to perform a leave-one-out cross-validated linear discriminant function analysis. Box's M test has been employed to test for homogeneity of covariance matrices and the multivariate Shapiro–Wilk test for checking normality. Here, we tested how well sex and status groups could be differentiated based on the behavioral measurements. Furthermore, we tested whether the animals could be reliably assigned to their respective family by using one-tailed exact binomial tests. Since only families with at least four members were considered for these group assignments, the respective dataset was reduced to 34 animals from five different families.

We calculated additive linear models with the behavioral measurements as dependent variables and sex, status (reproductive, non-reproductive, and immature), patch size and age as explanatory variables [*function lm()* in the R stats package]. The assumptions for linear models have been checked visually, and Gaussian distribution of the residuals was further confirmed by a Shapiro–Wilk test for normality ($p > 0.05$). We also ran an additive linear model with relative patch size as the dependent variable, taking sex, status, and body mass as explanatory variables into account. This way, we tried to identify factors which might bias head patch expression and thus would need to be addressed in analyses concerning this trait. Since the residuals were not normally distributed even after log transformation, we excluded three outliers. In addition to the linear model, we ran a t-test in order to compare relative patch size between males and females.

RESULTS

Behavioral Consistency Over Time, Habituation, and Interindividual Variation

The values of most behavioral variables measured in trial 1 and trial 2 (except CONTROL and AFF_CONTACT) were moderately but significantly correlated (**Table 1**) thus showing temporal consistency. Habituation only appeared to have occured for the two variables measured in the open-field test, but not for any other. The coefficients of variations calculated over the means were rather high for each test indicating high interindividual variance. The latencies for crossing the tunnel in the control test (set-up without bubble wrap) were in both trials significantly lower than in those with bubble wrap (paired Wilcoxon test, trial 1: $V = 889$, $p < 0.00001$; trial 2: $V = 822$, $p < 0.00001$) indicating that the bubble wrap can be considered an obstacle.

Stability of Behavioral Responses Across Contexts

In the social encounter assays, the time individuals spent near the metal grid when an unfamiliar same-sex conspecific was in the adjacent compartment (AGGR_GRID) did not correlate with the time near the grid when a family member was confined (AFF_GRID) ($p = 0.47$, $r = 0.11$). There was, however, a significant moderate correlation ($p = 0.035$, $r = 0.32$) for the contact time (i.e., when the other individual was in close contact to the grid as well) for the two set-ups (AGGR_CONTACT and AFF_CONTACT). Also, the number of contacts measured in the two set-ups (AGGR_#CONTACT and AFF_#CONTACT) were significantly correlated ($p < 0.0001$, $r = 0.6$).

Principal Component Analysis of Animal Personality Variables

We retained the first four principal components of the PCA that explained 76.4% of the total variance (**Table 2**). PC1 explained 26.1% of the total variance and loaded highest with handling score opposed to the number of contacts during social encounter assays (measured during

TABLE 1 | Recorded behaviors for n = 51 individuals in five tests (plus control), consistency over time (correlation between trial 1 versus trial 2, Spearman: rho, Pearson: R), variability (coefficient of variation CV measured over mean values), and habituation tests (paired t-test: t or Wilcoxon test: V).

Test	Recorded behaviors	Consistency over time (R, p or Rho, p)	Variability CV (range)	Habituation (t, p or V, p)
Control	Latency for crossing the tunnel without bubble wrap – CONTROL	Rho = 0.11 p = 0.49	1.89 (1.5–151.5)	V = 289.5 p = 0.3527
Bubble wrap	Latency to touching the foil (sec) – BW_LATENCY	Rho = 0.40 p = 0.008	1.05 (2.5–300)	V = 558.5 p = 0.1829
	Total time until all paws are on foil (sec) – BW_TOTAL	Rho = 0.47 p = 0.0013	0.85 (5.5–300)	V = 566 p = 0.154
Open-field (OF)	Distance covered in the OF (m) – OF_DISTANCE	R = 0.48 p = 0.0017	0.36 (8.3–55.0)	t = (3.2188 p = 0.003
	Time spent in the center of the OF (sec) – OF_CENTER	Rho = 0.13 p = 0.4417	0.63 (1.6–100.9)	V = 582 p = 0.02
Aggressive encounter	Time spent at the grid (sec) – AGGR_GRID	R = 0.51 p = 0.0017	0.33 (15–244)	t = 0.76861 p = 0.4473
	Time spent in contact with an unfamiliar conspecific (sec) – AGGR_CONTACT	R = 0.48 p = 0.0033	0.47 (0–186.8)	t = 1.0192 p = 0.3151
	Number of times the animal was in contact with the grid – AGGR_#CONTACT	R = 0.48 p = 0.003	0.37 (2–22.5)	t = 0.73415 p = 0.4677
Affiliative encounter	Time spent at the grid (sec) – AFF_GRID	R = 0.46 p = 0.003	0.33 (45.91–190)	t = 1.503 p = 0.1411
	Time spent in contact with a familiar conspecific (sec) – AFF_CONTACT	R = 0.18 p = 0.274	0.68 (8–133.54)	t = 1.0702 p = 0.2913
	Number of times the animal was in contact with the grid – AFF_#CONTACT	R = 0.49 p = 0.0015	0.30 (6–21.5)	t = 0.022 p = 0.9826
Handling	Handling score (escape, vocalizing, biting) – HANDLING	Rho = 0.75 p = 0.00001	1.43 (0–2.5)	V = 92 p = 0.4469

both aggressive and affiliative encounters) (**Figure 1A**). This dimension thus encompasses the shy-bold continuum (docility during handling) and exploration (number of visits at the grid). The second principal component explained 21.1% of variance and encompassed variables related to the bubble wrap assay, therefore aligning with neophobia/exploration. PC3 loaded highest with variables measuring aggressiveness, while the time spent near the grid during affiliative encounters were recovered as the one that loaded highest for PC4, which thus corresponded to sociability. PC5 (loadings for respective variables not shown) had an eigenvalue of 0.86 and loaded significantly with the time spent in the center of the OF (loading factor: −0.66) and the distance covered in the OF (loading factor: −0.44). PC5 can therefore be considered to align with the exploration tendency and general activity level of the subjects.

Linear Discriminant Function Analysis: Effects of Sex, Status, and Family Group

The PCA was not able to separate individuals well regarding sex or status based on our behavioral measurements (**Figure 1B**). LDA assigned 6% of the females and 89% of the males correctly which in sum did not deviate from chance levels (p > 0.05). For the three reproductive categories, LDA showed a weak trend for correct assignments [non-reproductive adults: 58.8% correct (prior probability 38.6%, p = 0.074), reproductive adults: 50% correct (prior probability 27.3%, p = 0.079), young: 53.3% (prior probability 34.1%, p = 0.099)]. Assignments of individuals to families with at least four family members (n = 5

families with 4–11 family members) yielded mixed results with proportions of correct assignments ranging between 33.3 and 100%. The mean proportion of 61.8% individuals correctly assigned to their respective family differed significantly from randomness (mean prior probability 22.1%, p < 0.000001). Thus, personalities in Ansell's mole-rats entail a notable family group signal, while effects of sex and reproductive status appear to be negligible overall.

Linear Models: Effects of Sex, Status, and Relative Head Patch Size on Animal Personality

As indicated by the results of the LDA, breeders and non-breeders showed generally similar personalities. However, behavioral responses of reproductive Ansell's mole-rats differed significantly from that of non-reproductive animals (but not from that of immatures) in the bubble wrap test and the handling assay but not in any of the other experiments (**Tables 3**, **4**). Breeders took about twice as much time to touch the bubble wrap with at least one paw compared to adult non-breeders (t = 2.257, p = 0.0298) and also to stand on the bubble wrap with all four paws (t = 2.273, p = 0.0288). During handling reproductive Ansell's mole-rats scored significantly lower compared to adult non-reproductive animals (t = −2.251, p = 0.03). Non-reproductive adults and immature animals differed significantly in the time they spent near the metal grid to which an unfamiliar conspecific was close (t = −3.291, p = 0.0021). According to the linear models, sex had no influence on any of the dependent variables except for

Recorded behavior	PC1	PC2	PC3	PC4
BW_LATENCY	−0.038	**0.577**	−0.252	0.124
BW_TOTAL	−0.038	**0.571**	−0.268	0.169
OF_DISTANCE	−0.393	−0.007	−0.187	−0.201
OF_CENTER	0.239	0.245	0.120	0.324
AGGR_GRID	0.122	−0.071	**−0.596**	−0.329
AGGR_CONTACT	0.363	−0.047	**−0.403**	**−0.415**
AGGR_#CONTACT	**−0.474**	−0.175	−0.231	−0.156
AFF_GRID	−0.043	−0.315	−0.324	**0.588**
AFF_CONTACT	0.208	−0.375	−0.335	0.332
AFF_#CONTACT	**−0.444**	−0.028	−0.122	0.215
HANDLING	**0.414**	−0.055	0.111	0.055
Eigenvalue	2.868	2.317	1.842	1.381
Cumulative% of explained variance	26.1%	47.1%	63.9%	76.4%

Loadings of the variables are obtained from the 11 × 11 correlation matrix. Values in bold indicate those loadings that contributed most to the respective PC and had absolute values >0.4. The importance of the four PCs is indicated by their eigenvalues and the cumulative proportion of variance explained.

FIGURE 1 | **(A)** Principal component analysis (PCA) plot showing the five variables that contributed most to the PCA. The arrows for both variables measured during the bubble wrap test, BW_LATENCY and BW_TOTAL, were parallel to each other and almost perpendicular to the variables constituting PC1. **(B)** PCA plot showing individuals grouped according to their social status. Enlarged symbols mark group centroids.

handling where we found a trend in the additive model (**Table 4**, $F = 3.914$, $p = 0.055$). Wilcoxon signed rank test recovered that males scored significantly higher than females in the handling test ($W = 303$, $p = 0.042$). There was a trend for age across all three status groups to have an effect on affiliative behavior (AFF_GRID: $t = −1.919$, $p = 0.062$; AFF_CONTACT: $t = −1.719$, $p = 0.093$; AFF_#CONTACT: $t = −1.848$, $p = 0.072$) but not on any of the other dependent variables. In addition, we used PC1 as a dependent variable including all behavioral measures. The linear model revealed no significant overall effect of sex, status or age ($F_{5,38} = 2.274$, $p = 0.067$), but immature individuals had significantly lower PC1 values compared to non-reproductive adults ($t = −2.038$, $p = 0.049$) while there were no significant

differences for the other status group comparisons. None of the interactions of sex and status yielded significant results for the behavioral measures or PC1.

Figure 2 gives an overall impression of the relative patch size distribution in our sample itemized for females and males. The respective linear additive model showed no significant effect of sex, status, or body mass on relative head patch size ($F_{4,38} = 1.663$, $p = 0.179$). However, the factor sex was close to the level of significance ($t = −2.01$; $p = 0.0516$). Therefore, we additionally conducted a t-test on all subjects for which the relative patch size was available, but again the factor sex was not significant ($t = 1.41$, p-value = 0.175). The white patch does not change its shape during development, and Ansell's mole-rats reach their

TABLE 3 | Mean values ± SD of 11 behaviors recorded in five tests for the three status groups (non-reproductive adults, reproductive adults, and young).

Test	Non-reproductive	Reproductive	Immature	Statistics
Recorded behavior				
Bubble wrap				
Latency (sec)	66.9 ± 78.8[a]	141.7 ± 114.4[a]	77.2 ± 84.1	$F_{5,38} = 1.8$, $p = 0.14$
Total time (sec)	93.1 ± 86.73[a]	170.8 ± 99.7[a]	101.2 ± 99.6	$F_{5,38} = 2.2$, $p = 0.08$
Open-field				
Distance (m)	28.0 ± 8.8	35.6 ± 11.4	30.2 ± 12.3	$F_{5,38} = 1.8$, $p = 0.14$
Center (sec)	24.0 ± 9.6	29.8 ± 14.2	32.8 ± 25.8	$F_{5,38} = 0.6$, $p = 0.71$
Aggressive encounter				
Time at grid (sec)	156.9 ± 46.0	167.0 ± 36.8	146.6 ± 61.6	$F_{5,40} = 1.4$, $p = 0.25$
Contact time (sec)	102.1 ± 42.5[a]	81.2 ± 25.8	60.9 ± 30.4[a]	$F_{5,40} = 2.6$, $p = 0.04$
No. of contacts	9.9 ± 3.6	13.1 ± 3.6	12.4 ± 5.0	$F_{5,40} = 1.4$, $p = 0.26$
Affiliative encounter				
Time at grid (sec)	107.3 ± 36.0	87.0 ± 24.3	128.6 ± 33.3	$F_{5,40} = 3.2$, $p = 0.01$
Contact time (sec)	38.0 ± 31.0	24.5 ± 13.5	38.9 ± 19.9	$F_{5,40} = 1.7$, $p = 0.16$
No. of contacts	11.1 ± 3.9	12.5 ± 4.0	12.3 ± 3.0	$F_{5,40} = 1.3$, $p = 0.30$
Handling	1.02 ± 0.96[a]	0.13 ± 0.17[a]	0.36 ± 0.60	$F_{5,38} = 3.0$, $p = 0.02$

Statistical results refer to the additive linear model using sex and status as fixed factors and patch size and age as covariates. [a] indicates significant differences between the subgroups.

adult body mass at an age of approximately 1 year (Begall and Burda, 1998). In a second analysis, we excluded juveniles younger than 4 months, but included juveniles older than 8 months as most of them were already fully grown. Here, the picture was the same (sex was at the border of significance in the linear additive model, $p = 0.064$; all other factors had no effect).

We were unable to find evidence for consistent links between relative head patch size and any of the quantified behavioral responses or PC1. Animals with larger relative patch size covered more distance in the open field ($t = 2.039$, $p = 0.049$) and tended to spend less time at the metal grid during aggressive encounters ($t = -2.02$, $p = 0.05$). However, further analysis revealed that for OF_DISTANCE patch size was only decisive in the group of reproductive adults [(patch size: reproductive adults): $t = 2.432$, $p = 0.02$] while for AGGR_GRID the group of immature animals skewed the outcome [(patch size: immatures): $t = -2.238$, $p = 0.031$]. For other variables, no significant effects of patch size were found.

DISCUSSION

Behavioral Stability

Most of the recorded behaviors (83.3%, 10 out of 12) in this study showed temporal consistency over a period of 4 weeks. Only the control trials (crossing the tunnel without obstacle) and the times an animal spent in close contact to the familiar conspecific in the affiliative behavior assay were not correlated for trial 1 and trial 2. In comparison, in Talas tuco-tucos, only about half of the recorded behaviors (55.5%) where temporally stable (Fanjul and Zenuto, 2020), and emotional behavior (e.g., OF test and dark-light test) in domestic guinea pigs was not consistent over time at all (Zipser et al., 2013). We expect that the time span of 4 weeks between experimental trials was sufficiently long for our subjects to forget about their former test experience.

For instance, Burda (1995) demonstrated that non-reproductive Ansell's mole-rats "forgot" their siblings and started courtship behavior and incestuous mating after a separation of 3 weeks – behaviors otherwise not observed due to a strict incest-avoidance based on individual recognition. We found a significant decrease of measured values, and thus an indication for habituation, only in the two parameters recorded in the open field test (distance traveled in the OF and time spent in the center). It is possible that Ansell's mole-rats can retain memories related to spatial orientation for a longer period of time. In line with that notion, it was shown that Natal mole-rats [*Cryptomys* (*hottentotus*) *natalensis*] were capable of remembering a relatively simple maze for at least 30 days (but not for 60 days – Du Toit et al., 2012).

We used all three recorded behaviors from the aggressive and affiliative encounter set-ups for testing context stability of responses. Two of these variables showed significant weak to moderate positive correlations. In other personality studies on rodents, context stability also appeared to be rather weak or absent, and it has been argued that it should not be a mandatory aspect to test since it is a rather volatile criterion for behavioral type assessment (Zipser et al., 2013). Overall, however, our data clearly suggest that Ansell's mole-rats exhibit individually stable behavioral responses and thus personalities.

Personality Dimensions, Sex and Status Dependent Patterns

Unexpectedly, sex did not have an effect on any of the recorded behaviors except for handling, as was shown by the linear models and further supported by the linear discriminant analysis. However, not too much importance should be attached to the higher aggressiveness during handling in males, because handling scores were generally low. Since males are significantly larger than females and also have more formidable weaponry (i.e., hypertrophied jaws and incisors) that presumably evolved in the

TABLE 4 | Results of the additive linear models for each behavioral measurement and principal component 1.

Dependent variable	
Explaining factor	**Statistics**
BW_LATENCY	
Patch size	$F = 0.505, p = 0.482$
Sex	$F = 0.141, p = 0.710$
Status	$F = 3.827, p = 0.031$
Age	$F = 0.679, p = 0.415$
BW_TOTAL	
Patch size	$F = 0.429, p = 0.516$
Sex	$F = 0.049, p = 0.826$
Status	$F = 4.953, p = 0.012$
Age	$F = 0.492, p = 0.488$
OF_DISTANCE	
Patch size	$F = 6.009, p = 0.019$
Sex	$F = 0.042, p = 0.839$
Status	$F = 1.485, p = 0.239$
Age	$F = 0.011, p = 0.916$
OF_CENTER	
Patch size	$F = 0.368, p = 0.548$
Sex	$F = 0.010, p = 0.921$
Status	$F = 1.242, p = 0.300$
Age	$F = 0.028, p = 0.869$
AGGR_GRID	
Patch size	$F = 2.912, p = 0.096$
Sex	$F = 1.872, p = 0.179$
Status	$F = 0.809, p = 0.453$
Age	$F = 0.602, p = 0.442$
AGGR_CONTACT	
Patch size	$F = 0.934, p = 0.340$
Sex	$F = 0.047, p = 0.830$
Status	$F = 5.826, p = 0.006$
Age	$F = 0.196, p = 0.660$
AGGR_#CONTACTS	
Patch size	$F = 0.526, p = 0.473$
Sex	$F = 2.870, p = 0.098$
Status	$F = 1.726, p = 0.191$
Age	$F = 0.017, p = 0.898$
AFF_GRID	
Patch size	$F = 1.611, p = 0.212$
Sex	$F = 0.002, p = 0.964$
Status	$F = 5.555, p = 0.007$
Age	$F = 3.681, p = 0.062$
AFF_CONTACT	
Patch size	$F = 0.612, p = 0.439$
Sex	$F = 0.526, p = 0.473$
Status	$F = 2.212, p = 0.123$
Age	$F = 2.954, p = 0.094$
AFF_#CONTACTS	
Patch size	$F = 0.470, p = 0.497$
Sex	$F = 1.470, p = 0.233$
Status	$F = 0.472, p = 0.627$
Age	$F = 3.416, p = 0.072$

(Continued)

TABLE 4 | (Continued)

Dependent variable	
Explaining factor	**Statistics**
HANDLING	
Patch size	$F = 1.251, p = 0.270$
Sex	$F = 3.914, p = 0.055$
Status	$F = 4.615, p = 0.016$
Age	$F = 0.439, p = 0.512$
Principal component 1	
Patch size	$F = 3.230, p = 0.080$
Sex	$F = 1.337, p = 0.255$
Status	$F = 3.354, p = 0.046$
Age	$F = 0.097, p = 0.758$

Significant results are marked in bold. The residuals of the models have been checked for normal distribution using Shapiro–Wilk test.

FIGURE 2 | Frequencies of individual relative patch sizes (x-axis) in Ansell's mole-rats. The patch size distribution in females is more variable than the one in males. Drawings of the heads by Kai R. Caspar.
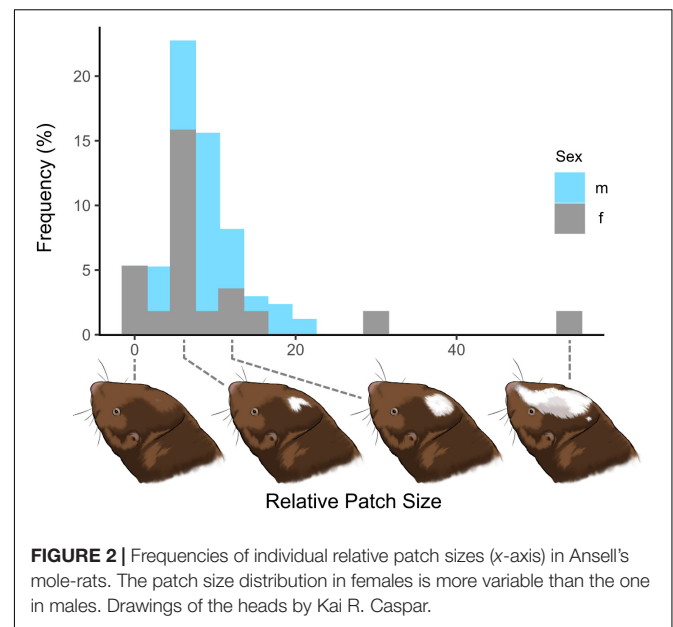
context of intrasexual competition (Caspar et al., 2021b), we expected that they would spend more time at the grid in the aggressive encounter set-up. On the contrary, males appear not to be generally more aggressive than females, even in situations in which they face unfamiliar conspecifics of the same sex. In line with that, a recent study on closely related giant mole-rats (*F. mechowii*) found that cortisol levels and thus likely reactive aggressiveness are not elevated in males compared to females in captivity (Begall et al., 2021b). In contrast to that, males of the highland tuco-tuco (*Ctenomys opimus*), a facultatively social South American subterranean rodent which also exhibits strong sexual dimorphism, show higher concentrations of fecal glucocorticoids than females in the wild (O'Brien et al., 2022). Future research might explore male-male tolerance in Ansell's mole-rats and their congeners in greater depth and should explore how it aligns with the expression of morphological traits which might aid in intrasexual combat.

The significant difference in the contact time during aggressive encounters found for the three social status groups was skewed by immature animals which spent significantly less time at the grid compared to non-reproductive breeders. This finding was not unexpected because young animals should avoid conflicts due to their greater physical vulnerability. Interestingly, the average time spent at the grid was higher during the aggressive encounter assay compared to the affiliative encounter assay in all three social status groups. Since the animal confined in the box was not in distress, it might not have been appealing for the focal subject to spend more time examining a familiar conspecific – a short olfactory inspection may have sufficed to recognize it as a family member (Heth et al., 2004). It should be noted that additional behaviors recorded during the aggressive and affiliative encounters clearly corresponded to the respective situation (e.g., biting into the grid, intensive sniffing, and vocalizations) but were not quantified here. Nevertheless, such social displays should be considered and analyzed in future analogous studies.

The LDA revealed a trend for correct assignments based on reproductive status. According to the linear models, breeders needed approximately twice as much time in the bubble wrap test as adult non-breeders and therefore appeared to be less explorative and more neophobic. This finding is reflected by the behavior of free-ranging breeders that spend most of the time in the nest area, avoid the inspection of opened tunnels and contribute little to the expansion of the burrow system (Šklíba et al., 2016). It also matches the fact that breeders are usually among the last group members (together with their young) to be captured in the wild (Jacobs et al., 1991; Moolman et al., 1998). Furthermore, breeders act less bold during handling where they scored even lower than immature Ansell's mole-rats. Otherwise, breeders and non-breeders showed a very similar performance across tasks, which is in line with the assumption that status-dependent behavioral differentiation in these rodents is overall weak (Zöttl et al., 2016) and thus not analogous to patterns found in eusocial invertebrates to which they have been compared to in the past (Burland et al., 2002).

Rather than sex or social status, the LDA recovered family group affiliation as the best available correlate of animal personality in Ansell's mole-rats. Similar patterns have been found in unpublished previous studies (bachelor theses) on the same species (Karnik, 2014) and also in the congeneric Micklem's mole-rat and giant mole-rat (*F. micklemi* – Czajkowski and Jakobi, 2015; *F. mechowii* – Padberg, 2016). These findings are consistent with the assumption that personality is to some extent inheritable (van Oers et al., 2005; van Oers and Mueller, 2010) and molded by social experience and other environmental conditions. Future work should focus on more detailed comparisons of family-level behavioral types in social mole-rats, particularly in the wild (see Bengston and Dornhaus, 2014, for analogous studies on ant colonies).

Finally, it is noteworthy (compare e.g., Fanjul and Zenuto, 2020) that recorded behaviors relating to social encounters contributed to a higher degree to individual differentiation in Ansell's mole-rat personality than those concerned with activity (**Figure 1A**). Our results might be explained by the fact that while these animals are highly sociable, their activity levels are generally low (in captivity, up to 90% of the time is spent resting, Dammann and Burda, 2006; Schielke et al., 2012).

Are Mole-Rats of the Genus *Fukomys* Self-Domesticated?

We were unable to find consistent correlations between the expression of the white head patch and personality dimensions in Ansell's mole-rats. For the two variables which showed a marginal significant association, closer inspection revealed individual status groups to skew population-level results (immatures in case of the time an animal spent near the grid in the aggressive encounter assay; breeders regarding the distance covered in the OF). In light of these findings, we currently see no indication of relative head patch size being notably linked to personality, particularly aggressiveness and affiliative tendencies in this species. Given that the self-domestication hypothesis assumes lowered aggression and increased pigmentation defects to be correlated due to their shared dependence on neural crest-derived cell-lines, our results do not conform to it. Available data on potential links between the two traits are currently scarce and inconclusive. Ghazanfar et al. (2020) recently reported a positive correlation between vocal responsiveness, a prosocial trait, and white head patch size in a very small sample of common marmosets. In contrast to that, a domestication study on rats found that although depigmentation evolved in a lab lineage selected for tameness, the quantitative trait locus for white spotting showed no genomic overlap with regions affecting tame behavior (Albert et al., 2009). Furthermore, the presence compared to the absence of spotting was not associated with increased tameness at the individual level (Albert et al., 2009). Apart from that, we are not aware of studies experimentally testing links between personality traits and the expression of depigmentation in species of domesticated or allegedly self-domesticated animals (different from coloration patterns in general, see e.g., Brunberg et al., 2013). More such research will be needed to conclude on how tightly these two factors covariate and whether their potential association differs among mammalian taxa.

Our findings on head patch expression and personality in Ansell's mole-rats, do not lend support to the idea that *Fukomys* might be a self-domesticated wild rodent. At the same time, we need to acknowledge that our results represent only a small initial contribution to this issue and cannot rule out a role of self-domestication in *Fukomys* evolution. An important theoretical restriction of our approach and similar research on other species (compare Ghazanfar et al., 2020) is its insensitivity to ceiling effects. When alleles underlying self-domestication become fixed in the population, it has to be assumed that the residual variation in traits determined by these alleles will become uninformative to identify their dependence on selection against aggression. Yet it is unclear, how to determine whether such a ceiling has been reached

in a wild species. Despite these limitations, there appears to be little indication overall that *Fukomys* is self-domesticated. In fact, we would argue that although *Fukomys* species do superficially mirror domesticated mammals in several traits summarized under the "domestication syndrome", they acquired them over the course of adaptation to subterranean life rather than as evolutionary byproducts for selection against aggression. Thus, *Fukomys* might act as an admonishing example for incautiously assigning self-domestication to lineages of wild mammals. Below, we discuss prevalent ideas and current evidence regarding the evolutionary background of relevant traits occurring in the genus.

The highly variable head patch in *Fukomys* is one of its most striking domestication-like traits but comparisons with other subterranean rodents suggest that natural selection on pigmentation can explain its presence more parsimoniously than the self-domestication concept would do. Variably expressed head spots and other forms of integumental depigmentation are commonly observed in a number of unrelated lineages of subterranean rodents that comprise diverse social systems. They are found in varying degrees of expression in pocket gophers (*Geomys* – McCarley, 1951), zokors (*Myospalax* – Bazhenov and Pavlenko, 2020), blind mole-rats (*Nannospalax* and *Spalax* – e.g., Festetics, 1965), root rats and relatives (*Cannomys* and *Tachyoryctes* – see e.g., Eisenberg and Maliniak, 1973), and in several lineages of bathyergids other than *Fukomys* (*Bathyergus*, *Georychus*, *Heliophobius*, rarely in *Cryptomys* – Bennett and Faulkes, 2000; pers. obs.). Interestingly, frequent light pelage spotting is also well documented in moles (Kamm et al., 2008). Thus, there appears to be a link to subterranean life but evolutionary drivers of depigmentation in *Fukomys* and other underground-living mammals are not trivial to identify.

Lovegrove et al. (1993) suggested that African mole-rats use the white head patch as a "photon window" in addition to their small eyes to aid the pineal gland in responding to changes in light levels, thus allowing the entrainment of circadian rhythms. To the present day, this idea has remained untested. However, since a bles is not expressed in a number of bathyergids and various other microphthalmic mammal lineages and because depigmentation in subterranean taxa is often not restricted to the forehead, this explanation appears to be unconvincing.

Given the cryptic life of burrowing mammals, a lack of predation pressure could be hypothesized to cause these aberrant pigmentation patterns. Yet, the observation that many subterranean rodents show remarkable pelage color variation that corresponds well to the soils they dwell in Nevo (1979) and evidence that they can fall prey to diverse avian as well as mammalian carnivores, at times at surprisingly high rates (De Graaff, 1964; Németh et al., 2016), argues against negligible predation pressure. In fact, a potential adaptive function of the head spot in some subterranean groups might be aposematism. Contrasting coloration, though particularly in medium-sized mammals, has been prominently hypothesized to aid in signaling feistiness to predators (Caro, 2009), which at least appears well justified for tooth-diggers such as bathyergids and spalacids that are equipped with powerful jaws. However, the extreme individual variability of the head spots in *Fukomys* and other

taxa argues against an aposematic function, since anti-predatory signals are expected to be selected for stability and unambiguity. Hence, although we suggest that the evolution of the *Fukomys* head patch relates to its ecology based on correlational evidence, we are currently unable to convincingly identify selection pressures (or a lack thereof) underlying its expression. This is possible, however, for other domestication-like traits of the genus.

The extremely low stress hormone levels in *Fukomys* and other bathergids, one order of magnitude below the ones of epigenetic rodents (Ganem and Bennett, 2004), are at least partially linked to their markedly low basal metabolic rate (Haase et al., 2016). Given that glucocorticoids regulate the mobilization of blood sugar, low levels of adrenal stress hormone release contribute to saving energy during resting between metabolically demanding phases of digging activity (Moshkin et al., 2007). Low metabolic rates and decreased levels of circulating stress hormones have evolved convergently in different lineages of subterranean rodents, irrespective of their social systems (e.g., Ganem and Nevo, 1996; Moshkin et al., 2007). Thus, low stress hormone levels in *Fukomys* constitute an adaptation to life underground that already evolved in the bathyergid stem-lineage (compare Ganem and Bennett, 2004) rather than from more recent selection against aggression. Nevertheless, although acts of severe conspecific aggression might still occur (though mostly reported from captive settings and so far, only inferred for natural populations – Caspar et al., 2021b) the low stress hormone levels in these animals likely provide a proximate explanation and possibly an exaptation for their remarkably harmonic intragroup lives (Ganem and Nevo, 1996; Ganem and Bennett, 2004; Begall et al., 2021b). Interestingly, social bathyergids (*Cryptoms*/*Fukomys*) behave agonistically when encountering a foreign conspecific, but do not show evidence for stress arousal, which contrasts with the responses of Cape mole-rats (*Georychus capensis*), a solitary species (Ganem and Bennett, 2004). Although the adrenal glands in Ansell's mole-rats only secrete low hormone concentrations, they are surprisingly large, with an adult mean length of 3.5 mm (Kowalski, 1996). Hence, the average adrenal gland size in Ansell's mole-rats exceeds that of full-grown laboratory rats (2.9 mm long – Siasios et al., 2021). This is the opposite of what would be expected if domestication-like evolutionary trajectories are assumed for *Fukomys* (Albert et al., 2009) and also appears to be add odds with the low stress hormone levels described for the genus. Adrenal morphology and physiology in African mole-rats might become an interesting subject for future research.

The subterranean lifestyle of *Fukomys* could also underlie their relatively small brains (Kruska and Steffen, 2009), because the disproportionate caloric demand of the central nervous system needs to be curbed to sustain energy-intensive digging activity (Kverková et al., 2018). It should be noted, however, that *Fukomys* brains are not smaller relative to body size or contain less neurons than the brains of solitary African mole-rats and that the bathyergid allometric brain-body ratio does not significantly differ from other rodents (Kverková et al., 2018).

Finally, sophisticated vocal behaviors have been discussed as indicators for self-domestication (Ghazanfar et al., 2020), although they are in fact not apparent in most domestic mammal lineages. Indeed, the vocal communication in *Fukomys*

and other social bathyergids is more elaborate than in other rodents (Bednářová et al., 2013), but this pattern follows a general trend linking increased sociality to greater vocal repertoires in the rodent order (Lima et al., 2018). Vocal communication in African mole-rats is a topic of ongoing research, with particular attention being paid to the naked mole-rat, in which a complex interplay between social variables and vocalizations has been described (Barker et al., 2021). However, since little comparative data on other bathyergids as well as cooperatively-breeding rodents in general is available, it is hard to assess how unusual these behaviors actually are.

CONCLUSION

Selected behaviors of Ansell's mole-rats demonstrated a high degree of intraindividual temporal stability, strongly indicating that this subterranean rodent species displays different personalities. In contrast to expectation, sex had no effect on the recorded behaviors, but reproductive status was decisive for some dimensions of personality, with breeders being less explorative than non-reproductive adults. Family affiliation was more strongly correlated with an animal's personality than sex or reproductive status were.

We found no consistent link between the expression of the white head patch and personality dimensions in Ansell's mole-rat and thus no indication that this morphological trait evolved in the framework of self-domestication. More data on this issue would nevertheless be crucial to confidently rule out this option. Although a number of traits in *Fukomys* appear to fit the "(self-)domestication syndrome," closer inspection suggests them being consequences of adaptation to life underground or extensions of patterns observable among African mole-rats or social rodents in general. Thus, *Fukomys* might serve as a reminder to be cautious, when trying to explain characteristics of wild mammals by means of self-domestication. This is especially true for cases in which selective comparisons of traits between disparate groups are attempted which might miss the ecological and phylogenetic context in which the respective species evolved.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because animal housing (approved by permit no. 32-2-1180-71/328 Veterinary Office of the City of Essen) as well as all experiments complied with the animal testing regulations of the country where they were performed and were approved by the animal welfare officer. No ethical permissions were necessary. All behavioral tests conformed to the relevant ethical standards and did not harm the animals.

AUTHOR CONTRIBUTIONS

SB and KRC conceived the study and its methodology. LB performed the behavioral tests. KRC photographed the heads, measured the relevant parameters, and prepared the figures. SB calculated the relative head patch size. SB and LB analyzed the data. All authors interpreted the findings. SB and KRC wrote the first draft, which was reviewed by LB. All authors contributed to the article and approved the submitted version.

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Energetics and Water Flux in the Subterranean Rodent Family Bathyergidae

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The doubly labeled water (DLW) technique and indirect calorimetry enable measurement of an animal's daily energy expenditure (DEE, kJ/day), resting metabolic rate (RMR, kJ/d), sustained metabolic scope (SusMS), body fat content (BF, %) as well as water turnover (WTO, ml/day), and water economy index (ml/kJ). Small mammals have been the primary focus of many of the DLW studies to date. From large multi-species analyses of the energetics and water flux of aboveground small mammals, well-defined trends have been observed. These trends mainly refer to an adaptive advantage for lower RMR, DEE, SusMS, WTO and WEI in more ariddwelling animals to increase water and energy savings under low and unpredictable resource availability. The study of the subterranean rodent family Bathyergidae (African mole-rats) has been of particular interest with regards to field metabolic rate and metabolic studies. Although a great deal of research has been conducted on the Bathyergidae, a complete overview and multi-species analysis of the energetics and water flux of this family is lacking. Consequently, we assessed DEE, RMR, SusMS, BF, WTO and WEI across several different species of bathyergids from various climatic regions, and compared these to the established patterns of energetics and water flux for aboveground rodents. There was notable variation across the Bathyergidae inhabiting areas with different aridities, often contrary to the variations observed in above-ground species. These include increased DEE and WEI in arid-dwelling bathyergid species. While the climate was not a clear factor when predicting the SusMS of a bathyergid species, rather the degree of group living was a strong driver of SusMS, with solitary species possessing the highest SusMS compared to the socially living species. We conclude that the constraints of the underground lifestyle and the consequent spectrum of social behaviors possessed by the family Bathyergidae are most likely to be more crucial to their energetics and water flux than their habitat; however other important unstudied factors may still be at play. More so, this study provides evidence that often unreported parameters, measured through use of the DLW technique (such as BF and WEI) can enable species to be identified that might be at particular risk to climate change.

Keywords: daily energy expenditure (DEE), resting metabolic rate (RMR), sustained metabolic scope (SusMS), water turnover (WTO), water economy index (WEI), body fat (BF), African mole-rats, aridity

INTRODUCTION

The doubly labeled water (DLW) technique has been a powerful tool for measuring free-living energy expenditure in animals since its development in 1955 (Lifson et al., 1955; Lifson and McClintock, 1966; Speakman, 1997a). This technique has enabled great strides in understanding how biotic and abiotic pressures shape the daily energy expenditure (DEE) of animals whilst undertaking natural behaviors in the field. However, many other important biological parameters can be calculated using DLW methodology, which involves isotope dilution and elimination. Such aspects include water turnover (WTO), which is the rate that water leaves the body of an animal ('efflux'), water economic index (WEI), which is the ratio of WTO to DEE (Nagy, 2004), and body fat content (BF) which can be calculated by the body water dilution space (e.g., Scantlebury et al., 2006a). Furthermore, when DEE is combined with the measurements of resting metabolic rate (RMR), through the use of indirect calorimetry, one can determine measures of sustained metabolic scope (SusMS, DEE/RMR), which is an independent index of how hard an animal is working (Drent and Daan, 1980; Peterson et al., 1990; Hammond and Diamond, 1997). Determinations of both WEI and SusMS are useful as they enable comparisons between different species to be made (Peterson et al., 1990; Hammond and Diamond, 1997; Nagy, 2004). However, in most cases, researchers only report some of the biological parameters that can be calculated through the use of DLW and RMR measurements, which can restrict our understanding of the selection pressures that shape an animal's energy balance (DEE, RMR, and SusMS), health (BF) as well as water balance (WTO and WEI).

In particular, small mammals, such as rodents, have been the primary focus of much of the DLW research due to their ease of capture in the wild and requirements for a lower (and less expensive) DLW dose (Speakman and Król, 2005). For several reasons, the rodent family Bathyergidae (African mole-rats) has been a group of animals of particular interest for researchers. Apart from being an exclusively subterranean rodent family, they also exhibit varying degrees of sociality ranging from solitary to social to even eusocial (Jarvis and Bennett, 1993; Faulkes et al., 1997; Bennett and Faulkes, 2000; Burland et al., 2002). Social and eusocial bathyergids exhibit cooperative breeding and a reproductive division of labor in which a single breeding female and one to three of the largest males breed within the colony (Bennett and Faulkes, 2000). The remaining colony members are reproductively suppressed, and a marked division of labor, with breeders spending approximately half as much time foraging as their non-breeding counterparts, is apparent (Scantlebury et al., 2006c; Lacey and Sherman, 2009; Oosthuizen and Bennett, 2015; Francioli et al., 2020; Houslay et al., 2020). Furthermore, they persist over a wide range of climates, elevations and substrates from both mesic and arid environments, with behavioral and physiological adaptations being suggested to correspond to the macroclimate they experience (Bennett et al., 1988; Faulkes et al., 2004; McGowan et al., 2020; Wallace et al., 2021). For example, species of African mole-rats that inhabit soft sandy substrates with a mesic climate are often solitary, possess a larger body masses and use both their teeth and forelimbs, "scratch digging",

to dig, such as those species belonging to the genus of *Bathyergus* (Bennett and Faulkes, 2000). In contrast, species that inhabit arid areas with harder substrates are often smaller, social, and utilize "chisel-tooth" digging to extend burrows (Bennett and Faulkes, 2000). The family also hosts a range of thermoregulatory abilities, from endothermic homeothermy (Šumbera, 2019) to heterothermy (Bennett et al., 1993; Boyles et al., 2012; Oosthuizen et al., 2021) and poikilothermy (Buffenstein and Yahav, 1991; Cheng et al., 2021) which has been observed to affect their behavior (Hart et al., 2021; Finn et al., 2022).

The subterranean niche provides a relatively thermostable environment (Bennett et al., 1988, 1993; Šumbera et al., 2004; Holtze et al., 2018; Šumbera, 2019) as well as a shelter from aboveground predators and temperature extremes (Bennett and Faulkes, 2000; Finn et al., 2020). However, burrow systems have hypoxic and hypercapnic atmospheres with poor ventilation, high humidity, and limited access to light (Kennerly, 1964; Darden, 1972; Roper et al., 2001; Ivy et al., 2020; Logan et al., 2020). In addition, excavation of tunnel systems is energetically more expensive than aboveground exploration (Vleck, 1979), with energy requirements of digging directly related to soil moisture and hardness (Lovegrove, 1989; Thomas et al., 2009, 2016). The added energy expenditure necessary for digging, in conjunction with the fact that African mole-rats obtain all their nutrient and water requirements from the storage organs of underground geophytes (Bennett et al., 1994; Bennett and Jarvis, 1995; Spinks et al., 1999; Malherbe et al., 2003), has been suggested to exacerbate the selection pressures placed upon African mole-rats to enable them to locate adequate resources for survival and reproduction (Bennett and Faulkes, 2000). The energetic cost of digging under the unique microclimate within the tunnels they inhabit has been hypothesized to lead to lower RMRs and body temperatures displayed by African mole-rats in comparison to aboveground rodent species (McNab, 1966; Shkolnik and Schmidt-Nielsen, 1976; Bennett et al., 1992, Bennett et al., 1993, 1994; McGowan et al., 2020) and a social and eusocial lifestyle in species inhabiting very arid environments [the 'Arid-Food-Distribution-Hypothesis' Jarvis et al. (1994); Lacey and Sherman (1997)].

Studies measuring the DEE and RMR in various species of African mole-rat (**Table 1**) have revealed insights into the biotic and abiotic pressures that influence the energy fluxes of these species. For example, Scantlebury et al. (2006c) revealed distinct physiological castes related to body fat and dispersal likelihood. Both Scantlebury et al. (2006c) and Zelová et al. (2011) showed that seasonal fluctuation in soil moisture due to the seasonal rainfall affects DEE and SusMS in a eusocial and solitary mole-rat species, respectively. To-date, only one study has directly compared the ecological relevance of energetic differences between two species of African mole-rat (Scantlebury et al., 2006b). This study concluded that the energy costs of sexual dimorphism are likely to be due to the maintenance of a larger body size in males, rather than sex-differences in behavior *per se*. This study also compared an exclusive chisel tooth digger with a forelimb (scratch digger) that also uses tooth digging and found that the latter species possessed a higher SusMS (i.e., it worked 'harder') than the

exclusive chisel tooth digger. However, as in other studies that measured DEE and RMR, not all possible biological parameters were investigated.

Although a great deal of research has been conducted on the Bathyergidae family, a complete overview and multi-species analysis of the energetics and water flux of the family is still absent. Consequently, we assessed a combination of energetic parameters, namely DEE, RMR, SusMS, BF, WTO, and WEI, across several different species of bathyergid mole-rats (**Tables 1**, **2**). We also include previously unpublished energetics data on the largest African mole-rat species, *Bathyergus suillus*, and data from two sympatric terrestrial aboveground rodent species for comparison (**Table 2**).

This overview aimed to highlight various features that drive the existing variation in energetics and water flux within the family Bathyergidae. As DEE, RMR, and SusMS of certain species have been described previously; we focus on inter-specific comparisons and the previously understudied biological parameters of BF, WTO, and WEI. Furthermore, this current study also attempts to compare the well-known patterns of energetics and water flux for aboveground rodents and other small mammals (Scantlebury et al. (2002, 2008); Scantlebury and Haim, 2012) against those found in the African mole-rats. Extensive multi-species analyses of both the energetics and water flux are present for aboveground small mammals (see Nagy et al. (1999) for an overview of the energetics of terrestrial mammals and Nagy (2004) for an overview of the water flux of terrestrial mammals). At both the inter- and intra-specific levels, aboveground mammals inhabiting drier (more arid) environments display consistent differences in their energetics and water flux compared to their counterparts in mesic or wetter (less arid) habitats (Nagy et al., 1999; Nagy, 2004). These differences include lower RMR, DEE and WEI in mammals inhabiting arider environments than those inhabiting more mesic environments (Speakman, 1997a; Nagy et al., 1999; Nagy, 2004). Even though African mole-rats are exclusively subterranean and thus are buffered from some of the macroclimatic (the region they inhabit broad climate) variations, recent studies have suggested that the macroclimatic differences experienced by different mole-rat species affect their physiology (Šumbera, 2019; McGowan et al., 2020; Wallace et al., 2021). As such it can be expected that similar trends in the energetic parameters, including RMR, DEE and WEI, between the African mole-rat species may be observed as seen between small aboveground mammals. This would include lower RMR, DEE, and WEI in mole-rats inhabiting arid regions compared to those inhabiting mesic environments (Speakman, 1997a; Nagy et al., 1999; Nagy, 2004). Alternatively, the microclimatic conditions found in African mole-rat burrows (the subterranean niche) may result in contrasting energetic patterns; specifically, all African mole-rats may possess similar energetic parameters regardless of their experienced macroclimate on account of their shared buffered lifestyle and behavioral adaptations (for example, social living). Furthermore, African mole-rats would then be expected to possess lower RMR, and similar WTO and WEI than the aboveground rodents in this study due to living in a buffered

microclimate. At the same time, African mole-rats would also be predicted to have higher DEE, SusMS and BF values compared to the aboveground rodents as the result of increased energy expenditure due to digging.

MATERIALS AND METHODS

Novel Data Analysis
Study Site and Animal Capture

A total of 11 Cape dune mole-rats (*B. suillus* – 'BS') were captured with live Hickman traps (Hickman, 1979) on two separate occasions, August 2003 and June 2007, near the village of Darling (33°22′S 15°25′ E) in the Western Cape, South Africa. The Western Cape region has predictable winter (May-September) rainfall and moderate temperatures throughout the year (Rutherford and Westfall, 1994). All individuals were captured prior to their respective breeding season (Hart et al., 2006). Using growth curve data (D. Hart unpublished work), all but one individual was defined as an adult (body mass > 350grams). Individuals were not anaesthetized and were handled in cotton bags to minimize stress. Trapping occurred throughout the day; animals were transported to a field laboratory close by (less than 2 km) upon capture. Animals were housed in plastic containers 30 cm in diameter, 50 cm in height, with sawdust as bedding and sweet potatoes provided *ad libitum* as food overnight. On completing the experiments, the animals were returned to their original capture sites. All experimental procedures and animal husbandry practices were approved by the Animal Ethics Committee, University of Pretoria.

Daily Energy Expenditure and Water Turnover Measurements in *Bathyergus suillus*

The DEEs (kJ d^{-1}) of 6 Cape dune mole-rats (*B. suillus*) were measured using the DLW technique (Lifson and McClintock, 1966; Speakman, 1997b). Upon capture, individuals were taken to a field laboratory, where their RMR values were determined (below). The following morning (c. 09:00), they were weighted ($\pm$ 0.1 g Sartorius balance), and a 0.2 ml blood sample was obtained from the cephalic vein in a foot to estimate the background isotope enrichments of ^{2}H and ^{18}O. Blood samples were immediately heat-sealed into 4 × 50 µL glass capillaries and stored at room temperature. Thereafter, a known mass of DLW (100 g 95% APE enriched ^{18}O water (Rotem Industries Ltd., Beer Sheva, Israel) and 50g 99.9% APE enriched ^{2}H water (Isotec, Inc. Miamisburg OH, United States) mixed with 342 g ^{1}H$_2$^{16}O) was administered (IP, 0.3 g/100 g body mass). Syringes were weighed before and after administration ($\pm$ 0.0001 g, Sartorius balance) to calculate the mass of DLW injected. Blood samples were taken after 2 h to estimate the initial isotope enrichments, and subsequently, the animals were released at their original capture sites. Animal recaptures (*via* re-setting traps) were attempted between 2 and 5 days post-injection of DLW. Traps were set at approximately 06:30 h on the intended day of recapture in an attempt to capture them before 09.00 hrs so that measurements of isotope

TABLE 1 | Overview of previously published and original data that utilized the DEE methodology on various African mole-rat species.

Species	"Caste" or sex	Season	Body mass (g)	Mass-corrected RMR (kJ g⁻¹ day⁻¹)	Mass-corrected DEE (kJ g⁻¹ day⁻¹)	SusMS	BF	WEI (ml.kJ⁻¹)	Publication	Raw data available
Fukomys damarensis	Infrequent workers	Dry	133	0.42	0.54	1.4	11.1	0.35	Scantlebury et al., 2006c, This study	Yes
		Wet	139	0.43	0.82	2.2	10.3			
	Frequent workers	Dry	78	0.57	0.90	1.9	8.9			
		Wet	93	0.47	0.88	1.9	7.7			
	Queen	Dry	124	0.39	0.63	1.6	10.9			
		Wet	110	0.44	0.97	2.5	8.5			
Bathyergus janetta	Male	Wet	423	0.27	0.89	3.2	4.3	0.22	Scantlebury et al., 2006b, This study	Yes
	Female	Wet	291	0.34	0.88	2.6				
Georychus capensis	Male	Wet	137	0.53	1.00	1.9	14.1	0.15	Scantlebury et al., 2006b, This study	Yes
	Female	Wet	127	0.41	1.10	2.8				
Cryptomys hottentotus natalensis	Both sexes	Both season	86	0.82	1.11	1.51	8.5	0.19	Lutermann et al., 2013, This study	Yes
Bathyergus suillus	Both sexes	Both seasons	943	0.23	2.06	2.40	10.3	0.18	This study	Yes
Heliophobius argenteocinereus	Both sexes	Dry	161	0.53	0.76	1.5	-	-	Zelová et al., 2011	No
		Wet	162	0.53	1.00	2.0				

Presented data includes resting metabolic rates (RMR), daily energy expenditures (DEE), sustained metabolic scopes (SusMS), water economy index (WEI) and body fat percentage (BF) in bathyergids with different social organizations or sexes over different seasons. If the raw data was available for this current study was also indicated.

TABLE 2 | Scientific names and life history characteristics of the five mole-rat species and two aboveground foraging rodent species used in this study.

Species	Abbreviation	n	Lifestyle	Sociality	Thermoregulation	Activity pattern	Digging method	Foraging tunnel depths (cm)	Nest depths (cm)	Capture area	GPS
Bathyergus suillus	BS	5	Subterranean [8,9]	Solitary[9]	Homeothermic[10]	Nocturnal[11]	Claw scratcher/chisel tooth digger[9]	30[13]	30[13]	Darling, Western Cape	33°24′ S 18°25′ E
Bathyergus janetta	BJ	13	Subterranean[9]	Solitary[9]	Homeothermic	Crepuscular	Claw scratcher/chisel tooth digger[9]	30[9]	30[9]	Kamieskroon, Northern Cape	30°12′ S 17°56′ E
Georychus capensis	GC	15	Subterranean[8,9]	Solitary[9]	Homeothermic[10]	Nocturnal[11]	Chisel tooth digger[9]	10-15[16]	10-15[16]	Darling, Western Cape	33°24′ S 18°25′ E
Cryptomys hottentotus natalensis	CHN	49	Subterranean[9]	Social[9]	Heterothermic[15]	Nocturnal[14]	Chisel tooth digger[9]	20[12]	30[12]	Glengarry, Kwa-Zulu Natal	29°45′S 29°13′E
Fukomys damarensis	FD	67	Subterranean[8,9]	Social[9]	Homeothermic[12]	Diurnal[11]	Chisel tooth digger[9]	5-25[12]	250[12]	Hotazel, Northern Cape	26° 46′ S 22° 34′ E′
Xerus inauris	XI	18	Fossorial[1]	Social[2]	Homeothermic[1]	Diurnal[3]	Claw scratch digger[3]	0 cm[4]	70 cm[4]	S. A. Lombard Nature Reserve, North-West	27°35′ S 25°23′E
Rhabdomys pumilio	RP	8	Terrestrial[5]	Social[5,6]	Homeothermic[7]	Diurnal[6]	N/A	0 cm[6]	0 cm[6]	Goegap Nature Reserve, Northern Cape	29°41′S 18°01′E

Subterranean rodents construct large and complex burrows and spend most of their lives underground, while fossorial species construct simpler burrows and are more active above ground. [4]Herzig-Straschil (1978), [3]Herzig-Straschil (1979), [7]Haim and Fourie (1980), [1]Haim Skinner and Robinson (1987), [13]Bennett et al. (1988), [2]Waterman (1995), [9]Bennett and Faulkes (2000), [16]Roper et al. (2001), [11]Oosthuizen et al. (2003), [6]Schradin and Pillay (2004), [14]Hart et al. (2004), [5]Scantlebury et al. (2006a), [8]Ivy et al. (2020), [12]McGowan et al. (2020), [10]Okrouhlík et al. (2021), [15]Oosthuizen et al. (2021).

turnover would be for whole 24 h periods (Speakman and Racey, 1988). Six out of the 11 injected individuals were recaptured. These were five adults (two males and three females) and one juvenile. Recaptured animals were weighed, and final blood

samples (0.2 ml) were taken. Capillary tubes that contained blood samples were then vacuum distilled (Nagy, 1983), and water from the resulting distillate was used to produce CO_2 and H_2 (methods in Speakman (1997b) for CO_2 and Speakman

TABLE 3 | Variables from ERA5-Land dataset for each site from 1981 to 2020 used for analysis.

Climate variable	Description	Darling	Kamieskroon	Glengarry	Hotazel	S.A. Lombard Nature Reserve	Goegap
Aridity index (AI)	Degree of dryness of the climate at a given location.	0.51 ± 0.12	0.13 ± 0.05	1.33 ± 0.29	0.17 ± 0.09	0.28 ± 0.11	0.09 ± 0.04
2m temperature (T_{air}) (°C)	Temperature of the air 2m above the soil surface	16.8 ± 3.52	16.8 ± 3.91	9.86 ± 4.24	19.8 ± 5.50	18.5 ± 5.00	16.9 ± 4.39
Soil temperature level 1 (T_{S1}) (°C)	Temperature of the soil in layer 1 (depth 0 – 7cm)	19.0 ± 5.01	20.3 ± 5.53	10.7 ± 5.19	22.7 ± 6.67	20.4 ± 5.66	21.03 ± 6.44
Soil temperature level 2 (T_{S2}) (°C)	Temperature of the soil in layer 2 (7 – 28cm)	18.9 ± 4.65	20.3 ± 5.16	10.7 ± 5.00	22.5 ± 6.11	20.3 ± 5.25	20.9 ± 5.88
Soil temperature level 3 (T_{S3}) (°C)	Temperature of the soil in layer 3 (28 – 100cm)	18.6 ± 3.65	20.2 ± 4.08	10.7 ± 4.08	22.4 ± 4.68	20.3 ± 4.20	20.9 ± 4.59
Total precipitation (tp) (cm)	Accumulated liquid and frozen water that falls to the Earth's surface	113 ± 101	578 ± 569	353 ± 285	108 ± 137	151 ± 153	452 ± 481
Volumetric soil moisture content 1 (M_{s1}) (m^3 m^{-3})	Volumetric soil moisture content of the soil in layer 1 (depth 0 – 7cm)	0.13 ± 0.06	0.17 ± 0.04	0.32 ± 0.07	0.09 ± 0.04	0.17 ± 0.05	0.10 ± 0.03
Volumetric soil moisture content 2 (M_{s2}) (m^3 m^{-3})	Volumetric soil moisture content of the soil in layer 2 (7 – 28cm)	0.12 ± 0.06	0.16 ± 0.04	0.32 ± 0.06	0.09 ± 0.04	0.19 ± 0.04	0.14 ± 0.22
Volumetric soil moisture content 3 (M_{s3}) (m^3 m^{-3})	Volumetric soil moisture content of the soil in layer 3 (28 – 100cm)	0.10 ± 0.04	0.14 ± 0.01	0.33 ± 0.05	0.08 ± 0.03	0.17 ± 0.02	0.14 ± 0.005

et al. (2004) for H_2). The isotope ratios $^{18}O{:}^{16}O$ and $^2H{:}^1H$ were then analyzed using gas source isotope ratio mass spectrometry (Optima, Micromass IRMS and Isochrom mG, Manchester, United Kingdom), before calculation of DEE.

Resting Metabolic Rate (RMR) Measurements in *Bathyergus suillus*

All 11 BS were weighed (± 0.1 g Sartorius balance), and their RMR was measured upon initial capture. An open-circuit respirometry system (Depocas and Hart, 1957; Hill, 1972) was used. A metabolic chamber (6912 cm^3) was immersed in a temperature-controlled water bath (Labotec) and maintained at 28–29°C (LAUDA, Konigshofen, Germany) within the thermoneutral zone of the species. Dried air was pumped into the chamber at a rate of approximately 900 ml min^{-1}. A flow regulator that controlled airflow (F900, Applied Electrochemistry, AEI Technologies, Inc. United States) was placed upstream of the metabolism chamber. Measurements of VO_2 were taken using an oxygen analyzer (S-2A Applied Electrochemistry, AEI Technologies, Inc. United States). The analyzer was calibrated to an upper value (20.95% O_2) prior to the measurement of each animal and a lower value (0% O_2 in N2 gas, AFROX, South Africa) every two weeks. After an initial hour in which animals were observed to settle in the respirometry chamber, measurements were taken every minute for half an hour. The mean of the lowest ten readings of oxygen consumption (mlO_2h^{-1}) was taken when animals were seen to be at rest (Bennett et al., 1992). Measurements were carried out between 11.00 and 17.00. Results were corrected to standard temperature and pressure.

Data Analysis

Sustained metabolic scope (SusMS = DEE/RMR) for each recaptured animal was calculated ($n = 6$) (Drent and Daan, 1980). The ratio of water efflux (ml/day) relative to energy expenditure (kJ/day), the Water Economy Index (WEI, ml/kJ), was calculated (Nagy, 2004). Body fat percentage (BF) was calculated using isotope dilution (Scantlebury et al., 2005). The juvenile BS was excluded from all statistical analyses, but the data are presented for visual comparison with the adult BS. The response variables' normality was determined using Shapiro Wilk (S–W) tests and residual plots. Homogeneity of all dependent variables was confirmed with a Levene's test. All variables were normally distributed. An two-sample t-test was used to compare the body mass of males and females.

Linear regression lines were fitted to determine the relationship between body mass and DEE, RMR and WTO, respectively, in adult BS. Due to the low sample size of male recaptures, only descriptive analyses were possible for DEE, SusMS, WTO, WEI and BF. The effect of sex on RMR of adult BS was investigated using a linear model (LM) with body mass as a covariate. Linear regression lines were fitted to explore the relationships between body mass and RMR in male and female adult BS. IBM SPSS 28 (IBM Corp., Armonk, NY, United States) was used for all statistical analyses and significance was assumed at $p \leq 0.05$. All data are presented as mean ± SE.

Multi-Species Analyses

Only studies that possessed both DEE and RMR were included in the analyses. **Table 1** shows the mole-rat species for which

raw data were available for analysis. Additionally, raw data for outgroup rodent species were available for this study, namely, the Cape ground squirrel (*Xerus inauris*; Scantlebury et al., 2007) and four-striped mouse (*Rhabdomys pumilio*; Scantlebury et al., 2006a). **Table 2** presents the general biological, ecological and behavioral information about each rodent species used in this study. IBM SPSS 28 (IBM Corp., Armonk, NY, United States) was used for all statistical analyses and significance was assumed at $p \leq 0.05$. The normality of the response variables (RMR, DEE, SusMS, WTO, WEI, and BF) was determined using Shapiro Wilk tests (S–W) and residual plots. Homogeneity of all dependent variables was confirmed with a Levene's test. All non-normally distributed dependent variables were log-transformed in an attempt to obtain a normal distribution.

Species Comparison

Linear regressions were fitted to examine the relationships between body mass with RMR, DEE and WTO, respectively, for all species combined and each species separately. We used generalized linear models (GLMs), with Gamma log-link distribution, or LMs, to examine differences in body mass, SusMS, WEI, and BF among species. Subsequently, GLMs or LMs, with body mass as a covariate, were used to examine differences in DEE, RMR, and WTO among species. Subsequent *post-hoc* analyses were conducted using LSD using estimated marginal means (EMMs). All data are presented as adjusted means (EMMs) ± SE.

Environmental Conditions

Climate data were gathered using the methods outlined by Wallace et al. (2021). All environmental conditions are presented in **Table 3**. Climate data for each site were retrieved from ERA5-Land of the European Centre for Medium-Range Weather Forecasts-the latest generation created by the Copernicus Climate Change Service (Muñoz-Sabater et al., 2021). The spatial (resolution is 0.1° by 0.1°). These data were used to calculate an annual aridity index (AI) (eq. (1)). Whereas total precipitation (tp) was directly obtained from ERA5-Land, potential evapotranspiration (PET) was calculated from the well-known Romanenko estimation (eq. (2)) (Romanenko, 1961). For eq. (2), relative humidity (RH) was calculated from ERA5-Land d2m (eq. (3)).

$$AI = \frac{tp}{PET} \qquad (1)$$

$$PET = 0.00006 \times (100 - RH) \times (25 + Tair)^2 \qquad (2)$$

$$RH = 100 \times 10^{7.591386\left(\frac{d2m}{d2m + 240.7263} - \frac{Tair}{Tair + 240.7263}\right)} \qquad (3)$$

Each environmental condition (**Table 3**) was analyzed separately using a GLM, Gamma log-link distribution, and the site as the primary predictor. Subsequent *post-hoc* analyses were conducted using LSD using estimated marginal means (EMMs). All data are presented as mean ± SD.

RESULTS

Novel Species Analysis - *Bathyergus suillus*

Male *B. suillus*, on average, were 165.2% heavier than female BS ($t = -5.406$, $p = 0.001$, **Figure 1A**). When both sexes were combined, there was a strong effect of body mass on RMR; the linear fitted regression

$$RMR\ (kJ.d^{-1}) = 102.0 + 0.1058\ X\ body\ mass\ (g)$$

significantly explained the variance ($F = 16.8$, $p = 0.004$, **Figure 2A**). Likewise, when both sexes were combined, there was a strong effect of body mass on DEE; the linear fitted regression

$$DEE\ (kJ.d^{-1}) = -127.0 + 0.6744\ X\ body\ mass\ (g)$$

significantly explained the variance ($F = 61.6$, $p = 0.004$, **Figure 2C**).

Furthermore, when both sexes were combined, there was a strong effect of body mass on WTO; the linear fitted regression

$$WTO\ (ml.d^{-1}) = -18.0 + 0.114\ X\ body\ mass\ (g)$$

significantly explained the variance ($F = 135.2$, $p = 0.001$, **Figure 2E**).

Neither sex (F = 9.78, p = 0.89, **Figure 1C**) nor body mass (F = 1291.1, p = 0.14) affected RMR. Interestingly, in females there was a strong effect of body mass on RMR;the linear fitted regression

$$RMR\ (kJ.d^{-1}) = 30.6 + 0.2026\ X\ body\ mass\ (g)$$

significantly explained the variance ($F = 18.9$, $p = 0.007$, **Figure 2B**). While, body mass did not have a significant effect on RMR in males ($F = 0.21$, $p = 0.73$, **Figure 2B**) but did possess a negative relationship with RMR (Slope:−0.04, **Figure 2B**).

A similar trend was seen in DEE (**Figure 1B**) as in RMR, with increased DEE in adult male *B. suillus* compared to adult female *B. suillus*. Likewise, adult male *B. suillus* possess a higher SusMS than adult female *B. suillus* (**Figure 1D**). Similar values of WEI occurred for both male and female adult *B. suillus* (**Figure 1F**), while higher WTO was observed in adult male *B. suillus* compared to adult female *B. suillus* (**Figure 1E**). In addition, both male and female adult *B. suillus* DEE and WTO showed a positive relationship with body mass (**Figures 2D,F**). Contrastingly, BF of adult female *B. suillus* was higher than adult male *B. suillus* (**Figure 1G**).

Multi-Species Analysis
Species Comparison
Body Mass

All species possessed significantly different body masses from each other (**Figure 3A** and **Supplementary Tables 1**, **2**).

FIGURE 1 | (A) Body mass (g), **(B)** daily energy expenditure (DEE- kJ.d^{-1}), **(C)** resting metabolic rate (RMR- kJ.d^{-1}), **(D)** sustained metabolic scope (SusMS), **(E)** water turnover (WTO- ml.d^{-1}), **(F)** water economy index (WEI- ml.kJ^{-1}), and **(G)** body fat percent (BF-%) of adult (male and female) and juvenile *Bathyergus suillus* (BS). Data are shown as Adjusted mean (EMMs) ± SE. An asterisk indicates significance ($p \leq 0.05$).

FIGURE 2 | (A) Resting metabolic rate (RMR- kJ.d^{-1}), **(C)** daily energy expenditure (DEE- kJ.d^{-1}) and **(E)** water turn over (WTO- ml.d^{-1}) against body mass (g) of adult *Bathyergus suillus* (BS). **(B)** RMR, **(D)** DEE and **(F)** against male and female adult *Bathyergus suillus* (BS) body mass. Solid circles (•) and solid squares (■) represent female and male adult *Bathyergus suillus* (BS), respectively.

Daily Energy Expenditure

Both species and body mass significantly affected DEE (**Supplementary Table 1**). When all species were combined, there was a strong effect of body mass on DEE; the linear fitted regression

$$DEE \left(kJ.d^{-1} \right) = 34.7 + 0.5897 \, X \, body \, mass \, (g)$$

significantly explained the variance ($F = 1222,0$, $p < 0.0001$, **Figure 4A**).

All species, but *G. capensis*, had DEE values that were significantly affected by body mass (**Supplementary material Table 3** and **Figure 4B**).

Daily energy expenditure differed significantly among species (**Supplementary material**, **Table 1**). *Bathyergus janetta* had significantly higher DEE than all other mole-rat species and *R. pumilio* and *X. inauris* (**Figure 3B** and **Supplementary Table 4**). While *B. suillus* had significantly lower DEE than all mole-rat species, *F. damarensis* had a similar DEE to *B. suillus* (**Figure 3B** and **Supplementary Table 4**). Furthermore, *B. suillus* possessed a similar DEE to *R. pumilio,* but a lower DEE than *X. inauris* (**Figure 3B** and **Supplementary Table 4**). *Georychus capensis* possessed the second-highest DEE for the mole-rats species and a similar DEE to *X. inauris* but a lower DEE than *R. pumilio* (**Figure 3B** and **Supplementary Table 4**). *Cryptomys h. natalensis* and *F. damarensis* possessed similar DEE to one another (**Figure 3B** and **Supplementary Table 4**). Additionally, *C. h. natalensis* and *F. damarensis* possessed similar DEE to *X. inauris* and *R. pumilio* (**Figure 3B** and **Supplementary Table 4**). Lastly,

X. inauris a higher DEE than *R. pumilio* (**Figure 3B** and **Supplementary Table 4**).

Resting Metabolic Rate

Both species and body mass significantly affected RMR (**Supplementary Table 1**). When all species were combined, there was a strong effect of body mass on RMR; the linear fitted regression

$$RMR \left(kJ.d^{-1} \right) = 29.0 + 0.2979 \, X \, body \, mass \, (g)$$

significantly explained the variance ($F = 431.9$, $p < 0.0001$, **Figure 4C**).

Body mass significantly affected all species' RMR (**Supplementary Table 3** and **Figure 4D**).

The RMR differed significantly among species (**Supplementary Table 1**). For example, *Xerus inauris* possessed significantly higher RMR values than all mole-rat species and *R. pumilio* (**Figure 3C** and **Supplementary Table 4**). Contrastingly, *R. pumilio* possessed a similar RMR to all mole-rat species (**Figure 3C** and **Supplementary Table 4**). Similarly, *B. suillus* possessed similar RMR to all other mole-rats species (**Figure 3C** and **Supplementary Table 4**). Likewise, *B. janetta* possessed a similar RMR to all other mole-rats species, but *F. damarensis* possessed a lower RMR to *B. janetta* (**Figure 3C** and **Supplementary Table 4**). *Fukomys damarensis* also possessed a lower RMR than *C. h. natalensis* but a similar RMR to *G. capensis* (**Figure 3C** and **Supplementary Table 4**). *Georychus capensis* possessed a lower RMR than *C. h. natalensis* (**Figure 3C** and **Supplementary Table 4**).
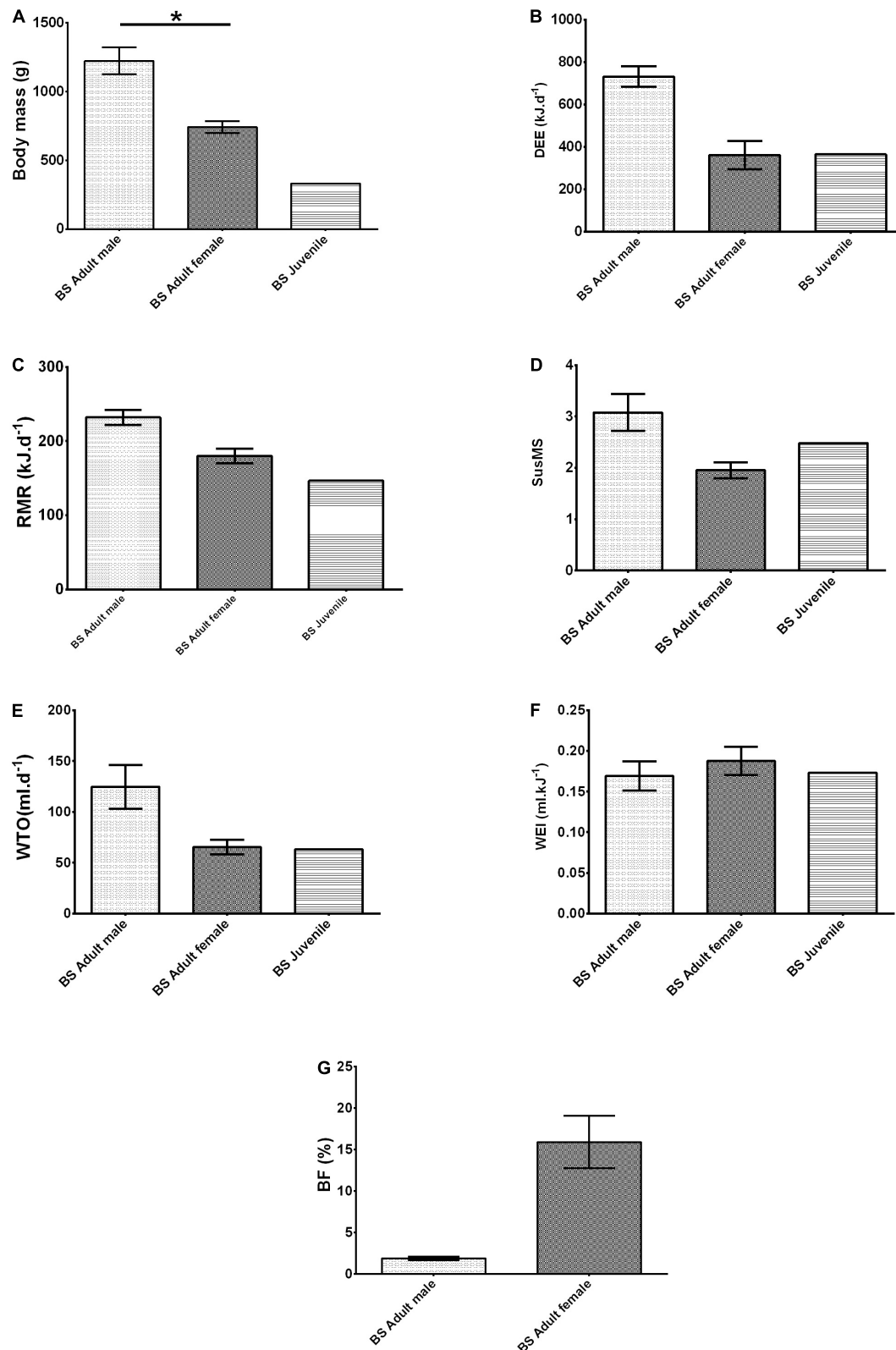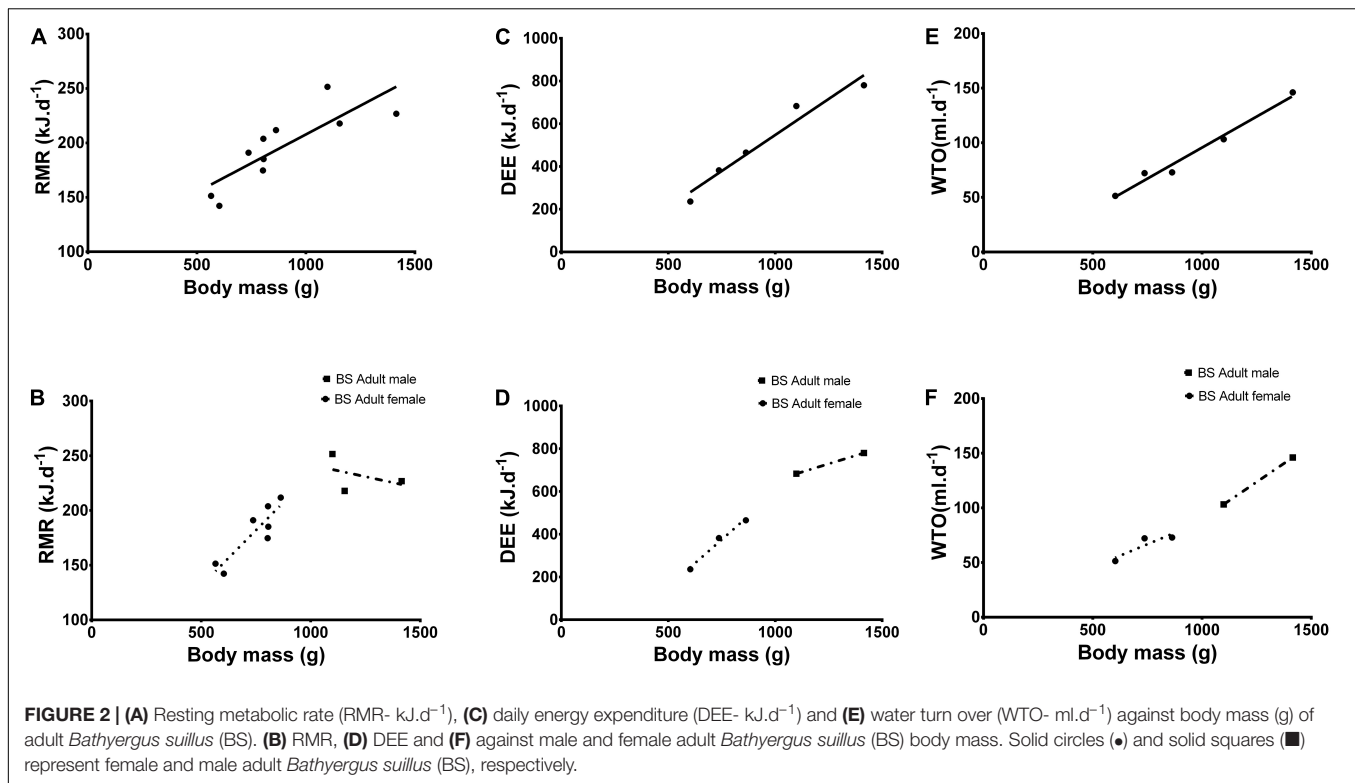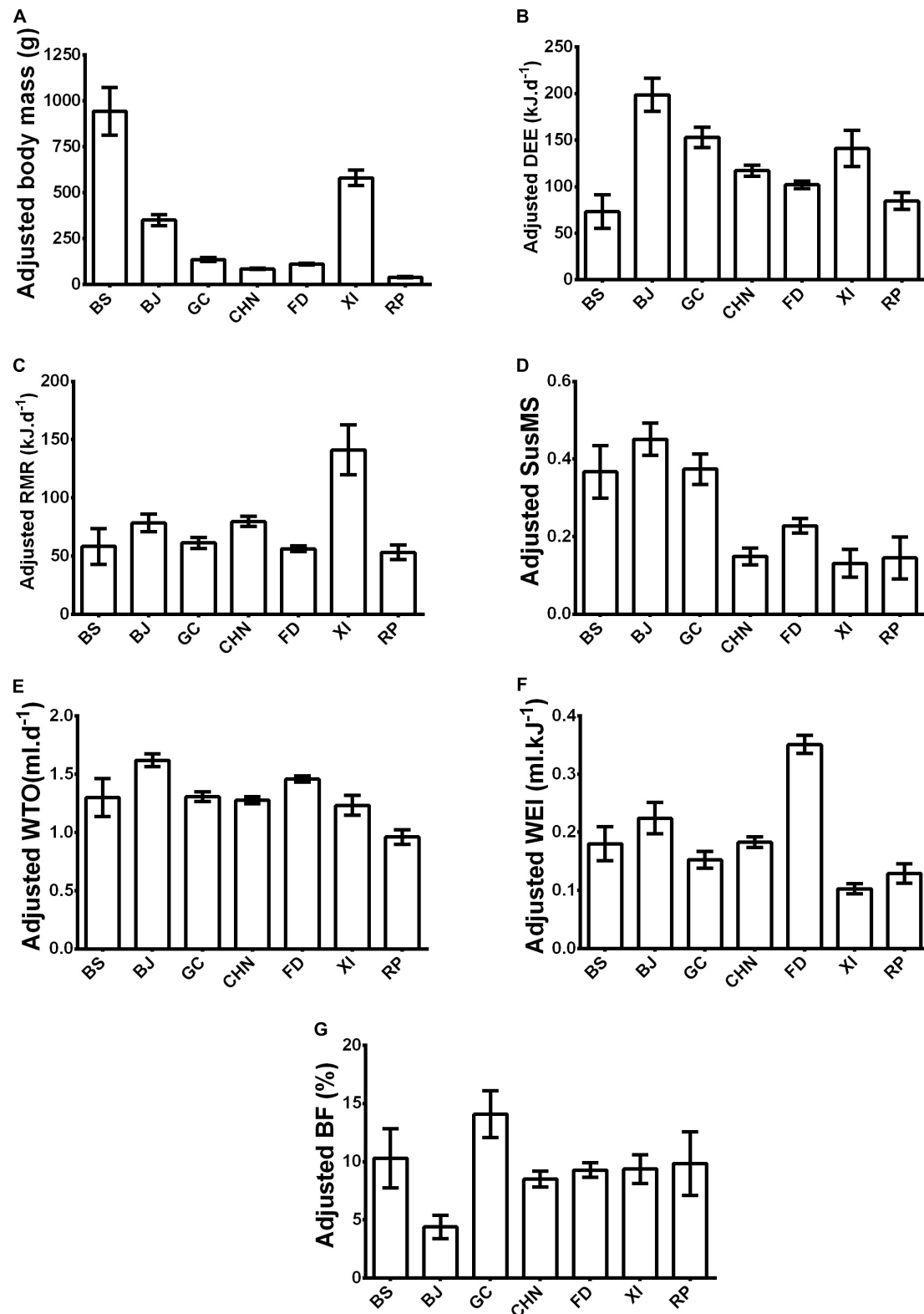
FIGURE 3 | (A) Body mass (g), **(B)** daily energy expenditure (DEE- kJ.d^{-1}), **(C)** resting metabolic rate (RMR- kJ.d^{-1}), **(D)** sustained metabolic scope (SusMS), **(E)** water turnover (WTO- ml.d^{-1}), **(F)** water economy index (WEI- ml.kJ^{-1}), and **(G)** body fat percent (BF- %) of all seven species of rodent featured in this study. BS - *Bathyergus suillus;* BJ: *Bathyergus janetta*; GC - *Georychus capensis*; CHN - *Cryptomys hottentotus natalensis*; FD - *Fukomys damarensis*; XI - *Xerus inauris*; RP - *Rhabdomys pumilio*. Data are shown as adjusted mean(EMMs) ± SE.

FIGURE 4 | Daily energy expenditure (DEE- kJ.d^{-1}) against body mass (g) of **(A)** all seven species of rodent combined and **(B)** all seven species of rodent separated. Resting metabolic rate (RMR- kJ.d^{-1}) against body mass **(C)** all seven species of rodent combined and **(E)** all seven species of rodent separated. Water turnover (WTO- ml.d^{-1}) against body mass **(D)** all seven species of rodent combined and **(F)** all seven species of rodent separated. BS - *Bathyergus suillus;* BJ: *Bathyergus janetta*; GC - *Georychus capensis*; CHN - *Cryptomys hottentotus natalensis*; FD - *Fukomys damarensis*; XI - *Xerus inauris*; RP - *Rhabdomys pumilio*.

Sustained Metabolic Scope

Sustained metabolic scope differed significantly among species (**Supplementary Table 1**). All solitary mole-rat species, namely *B. suillus*, *B. janetta* and *G. capensis*, possessed similar SusMS values (**Figure 3D** and **Supplementary Table 2**). All solitary mole-rat species had SusMS values that were significantly higher than the social mole-rat species, namely *C. h. natalensis* and *F. damarensis*, and the two aboveground rodent species (**Figure 3D** and **Supplementary Table 2**). However, the social mole-rat species were found to be significantly different from

one another, with *F. damarensis* possessing a higher SusMS than *C. h. natalensis* (**Figure 3D** and **Supplementary Table 2**). Likewise, *F. damarensis* possessed significantly higher SusMS than *X. inauris*, but similar SusMS to *R. pumilio* (**Figure 3D** and **Supplementary Table 2**). In contrast, *C. h. natalensis* shared a similar SusMS to both *X. inauris* and *R. pumilio* (**Figure 3D** and **Supplementary Table 2**).

Water Turn Over

Both species and body mass significantly affected WTO (**Supplementary Table 1**). When all species were combined, there was a strong effect of body mass on WTO; the linear fitted regression

$$WTO \ (ml.d^{-1}) \ = \ 14.8 + 0.0.067 \ X \ body \ mass \ (g)$$

significantly explained the variance (F = 124.3, p < 0.0001, **Figure 4E**).

All solitary mole-rat species, namely *B. suillus*, *B. janetta* and *G. capensis*, had WTO values that were not significantly related to body mass (**Supplementary Table** and **Figure 4F**). Similarly, *R. pumilio* WTO was unaffected by body mass (**Supplementary Table 5** and **Figure 4F**). The social mole-rat species, namely *C. h. natalensis* and *F. damarensis,* and *X. inauris* WTO were significantly affected by body mass (**Supplementary Table 5** and **Figure 4F**).

Water turnover differed significantly among species (**Supplementary Table 1**). *Bathyergus janetta* possessed a significantly higher WTO in comparison to all other African mole-rat species and the two aboveground rodent species (**Figure 3E** and **Supplementary Table 6**). *Fukomys damarensis* possessed the second-highest WTO, with *F. damarensis* possessing significantly higher WTO in comparison to the two aboveground rodent species and all other African mole-rats species but *B. suillus* (**Figure 3E** and **Supplementary Table 6**). Additionally, *G. capensis*, *C. h. natalensis* and *B. suillus* possessed similar WTO species (**Figure 3E** and **Supplementary Table 6**). At the same time, *R. pumilio* possessed significantly lower WTO values in comparison to all species except *B. suillus* species (**Figure 3E** and **Supplementary Table 6**). Lastly, *X. inauris* possessed similar WTO values to *C. h. natalensis* and *G. capensis* species (**Figure 3E** and **Supplementary Table 6**).

Water Economy Index

Water economy index differed significantly among species (**Supplementary material Table 1**). *Fukomys damarensis* possessed the highest WEI of all species (**Figure 3F** and **Supplementary Table 6**). Alternatively, *X. inauris* possessed the lowest WEI of all species, besides *R. pumilio*, which possessed a similar WEI (**Figure 3F** and **Supplementary Table 6**). *Bathyergus janetta* possessed higher WEI than *G. capensis* and *R. pumilio*, but similar WEI to *B. suillus* and *C. h. natalensis* (**Figure 3F** and **Supplementary Table 6**). *Georychus capensis*, *C. h. natalensis* and *B. suillus* possessed similar WEI (**Figure 3F** and **Supplementary Table 6**). Both *G. capensis* and *B. suillus* possessed similar WEI to *R. pumilio*; however, *C. h. natalensis* possessed a higher WEI than *R. pumilio* (**Figure 3F** and **Supplementary Table 4**).

Body Fat Percent

The terrestrial rodent species, *R. pumilio*, possessed similar BF to all other species (**Supplementary Tables 1**, **7** and **Figure 4E**). *Bathyergus janetta* possessed a significantly lower BF when compared to the other African mole-rat species and *X. inauris* (**Supplementary Table 7** and **Figure 4E**). The solitary *B. suillus* possessed similar BF to the other African mole-rat species and *X. inauris* (**Supplementary Table 7** and **Figure 4E**). In contrast, *G. capensis* possessed higher BF than the social African mole-rat species and *X. inauris* (**Supplementary Table 7** and **Figure 4E**). *Cryptomys h. natalensis* and *F. damarensis* possessed similar BF to one another and *X. inauris* (**Supplementary Table 7** and **Figure 4E**).

Environmental Conditions

Environmental conditions are presented in **Table 3**. The sites exhibited significant differences in environmental conditions ($\chi^2 \geq 32.2$, $p \leq 0.0001$). The locations of Darling and Glengarry share similar aridity indices ('AIs') (**Table 3** and **Supplementary Table 8**). Individuals from both Darling and Glengarry possessed significantly higher AIs than mole-rats from all other sites (**Table 3** and **Supplementary Table 8**). Individuals from Goegap possessed a similar AI to Hotazel and Kamieskroon, but a lower AI to S.A. Lombard Nature Reserve (**Table 3** and **Supplementary Table 8**). Hotazel and Kamieskroon possessed similar AIs, but both these sites had a lower AI than S. A. Lombard Nature Reserve (**Table 3** and **Supplementary Tables 7**, **8**). Air temperature (T_{air}) was similar between Darling, Goegap and Kamieskroon but different at all other sites (**Table 3** and **Supplementary Table 9**). Soil temperatures (T_{s1}, T_{s2} and T_{s3}) showed a similar pattern with S. A. Lombard Nature Reserve, Goegap and Kamieskroon being similar, while all other sites were significantly different from one another (**Table 3** and **Supplementary Tables 9**, **10**). Darling and Goegap possessed similar total precipitations (tp), while, Goegap and Kamieskroon possessed similar tps (**Table 3** and **Supplementary Table 11**). All other sites possessed tps which were significantly different from one another (**Table 3** and **Supplementary Table 11**). Soil moistures (M_{s1}, M_{s2} and M_{s3}) were significantly different among all sites, respectively, apart from S. A. Lombard Nature Reserve and Kamieskroon, which were found to have similar M_{s1}, and Kamieskroon and Goegap had similar M_{s3} (**Table 3** and **Supplementary Tables 11**, **12**).

DISCUSSION

Daily Energy Expenditure

Speakman (1997a) suggested that field metabolic rate (FMR), the DEE of animals in the wild or "field", is affected by environmental conditions. For example ambient temperature affects DEE with the trend of animals inhabiting a cooler climate (a characteristic of a mesic environment) possessing a higher FMR to animals that persist in hotter climates (a characteristic of an arid environment). A similar pattern found in other desert-dwelling vertebrates (see Nagy et al. (1999) for review). However, a contrasting pattern was observed within the African mole-rat

family, particularly in the solitary genus *Bathyergus*. *Bathyergus janetta,* which inhabits an arid and hot region of South Africa, possessed a greater DEE than its mesic-dwelling cousin, *B. suillus*. Indeed, *B. janetta* possessed the highest DEE compared to other arid dwelling mole-rat species, including *F. damarensis*, and arid dwelling aboveground species, namely *X. inauris* and *R. pumilio*. African mole-rats need to forage for geophytes through the energetically expensive method of digging (Vleck, 1979), which becomes energetically more expensive the drier the soils become (Lovegrove, 1989). It would, therefore, be expected that the arid-dwelling species, such as *B. janetta* and *F. damarensis,* would possess the highest DEE. Our results suggest that *B. janetta* follows this expected trend, but the social *F. damarensis* does not, as *F. damarensis* appears to possess similar DEE to the mesic dwelling solitar*y B. suillus* and the social *C. h. natalensis*. As proposed by the aridity food distribution hypothesis (Jarvis et al., 1994), the evolution of social living may negate or ameliorate the need for increased physiological adaptations to an arid environment (Scantlebury et al., 2006a, 2008). In species that form large groups, such as *F. damarensis*, animals will not have to expend as much energy daily, even in arid environments, as the increased group size allows for the efficient locating and retrieval of food. Group-living allows for such substantial reduction of DEE in *F. damarensis* that their DEE is similar to aboveground rodent species, both *X. inauris* and *R. pumilio*, who do not need to dig to forage and thus should possess a lower DEE. The benefit of group living is absent in the arid dwelling (solitary) *B. janetta*, in an increased DEE to allow them to locate and retrieve the supply of sufficient resources. By comparison, even though *B. suillus* lives solitarily, it exists in much softer soils and with access to a more abundant food resources, resulting in less energy being spent daily foraging (Bennett and Faulkes, 2000).

Both Speakman (1997a) and Nagy et al. (1999) revealed a strong correlations between body mass and DEE. As expected, in this study, body mass played a significant role in predicting an animal's DEE, with larger animals expending more energy per day when performing their natural behaviors compared to smaller animals. Interestingly, *G. capensis* was the only species not to show a significant relationship with body mass. Unlike *G. capensis*, both *B. janetta* (Scantlebury et al., 2006b) and *B. suillus* (this study) exhibit sexual dimorphism in body mass that leads to sexual dimorphism in DEE, lending added support to the body-maintenance hypothesis for sexual dimorphism (see Key and Ross (1999)). The lack of sexual dimorphism in *G. capensis* may be the leading cause for the absence of association between body mass and DEE, as even though equal numbers of males and females were captured, they were all adults (Bennett et al., 1991) and thus, all individuals were of similar mass resulting in no effect of body mass on DEE (Scantlebury et al., 2006b). We would expect that if smaller *G. capensis* were captured and compared with larger individuals, an effect of body mass would be seen.

Resting Metabolic Rate

It has long been thought that with increasing aridity (decreasing A.I.), there is an adaptive advantage for a lower RMR (Haim, 1987; Haim Skinner and Robinson, 1987; Tieleman et al., 2003;

Williams et al., 2004; Van Sant et al., 2012; Klüg-Baerwald and Brigham, 2017), as the adaptive value of reduced metabolic rates in more arid dwelling animals is thought to be related to increased water and energy savings under low and unpredictable resource availability (Lovegrove, 2000; Tieleman et al., 2003). Along this line of thinking, the arid dwelling mole-rat species (*F. damarensis, B. janetta*) are predicted to possess lower RMR than the mesic dwelling species (*G. capensis; B. suillus, C. h. natalensis*). Alternatively, African mole-rat are known to possess low RMRs in response to inhabiting an environment where they are exposed to hypercapnic and hypoxic conditions, whereby their low RMRs allows an equilibrium to be established between the partial pressures of oxygen and carbon dioxide in their blood and tissues, and in their external environment (Ar et al., 1977; Lovegrove, 1986; Ivy et al., 2020; Wallace et al., 2021). If this hypothesis were true, we would expect the aboveground foraging species, namely *X. inauris* and *R. pumilio*, and African mole-rat species with poor hypoxia tolerance, indicating that they are not routinely exposed to hypoxia in their burrow system, namely *B. suillus* (see Logan et al. (2020) for review), to possess the highest RMR. Nevertheless, both these predictions were not supported in this study. In this study, most mole-rat species possessed similar RMRs to one another. While the less hypoxic-tolerant African mole-rat species, *B. suillus*, possessed a similar RMR to both other solitary (*G. capensis*) and social (*F. damarensis*) mole-rat species. Therefore, other explanations, such as physiological and behavioral thermoregulatory strategies (Mota-Rojas et al., 2021), may be driving the pattern of RMR seen within these rodent species (see Speakman (1999) for review).

As with DEE, body mass seems to be the primary factor determining RMR in the current set of rodents, rather than the behavior or the micro- or macro-habitat of the animal. This conclusion is clearly evident by the strong relationship between RMR and body mass overall and within species.

Sustained Metabolic Scope

It has long been hypothesized that vertebrates that inhabit arid regions would work less (SusMS) than those inhabiting mesic regions to reduce energy expenditure and increase water and energy savings under low and unpredictable resource availability (Tieleman et al., 2003). However, as with DEE, the African mole-rat family does not follow this trend. Even though beneficial in some circumstances, an exclusively subterranean lifestyle imposes extremely high energetic demands on the animals inhabiting these ecotopes (Vleck, 1979). These energetic digging demands become greater as soils become drier and thus harder (Lovegrove, 1989). These demands are one of the fundamental suggested pressures that have resulted in some African mole-rat species evolving group-living that leads to them sharing the workload amongst group members; this sharing is especially important in more arid environments as posited by the aridity food distribution hypothesis (Jarvis et al., 1994; Lacey and Sherman, 1997).

The current analysis suggests that the aridity food distribution hypothesis proposes selective pressures for group living in African mole-rats (Jarvis et al., 1994; Lacey and Sherman, 1997). Regardless of environmental conditions and digging method,

all solitary mole-rat species possessed equally high SusMS compared to individuals of a colony-forming subterranean species (*C. h. natalensis*; *F. damarensis*) and fossorial and terrestrial group-living species (*X. inauris*; *R. pumilio*). This finding suggests that if an animal is solely responsible for underground foraging and burrow construction, whether the animal is digging in an arid (*B. janetta*) or mesic (*G. capensis*; *B. suillus*) environment, or with teeth (*G. capensis*), or a combination of teeth and forelimbs (*B. suillus*; *B. janetta*), they will be working harder than animals that live in groups in similar environments and/or use similar digging methods. For example, *F. damarensis* and *B. janetta* inhabit similar climatic conditions (see **Table 3**), but *F. damarensis* lives in large groups and thus possesses a lower SusMS.

Group living, however, does not mitigate all pressures placed upon subterranean animals by their environment. Group living mole-rat species that inhabit arid areas, namely *F. damarensis*, with drier soils still possess a higher SusMS than those group-living species living in mesic conditions, namely *C. h. natalensis*. Lovegrove (1989) observed that animals digging in moist soils use less energy than those digging in drier soils. As expected, the outgroup species, *X. inauris* and *R. pumilio*, that foraged aboveground (**Table 3**) possessed some of the lowest SusMS as predicted by Vleck (1979). Interestingly, *C. h. natalensis*, under the added benefits of group living and inhabiting a mesic environment, possessed equally low SusMS to the above ground-foragers, indicating that group living in a mesic environment could overcome the energetic demands of a subterranean lifestyle. The aridity food distribution hypothesis is often contested as several species of the genus *Fukomys* and *Cryptomys* species are found in mesic conditions (Bennett and Faulkes, 2000). These mesic conditions are predicted to be a driving force in selecting a solitary lifestyle, yet, some species still persist as group-forming mole-rat species. Using the patterns investigated in this current study, we hypothesize that the ancestors of mesic-dwelling mole-rat species may have been arid-dwelling social species that moved into a mesic environment and since there is an energetic benefit to remaining as group-forming species, as indicated by the similar SusMS to above ground-foragers, they retained a social lifestyle.

Water Turnover and Water Economy Index

Surprisingly, all studies using DLW techniques on African mole-rats have not investigated WTO or WEI. As a family, the African mole-rats occupy a range of environmental conditions, from hyper humid to hyper-arid, and as eutherian mammals, one would expect a trend in WEI ratios as seen in Nagy (2004). Nagy (2004) observed that eutherian mammals that occupy arid regions require less water per day (WTO) to achieve a water balance compared to their mesic dwelling relatives. Once Nagy (2004) adjusted for their metabolic rates (i.e., the WEI ratio), which is often lower in arid dwelling mammals (Nagy et al., 1999), arid-dwelling eutherians had significantly lower WEI compared to non-arid dwelling species. One might interpret this finding as arid-dwelling mammals using less water to perform their daily behaviors, which is expected as arid environments

are water restricted. Reduced metabolism and physiological (lower evaporation, increased water retaining abilities of kidneys) or behavioral (less time spent actively above ground, being relatively more nocturnal) adaptations may be responsible for the reduction in WEI ratios of Nagy's (2004) above ground eutherian mammals.

African mole-rats do not share this pattern as the more arid dwelling species possessed higher WEI than the more mesic dwelling species. A more surprising fact is that African mole-rats do not drink freestanding water but obtain all water requirements from underground geophytes, which become more dispersed in arid regions (Bennett et al., 1994; Bennett and Jarvis, 1995; Spinks et al., 1999; Malherbe et al., 2003). African mole-rats need to forage for geophytes through the energetically expensive method of digging (Vleck, 1979), which becomes energetically more expensive the drier the soils become (Lovegrove, 1989). It would, therefore, be expected that arid-dwelling species, such as *F. damarensis*, would require a lower WEI to survive their water restricted environment as water is less available and therefore they would need to conserve water the best they could. Yet, in this study, we see that *F. damarensis* possesses the highest WEI ratio of all species tested. The decreases in water availability and aridity seem to drive the WEI ratio higher in subterranean mole-rat species.

Several reasons for this surprising reversal in pattern could be at play. Physiological differences between arid-dwelling small mammals and mesic-dwelling small mammals that allow arid-dwelling small mammals to survive and thrive in an arid environment, such as lower RMR, body temperature and rates of evaporative water loss (Haim, 1987; Haim Skinner and Robinson, 1987; Tieleman et al., 2003; Williams et al., 2004; Van Sant et al., 2012; Klüg-Baerwald and Brigham, 2017), have been lost in African mole-rats species due to the shared constraints of their microclimate (burrow system) (Wallace et al., 2021). This hypothesis is supported by similar high water turnover rates in the arid dwelling subterranean species of *Spalax ehrenbergi* and *Thomomys bottae* (Gettinger, 1984; Yahav et al., 1989).

Furthermore, as proposed by the aridity food distribution hypothesis, the evolution of social living may negate the need for physiological adaptations to an arid environment (Jarvis et al., 1994). In species that form large groups, such as *F. damarensis*, animals can increase WEI as water is not a limited resource as the increased group size allows for the efficient locating and retrieval of food and water (behavioral osmoregulation). To date, no comprehensive multi-species study has been conducted on African mole-rats osmoregulatory abilities; however, anecdotal evidence has pointed to no differences in urine concentrating abilities of kidneys in different mole-rats species from different environments, again pointing toward behavioral osmoregulation to allow for the survival of social mole-rats in arid environments (M. van Dyk and D.W. Hart unpublish data).

However, this evidence begs the question of how *B. janetta*, possessing the second-highest WEI value, persists in their arid environment, while possessing a similar WEI to their mesic-dwelling cousin *B. suillus*, is not group living. On a recent research field trip (2021), a noticeable reduction in the *B. janetta* population in the areas of succulent Karoo (Kamieskroon, Garies

and Groenrivier) was observed by one of the authors who have been working on *B. janetta* for the last 30 years (N.C. Bennett per comm). It is believed that due to the effects of climate change, which has seen the areas of Kamieskroon and Garies regions in the Northern Cape area receive below-average rainfall and increased temperatures resulting in desertification (increased aridity) (see **Supplementary Figure 1**), in combination with poor water retention abilities of solitary African mole-rats species, this has driven this population decline. Even though lacking behavioral osmoregulatory benefits of social living, the solitary African mole-rats that inhabit mesic areas (*G. capensis*; *B. suillus*) still have fewer water demands placed on them as food is more abundant and equally dispersed in the softer and wetter soils of their environment. At the same time, *C. h. natalensis* possesses similar WEI to the solitary mole-rat species due to living in a hyper-mesic environment (Colantoni et al., 2015).

Percent Body Fat, Body Fat Composition

Body fat composition (BF) of an animal can be linked to its resource availability and amount of work (DEE and SuSMS) it does. Thus, animals which inhabitat arid regions are often expected to possess lower body conditions, including lower BF. Once again, the African mole-rat family does not fit that trend. Only one study (before the current study) on African mole-rats using the DLW technique has reported BF. Scantlebury et al. (2006c), using BF, discovered distinct physiological castes in a social mammal, *F. damarensis*, which solidified the concept that *F. damarensis* could be classed as a eusocial organism with similar traits to bees and termites. Scantlebury et al. (2006c) showed that infrequent workers in an *F. damarensis* colony also possess higher BF than frequent workers (particularly in the dry season), which suggested that infrequent workers constitute a physiologically distinct dispersing caste who contribute less to cooperative behaviors in the colony and focus more on building up their body reserves in preparation for dispersal and reproduction when environmental conditions are suitable.

In the current study, all social mole-rat species (*C. h. natalensis*; *F. damarensis*) and aboveground foragers (*R. pumilio*; *X. inauris*) possessed similar BF to one another. It should be noted that within this current study, all physiological castes, namely breeding individuals, and infrequent and frequent workers, were combined. The Cape mole-rat, *G. capensis* possessed the highest BF of all African mole-rat investigated a surprising find considering *G. capensis* is a solitary species and needs to forage (indicted by their high SusMS and DEE) and thermoregulation alone which should result in a lower BF. Surprisingly, this finding also includes the solitary *B. suillus*. *Georychus capensis* and *B. suillus* occur sympatrically, but *B. suillus* lives in much softer, easier to dig sandy soils while *G. capensis* inhabit a harder clay soil in vlei areas (Bennett and Faulkes, 2000). This should lead to the hypothesis that *B. suillus* should possess a higher BF than *G. capensis*, mainly because this species shows a significantly lower DEE than *G. capensis* as digging in softer sands is less energetically demanding (Lovegrove, 1989; this study). However, a possible lack of sexual dimorphism in *G. capensis* (Scantlebury et al., 2006b) and an apparent sexual dimorphism in *B. suillus* (this study) may cause

the differences in BF between species. *Bathyergus suillus* males show indications of increased DEE and SusMS, suggesting that males are far more active than females resulting in less stored fat in males. This sex bias is possibly due to the larger male *B. suillus* body size, which increases energy expenditure while constructing larger tunnels and nest complexes. Furthermore, males have been documented to search for female tunnel systems and dig into them, searching for a mate (Hart et al., 2006; Thomas et al., 2009); this searching would require more activity and energy expenditure in the males, while the females are less active and store fat in preparation for offspring care.

A finding of great concern is that the solitary arid-dwelling *B. janetta* possessed the lowest BF of all species tested in this study, indicating low resource reserves and possibly poor body condition. The WEI already indicated that the possible decline of the *B. janetta* population observed in 2021 is possibly due to desertification due to climate change and overgrazing by domestic animals on land in their distributional range (**Supplementary Figure 1**). The BF adds to this claim, as in 2006, when these data were collected, to 2021 there has been a drastic increase in aridity (a decrease in AI) (see **Supplementary Figure 1**). If *B. janetta* already showed reduced body fat reserves in 2006, possibly due to being a large, solitary subterranean mole-rat in an arid environment with dispersed food sources. Then as the area becomes more arid and food becomes scarcer due to geophytes drying out as well as the ground becoming harder, *B. janetta* may no longer obtain enough food to maintain a healthy body condition, which could drive local extinction of this species.

CONCLUSION

This current study attempted to provide an overview of the energetic and water flux (including previously unreported energetic parameters) in the African mole-rats (see **Table 1**). Furthermore, we attempted to test well established energetic and water flux trends for aboveground small mammals and rodents against trends observed within the African mole-rat family. We conclude that the unique underground lifestyle and spectrum of social behaviors possessed by the family Bathyergidae are most likely more crucial to their energetics and water flux than their habitat; however other important unstudied factors may still be at play as well. Researchers often under report all the energetic parameters that can be measured using (DLW) technique and indirect calorimetry, namely WTO, WEI, and BF. These measures are vitally important in understanding how well an animal is suited for their current and future predicted environmental conditions they experience. Therefore, we urge researchers who use the DLW technique and open-circuit respirometry to report all parameters; as with this knowledge available in the literature, even greater strides in our understanding of animal energetics and species protection can be made. These parameters can be used as important indicators of which species may be particularly vulnerable to local extinction, like *B. janetta*, due to the changing climate.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because rights to this raw data are held by the institute and further permission is needed to release such data. Requests to access the datasets should be directed to DH, u10022725@tuks.co.za.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee, University of Pretoria.

AUTHOR CONTRIBUTIONS

DH, NB, and DS conceived and designed the manuscript. DH analyzed the data and wrote the initial draft. DS, MO, JW, and CH performed experiments and sample analysis. All authors contributed to the final and revised manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.867350/full#supplementary-material

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Subterranean Life-Style Does Not Limit Long Distance Dispersal in African Mole-Rats

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Dispersal from the natal site to breeding sites is a crucial phase in the life history of animals and can have profound effects on the reproductive ecology and the structure of animal societies. However, few studies have assessed dispersal dynamics in subterranean mammals and it is unknown whether dispersal distances are constrained by living underground. Here we show, in social, subterranean Damaraland mole-rats (*Fukomys damarensis*), that a subterranean lifestyle does not preclude long distance dispersal and that both sexes are capable of successfully dispersing long distances (>4 km). Body condition did not predict dispersal distance, but dispersers from larger groups traveled farther than individuals from smaller groups. Subsequently we show in a phylogenetically controlled comparative analysis of dispersal distances in subterranean and surface-dwelling rodents that living underground does not constrain dispersal distances and that dispersal capacity is mainly a consequence of body size in both lifestyles.

Keywords: natal dispersal, ecological constraints, subterranean, fossorial, rodents, Damaraland mole-rat, *Bathyergidae*, cooperative breeder

INTRODUCTION

The decision by individuals to disperse – leaving their natal territory in search of breeding opportunities (Greenwood, 1980) – and the distance that they travel, can be affected by inbreeding avoidance, by competition for mates, and by the distribution of resources in the environment (Gaines and McClenaghan, 1980; Bowler and Benton, 2009; Clutton-Brock, 2016). During dispersal, individuals must navigate through an unfamiliar environment, and for most terrestrial mammals this presents considerable mortality risks (Lambin et al., 2001; Maag et al., 2022). Such risks have been suggested to be particularly high in subterranean species whose morphological and ecological adaptations to underground living may make them ill-suited to dispersing long distances (Buffenstein, 2000; Stein, 2000). Because of their morphological adaptations and reliance on underground burrows, the natal dispersal distances of subterranean mammals have been suggested to be shorter than those of surface-dwelling species (Nevo, 1979).

Dispersal distances are known to vary widely within and between species, and the distribution of dispersal distances can provide information on a species' ecology (Paradis et al., 2002; Nathan et al., 2012; Whitmee and Orme, 2012). For example, by comparing sex differences in dispersal across

species it becomes apparent that the relative distance that males and females travel when dispersing is not necessarily directly associated with their overall tendency to disperse (Pope, 2000; Hanski and Selonen, 2009; Clutton-Brock, 2016). Despite such insights, collecting data on dispersal dynamics remains challenging. Following dispersing individuals is time and labor intensive and often requires tracking individuals beyond the bounds of a study area (Tomkiewicz et al., 2010; Maag et al., 2022), or relies on the deployment of biologgers which makes tracking possible without the need for direct resighting or recapture (Wilmers et al., 2015; Cozzi et al., 2020). These challenges have rarely been met for subterranean mammals and quantitative data on dispersal are relatively uncommon in these taxa (Busch et al., 2000). Recent molecular studies on subterranean rodents have shown that not all species have low vagility (Bray et al., 2012; Welborn and Light, 2014; Visser et al., 2018), but whether the dispersal distances of subterranean rodents are generally lower than those of surface-dwelling rodents remains unclear.

Damaraland mole-rats (*Fukomys damarensis*) are cooperatively breeding subterranean rodents (Family *Bathyergidae*) with high reproductive skew: a single reproductive pair monopolize reproduction, and their philopatric offspring delay dispersal until ecological constraints on burrowing (e.g., hard soils) are relaxed (Jarvis and Bennett, 1993; Bennett and Faulkes, 2000). Breeders can have long life spans, and non-breeders have a low chance of inheriting the burrow system (<1%; Schmidt et al., 2013; Torrents-Ticó et al., 2018; Thorley et al., 2021). Thus offspring of either sex must disperse from the natal group to acquire reproductive opportunities, even though strong ecological constraints make successful settlement rare. Indeed, very few individuals within a Damaraland mole-rat populations breed at some point in their lives (<8%; Jarvis and Bennett, 1993; Torrents-Ticó et al., 2018; Mynhardt et al., 2021; Thorley et al., 2021). Previous field studies of social mole-rats have shown that when individuals disperse, they frequently do so alone and rarely in coalitions (Jarvis and Bennett, 1993; Torrents-Ticó et al., 2018), and evidence suggests that dispersal occurs both above and below ground (Hazell et al., 2000; Bray et al., 2012; Patzenhauerová et al., 2013; Finn, 2021). By blocking themselves off into a section of the natal burrow, individuals can form a new territory *via* "territory budding" (Jarvis and Bennett, 1993; Lövy et al., 2013; Mynhardt et al., 2021). Genetic and re-trapping data suggest that new groups often form from unrelated pairs (Burland et al., 2002). Taken together, this demographic information identifies features that are likely to have been important selective forces in the formation of social groups in mole-rats. For example, the fact that males sometimes immigrate into established groups, whereas females rarely do (Patzenhauerová et al., 2013; Torrents-Ticó et al., 2018; Thorley et al., 2021), might explain the relatively large male-biased sexual size dimorphism in *Fukomys* mole-rats (Caspar et al., 2021). However, mole-rat populations are known to occupy habitats with varying climate and geology and the structure of groups often varies (Spinks et al., 2000; Finn et al., 2018). In this context, dispersal could play a crucial role in structuring population differences and it

is important to understand sex-specific dispersal patterns across mole-rat populations.

In this study we investigate the dispersal distances of Damaraland mole-rats in the southern Kalahari Desert. Our study serves two aims. First, we use longitudinal data from a capture-mark-recapture study to test whether the distances traveled vary between the sexes or as a result of body condition or group size. Second, we conducted a phylogenetically controlled comparative analysis of published dispersal distances in rodents to test whether the below ground lifestyle constrains dispersal distances.

METHODS

Study Site and Animal Capture Methods

We monitored 48 Damaraland mole-rat groups and 54 single individuals at the Kalahari Research Centre and an adjacent farm (26.98706°S, 21.81229°E; total area 1,850 ha) in the Northern Cape Province of South Africa between November 2013 and May 2020 (**Supplementary Material**). Groups are defined as two or more individuals which were recaptured repeatedly in the same location (**Supplementary Material**). Groups were recaptured at approximately 6-month intervals (mean ± SD = 7.5 ± 5.0 months, n = 403) and were recaptured 5.3 ± 3.5 (mean ± SD) times during the study period.

Animals were caught using Hickman traps baited with sweet potato (see **Supplementary Material**) for a total of 899 individual mole-rats (2,305 distinct animal captures across 403 group capture events). All individuals in a group were assumed to be captured when no activity was detected in the traps for 24 h, and these likely complete captures comprised 81.9% of capture events (n = 330; see **Supplementary Material**). Captured individuals were sexed, weighed to the nearest gram, and their total length was measured dorsally from nose to tip of tail with a ruler (±1 mm), and upper incisor width was measured with digital calipers (±0.01 mm). Animals were made individually identifiable by implanting them with a passive integrated transponder tag (Trovan Unique, DorsetID, Aalten, Netherlands). Thus, *known* and *unknown* individuals refer to animals with or without a transponder tag upon capture. Captured mole-rats were housed together with group members in artificial tunnel systems made from PVC pipes and provided with sweet potato *ad libitum* before they were released again in their burrow system (see Thorley et al., 2021).

The study site is divided into a core area where we focused most of our efforts and a western area where trapping was less intensive (**Figure 1** and **Supplementary Material**). New groups were detected by the presence of mounds created during burrow construction while walking between groups when checking traps. To further detect new groups, the study area was systematically searched biannually by walking transects in areas where mole-rat mounds were absent. These searches maximized our likelihood of identifying dispersing individuals. Our study area was increased over time as a result of these searches (**Figure 1**).

When new locations of mounds were found, we captured the individuals present, and many dispersers were found in this
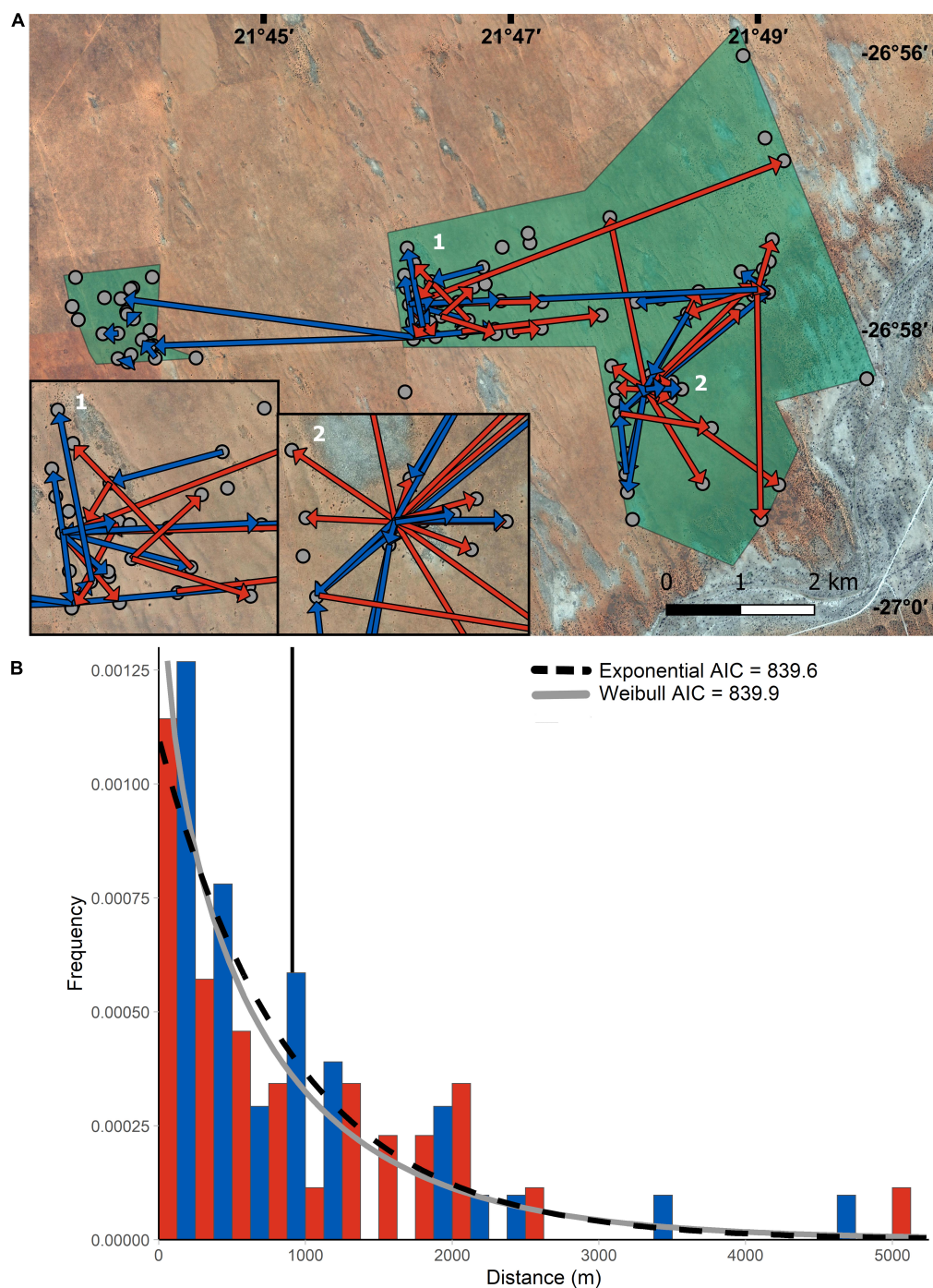
FIGURE 1 | Dispersal distances in Damaraland mole-rats. The distance traveled was measured as the Euclidean distance between the natal group and new location. (A) Direction of travel in female (red), and male dispersers (blue) at the study site (green boxes). Group locations are indicated as gray circles. Inset 1 and 2 enlarge areas of multiple overlapping arrows. The areas we focused our search for dispersers is highlighted in green, with the core area being the larger area to the east. (B) Histogram of distances traveled during natal dispersal by Damaraland mole-rats (males in blue and females in red). The exponential (dashed) and Weibull (gray) density curves are overlaid on the graph. The solid vertical line indicates the mean distance traveled (910 ± 1,004 m). There was no significant difference in the mean distance traveled between the sexes ($n = 76$, p-value = 0.30) (doi:10.1093/jmammal/gyaa163).

way. Individuals with a transponder that were captured in a new location were considered as *known* dispersers. If adult individuals (>90 g) lacking a transponder were captured in a group within

6 months of a previous capture they were most likely *unknown* dispersers because wild juveniles will not reach 90 g in 6 months (Zöttl et al., 2016; Thorley et al., 2021). Due to the chance that

some individuals may evade capture adult *unknown* individuals are therefore likely dispersers. When the capture interval was >6 months, new individuals >90 *g* may be the offspring of the dominants, and these individuals were therefore excluded from the analyses as we could not distinguish between in-group recruitment and out-of-group immigration. Our demographic data suggested that female dispersers frequently settle singly in a new location after dispersal, and rarely (*n* = 3) represent the last remaining individual in a previously established group (Thorley et al., 2021). Thus, we assume that *unknown* single individuals were also likely dispersers.

Dispersing mole-rats may block-off an unused section of their natal burrow, creating a new burrow adjacent to their natal burrow (Jarvis and Bennett, 1993; Lövy et al., 2013). We refer to these instances as "territory budding" (**Supplementary Material**). A radiofrequency identification (RFID) reader array was deployed in cases of suspected territory budding to ensure that the new group was physically separated from the natal group (Francioli et al., 2020). In short, this involved the placement of multiple RFID antennas above active foraging tunnels for 2–5 days. The antenna detects the transponder tag of individuals as they move through their burrow system. If individuals were living in physically separated burrow systems, then they would be detected on one antenna but not others with no overlap of individuals across antennas, thus implying the budding territory was distinct from the natal territory.

Dispersal Distances in Damaraland Mole-Rats

All statistical analyses were performed with R version 4.0.5 (R Core Team, 2021). Data are reported as mean ± 1 SD. A single GPS point was taken between trap sites at each group capture (**Supplementary Material**), and distances between capture locations of *known* dispersers were measured (±1 m) as an estimate of dispersal distance between locations with the *Geosphere* package (Hijmans et al., 2019). All *unknown* dispersers were removed from the distance analysis because they originated from unknown locations. We assigned each *known* disperser a body condition score which was computed as the residuals from a linear regression where body mass was the response and upper tooth width and body length were predictors. We analyzed the effects on dispersal distance in a linear mixed model with the natural log transformed dispersal distance as the response and sex, natal group size (number of individuals at last capture in their natal group), and the interaction between body condition and sex as predictors using *nlme* (Pinheiro et al., 2021). Group identity was added as a random effect to control for non-independence arising from dispersers that originated from the same group, and after fitting the model, we validated that model assumptions were met (**Supplementary Material**). Fisher's exact tests were used to compare instances of territory budding to natal dispersal.

Dispersal Distances in Subterranean and Surface-Dwelling Rodents

To place the dispersal distance of Damaraland mole-rats into a broader context, we collated mean and maximum dispersal distances of rodents from the literature. We used the comparative analyses of Whitmee (2010) as the starting point for our literature search, and supplemented with further information, targeting subterranean and fossorial rodents in particular (**Supplementary Table 4**). We classed rodent species as fossorial if they spent nearly their entire lives underground and possess morphological adaptations for a burrowing lifestyle (Buffenstein, 2000; Stein, 2000; Gomes Rodrigues et al., 2016; Montoya-Sanhueza et al., 2019; see **Supplementary Material**). We further classified fossorial species into subterranean or not depending on whether they forage primarily below or above ground (Thorley, 2020). We added mean body mass of each species using the AnAge (de Magalhães and Costa, 2009) and Pantheria databases (Jones et al., 2009). In total, our data set included information from 57 studies, covering 53 species (**Supplementary Table 4**).

We fitted a series of Bayesian phylogenetic mixed models on our data set (PLMM) using the *MCMCglmm* package (Hadfield, 2010). PLMMs incorporate a phylogenetic tree as a random effect to control for the non-independence of comparative data due to the shared ancestry among species (Hadfield and Nakagawa, 2010). First, we explored the phylogenetic signal in both the mean and maximum dispersal distance by fitting a simple PLMM in which the natural log of the dispersal distance [ln(mean) or ln(maximum)] was modeled against the intercept – an intercept-only model. The phylogenetic variance was fitted as a phylogenetic covariance matrix and included as a random effect, and was derived from a randomly chosen rodent phylogeny taken from Vertlife[1]. The phylogenetic signal was estimated as the phylogenetic heritability (h^2_{phy}) – the ratio of the phylogenetic variance to the total trait variance. Additional models then included predictors of the mean and maximum dispersal distance: ln(body mass), subterranean (categorical: yes or no), dispersal event sample size (categorical: very low, <10; low, 10–25; medium, 25–50; and >50, high). All models were fitted to a Gaussian error distribution and ran for 205,000 iterations, with a burn-in of 5000 and a thinning interval of 100; generating a posterior sample of 2,000 for all model estimates. A parameter expanded prior was specified for the phylogenetic covariance matrix (*V* = 1, nu = 1, alpha.mu = 0, and alpha.V = 1,000). Diagnostic checks indicated that the level of auto-correlation in chains was very low and that model residuals conformed to assumptions of normality and homogeneity of variance.

RESULTS

Dispersal Distances of Damaraland Mole-Rats

A total of 76 *known* (35 females, 41 males) and 37 *unknown* (29 females, 8 males) dispersers were captured during the study, comprising 12.5% of the population and 4.9% of the total capture effort. The sex ratio of dispersers (males divided by total dispersers, 0.44) was the same as the mean population adult sex ratio (0.44 ± 0.23). We found four suspected dispersal coalitions

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[1] http://vertlife.org/phylosubsets/

(**Supplementary Material**). All *known* dispersers were never recaptured with members of their natal burrow after dispersal.

We found that the mean dispersal distance was not significantly different between the sexes (males = 834 ± 980 m; females = 998 ± 1,038 m; and LMM: Est. = −0.26, S.E. = 0.24, p-value = 0.30; **Supplementary Table 2**) and the majority of *known* dispersers traveled less than 1,000 m (68.8%; **Figure 1B**). However, two individuals traveled over 4.5 km during natal dispersal (female 4,597 m, male 5,232 m). We found that 57.1% of female dispersers (n = 20), and 48.8% of male dispersers (n = 20) moved >500 m, while 22.9% of females (n = 8) and 29.3% of males (n = 12) settled within 200 m of their natal group. Territory budding was rare and occurred less frequently than other forms of dispersal (5 instances; 11.6%; Fisher's: df = 1, p < 0.001). When it occurred, individuals usually settled in parts of the natal territory and blocked of a subsection of the groups burrow (**Supplementary Material**).

We found that only natal group size (LMM: Est = 0.08, S.E. = 0.02, and p-value = 0.002) and not body condition (LMM: Est. = −0.01, S.E. = 0.01, and p-value = 0.45) were significant predictors of dispersal distance, where dispersal distance increased with group size for both sexes (**Supplementary Figure 2** and **Supplementary Table 2**).

Dispersal Distances in Subterranean and Surface-Dwelling Rodents

The dispersal distances of subterranean rodents were similar to those of surface-dwelling rodents (**Figure 2**). When body mass was excluded, both the mean and maximum dispersal distance showed a strong phylogenetic signal (**Table 1**). After the strong positive association with body mass was included, the phylogenetic signal dropped substantially and was estimated with wide credible intervals. Sample size did not affect either the mean or maximum dispersal distance in rodents given the available data (**Table 1**).

DISCUSSION

Our study investigated the dispersal dynamics of the Damaraland mole-rat. We did not find a significant difference in the dispersal distances of males and females. Both sexes were capable of traveling well over 1,000 m, and in two cases over 4,500 m. This similarity may be due to the social structure and mating system of social mole-rats, where mole-rat families are composed of an unrelated monogamous pair (Bennett and Faulkes, 2000). This structure forces both sexes to disperse in order to reproduce with unrelated individuals, rather than inheriting breeding positions in their natal burrow. Females can travel long distances searching for new territories with food resources (i.e., plant tubers), and males can travel long distances searching for mates.

In most monogamous and cooperatively breeding mammals, like the Damaraland mole-rat, females often settle in close proximity to their mother's territory (Dobson, 2013). Therefore, we expected that females would also follow this pattern as observed in a related social mole-rat (Lövy et al., 2013). Whereas short dispersal distances do occur, we found that females mostly

traveled more than 500 m from the natal burrow, and as shown previously, establish a new burrow system while waiting for dispersing males to join them (Thorley et al., 2021). The widely scattered food resources may force females to travel far to find suitable territory. Alternatively, females may disperse farther from the natal group to avoid competing with neighboring groups since over time burrow systems can become quite large. When the population density is low, the chance of an immigrant male locating a single female is low and therefore single females may have to wait several years before finding a mate (Thorley et al., 2021). The exceptional longevity of mole-rats for their size (Buffenstein et al., 2020; Thorley, 2020), combined with low predation rates once settled in a burrow, may allow females to endure these considerable periods without reproductive opportunities while waiting for male dispersers to find them.

Dispersal distance can be affected by body mass, territory size, availability of resources or vacant territories, and breeding opportunities (Bowman et al., 2002; Clobert et al., 2009; Whitmee and Orme, 2012). In our study, we found that animals from larger groups traveled further, and that natal group size had a stronger effect on dispersal distance than the body condition of dispersing individuals (**Supplementary Material**). The cause of this pattern is unclear, but one possibility is that group and burrow size may be influenced by the distribution of food resources in the landscape. In the Kalahari, the food resources for mole-rats exhibit a scattered but patchy distribution which may affect the dispersion and size of family groups in the landscape (Lovegrove, 1988). In particular, one could hypothesize that large groups occupy larger burrow systems and that this may force females to move further to find a vacant habitat with sufficient food resources. Thus, since natal group size and burrow size are closely related (Lovegrove, 1988; Thomas et al., 2016) the effect of group size may indicate that burrow size affects dispersal distances in mole-rats. Variation in group size might also reflect the localized abundance of plant tubers, and this in turn might affect the distances that individuals are required to move to find vacant habitat or receptive mates. Lastly, increases in group size may also lead to increased competition for food or space between individuals within a group (Cooney, 2002; Zöttl et al., 2016), which may cause heightened stress levels and trigger dispersal to avoid competition (Quirici et al., 2011). Heightened stress levels from increasing group size (and limited burrow capacity) may cause animals to disperse farther to escape a perceived stressor. Adult Damaraland mole-rats exhibit higher glucocorticoids than juveniles (Medger et al., 2018), and cortisol levels increase during the rainy season likely due to dispersal opportunities (Young et al., 2010), but the effects of group size on stress levels has not been investigated in mole-rats.

Despite the challenges of following dispersal in a subterranean rodent, the low rate of successful dispersal conforms to the general view that dispersal in mole-rats and other subterranean rodents carries a high mortality risk. Mole-rats are specialized diggers with low visual acuity (Kott et al., 2010; Gomes Rodrigues et al., 2016; Montoya-Sanhueza, 2020), and like other similarly adapted species, it has long been assumed that an underground lifestyle limits vagility (Nevo, 1979). Adaptations for subterranean life may make mole-rats particularly susceptible
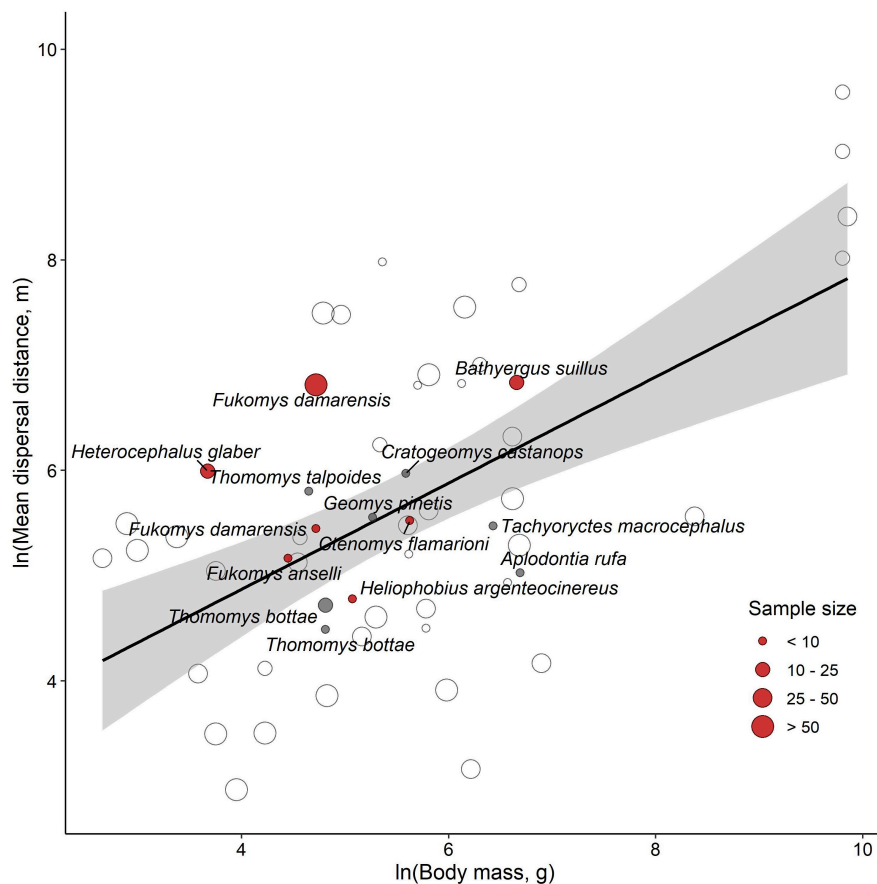
FIGURE 2 | The relationship between the mean dispersal distance and body mass in rodents. Data points are separated according to whether the species is subterranean (red), fossorial (gray), or surface-dwelling (clear). Points are sized according to the number of observed dispersal records in the literature review as shown in the legend. The large *Fukomys damarensis* circle is from this study. The solid line indicates the line of best fit through the raw data for surface-dwelling rodents with shading denoting the 95% confidence intervals.

to predation while dispersing. Indeed, diet studies indicate that mole-rats may present a seasonally available prey item to jackals (*Canis mesomelas*) and serval (*Leptailurus serval*; Humphries et al., 2015; Ramesh and Downs, 2015). Despite the apparent constraints on dispersal, we present evidence showing that dispersal distances in subterranean rodents fall within the expected range of rodents based on their body size. Within the Family *Bathyergidae* previous studies have recorded a maximum dispersal distance of 2,149 m in Cape dune mole-rats (*Bathyergus suillus*; Bray et al., 2012), and 2,400 m in naked mole-rats (*Heterocephalus glaber*, Braude, 2000). Our study found that 5 dispersers, including individuals of both sexes, traveled over this distance, with the greatest distance traveled being 5,232 m from the natal burrow. Our study shows that the subterranean niche does not necessarily limit long distance dispersal, and some individuals can settle successfully far away from their natal territory. The long distances observed in this study suggest that traveling above ground may be the primary method of natal dispersal for Damaraland mole-rats and perhaps many other subterranean rodents, as previously suggested in geomyids (Welborn and Light, 2014).

Like in other field studies of natural populations the estimations of dispersal frequency and distance are likely to be conservative underestimates. The estimation of dispersal distances are based on the recapture of *known* dispersers and it is likely that the likelihood of finding these individuals decreases with increasing dispersal distance (Tomkiewicz et al., 2010; Maag et al., 2022). The number of *unknown* dispersers is also an approximation because we were unable to assess relatedness among individuals in each group. Some *unknown* dispersers found in established groups during the study may be recruits born to the reproductive pair which evaded capture and were not immigrants. However, our data suggests that these are rare occurrences because longitudinal resampling suggests that we rarely miss individuals repeatedly. Because of these reasons we believe that both of these sources of bias either lead to conservative underestimates or to very minor inaccuracies in frequencies of immigration.

By focusing on successfully established dispersers and the distances they moved, our study can only get us part way toward a complete understanding of sex-specific dispersal distances

TABLE 1 | Results of Bayesian phylogenetic mixed models fitted to comparative data on rodent dispersal.

	Ln(mean dispersal distance)		Ln(maximum dispersal distance)	
Intercept	5.65 [4.17, 7.27]	3.48 [1.09, 6.00]	6.83 [5.72, 8.08]	3.42 [0.60, 6.19]
ln(Body mass)	–	0.40 [0.05, 0.75]	–	0.54 [0.14, 0.89]
Subterranean: Yes	–	0.17 [-3.11, 3.09]	–	−0.02 [-3.70, 3.12]
Sample size: low	–	0.25 [-0.67, 1.32]	–	0.44 [-0.58, 1.54]
: medium	–	−0.48 [-1.36, 0.51]	–	−0.02 [-1.15, 1.19]
: high	–	−0.05 [-0.92, 0.86]	–	0.98 [-0.11, 2.00]
Phylogenetic variance	3.87 [0.81, 8.26]	2.88 [1.24e-05, 7.07]	2.32 [0.32, 4.71]	3.30 [1.11e-06, 8.82]
Residual variance	0.85 [0.33, 1.48]	0.99 [0.33, 1.85]	1.05 [0.43, 1.74]	1.20 [0.35, 2.26]
Phylogenetic signal (h^2_{phy})	0.78 [0.50, 0.98]	0.49 [5.29e-06, 0.89]	0.65 [0.35, 0.91]	0.61 [6.29e-07, 0.94]
Sample size	n = 57 values (50 species)		n = 55 values (47 species)	

Estimates provide the posterior mean [L95% CI, U95% CI].

in Damaraland mole-rats. Dispersal is a three-stage process covering emigration, transience, and settlement, and different factors (e.g., mortality) may affect the sexes at each of these stages (Clobert et al., 2009; Maag et al., 2022). The examination of settlement patterns provides information on the probability that members of either sex acquire breeding opportunities, and the circumstances under which they do so, but additional sources of information must also be used to understand individual decisions during emigration and transience. As mole-rats cannot be observed continuously, emigration patterns have often been inferred from the duration of philopatry (Torrents-Ticó et al., 2018; Thorley et al., 2021), under the assumption that a sizeable proportion of disappearances are due to dispersal rather than *in situ* mortality. Such data suggests that in the social mole-rats, males emigrate from groups earlier than females (Torrents-Ticó et al., 2018). However, there is also no clear evidence of lifelong philopatry of non-breeding females (Torrents-Ticó et al., 2018; Thorley et al., 2021). While males may disperse earlier, the difference in the absolute number of male and female emigrants may be very small, at least in our population where the adult sex ratio is equal to that of dispersers (cf. Hazell et al., 2000). Direct information on the transience phase of dispersal in mole-rats is largely non-existent, save for one individually tracked animal (Lövy et al., 2013). It remains to be seen whether the two sexes face differing mortality risk during dispersal, such as from competitive aggression (Busch et al., 2000), or through increased search times (Šumbera et al., 2008). Molecular techniques have advanced our understanding of dispersal patterns in fossorial rodents by using microsatellite markers to determine the presence of sex biases in dispersal, gene flow between populations, and allow for an estimation of dispersal distances without the need for longitudinal sampling (Cutrera et al., 2005; Fernández-Stolz et al., 2007; Bray et al., 2012; Patzenhauerová et al., 2013; Welborn and Light, 2014; Visser et al., 2018; Mynhardt et al., 2021). Comparing variation in dispersal patterns within a single species across populations living in varying population densities, or environmental conditions (e.g., rainfall or soil type) would greatly assist in determining the extent to which demographic and ecological factors affect dispersal opportunities, frequency of intergroup movement, and gene flow in subterranean rodents.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found on the online repository FigShare: https://doi.org/10.6084/m9.figshare.19196732.v1.

ETHICS STATEMENT

The study was approved by the University of Pretoria Ethics Committee (EC032-13, EC006-15, EC050-16, and EC059-18) and subsequently approved by Northern Cape Nature Conservation.

AUTHOR CONTRIBUTIONS

KF, JT, and MZ conceived and designed the study. KF, JT, HB, and MZ collected the data. KF and JT analyzed the data. KF wrote the first draft of the manuscript. All authors made significant contributions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.879014/full#supplementary-material

Montoya-Sanhueza, G., Wilson, L. A. B., and Chinsamy, A. (2019). Postnatal development of the largest subterranean mammal (*Bathyergus suillus*): morphology, osteogenesis, and modularity of the appendicular skeleton. *Dev. Dyn.* 248, 1101–1128. doi: 10.1002/dvdy.81

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Spatial Learning and Memory in the Naked Mole-Rat: Evolutionary Adaptations to a Subterranean Niche

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Evolutionary adaptation to a subterranean habitat consisting of extensive underground tunnel systems would presumably require adept spatial learning and memory, however, such capabilities have not been characterized to date in naked mole-rats (*Heterocephalus glaber*) which, like other members of Bathyergidae, are subterranean rodents. The goal of this study was to develop a method for effectively assessing spatial learning and memory by modifying a Hebb-Williams maze for use with these subterranean rodents. Established behavioral tests to assess spatial learning and memory have primarily focused on, and have been optimized for, more typical laboratory rodent species such as mice and rats. In the current study, we utilized species-appropriate motivators, analyzed learning curves associated with maze performance, and tested memory retention in naked mole-rats. Using a modified Hebb-Williams maze, naked mole-rats underwent 3 days of training, consisting of five trials per day wherein they could freely explore the maze in search of the reward chamber. Memory retention was then tested 1 day, 1 week, and 1 month following the last day of training. Performance was analyzed based on latency to the reward chamber, errors made, and distance traveled to reach the reward chamber. Overall, this study established a behavioral paradigm for assessing maze navigation, spatial learning, and spatial memory in subterranean rodents, including optimization of rewards and environmental motivators.

Keywords: naked mole-rat, learning, memory, spatial navigation, rodent, reward

INTRODUCTION

The elaborate tunnel systems maintained by bathyergids are indicative of adept spatial navigation skills. This is supported by studies that have been conducted in other subterranean species (Kimchi and Terkel, 2001, 2004; Costanzo et al., 2009; Oosthuizen et al., 2013; Brachetta et al., 2014; Oosthuizen, 2020), including one study that demonstrated that Damaraland mole-rats outperformed voles and rats in long-term memory tests (Kimchi and Terkel, 2001). However,

limited research has focused on maze optimizations for testing spatial learning and memory in subterranean species, and no such studies have been conducted in naked mole-rats to date.

Naked mole-rats are a rodent species native to eastern Africa (Sherman et al., 1991). Like other bathyergids, they are subterranean rodents that thrive in hot, humid environments and construct complex burrows of interconnected underground tunnels (Sherman et al., 1991). More extensive burrow systems are associated with greater spatial memory performance in other mole-rat species, likely due to the daily reliance on spatial cues required to navigate these underground labyrinths (Costanzo et al., 2009). These systems include designated living areas that consist of toilet chambers, food chambers, and nesting chambers (Faulkes and Bennett, 2021; Smith and Buffenstein, 2021), the complexity of which may also warrant well-developed spatial memory. Naked mole-rats establish subterranean tunnel systems that can extend several kilometers in length (Brett, 1991), which may be attributable in part to their large colony sizes of up to approximately 300 animals (Faulkes and Bennett, 2021). Both naked mole-rats and Damaraland mole-rats exhibit a social hierarchy characterized by set divisions of labor, as well as generally having one queen as the only reproductively active female (Wilson, 1971; Buffenstein et al., 2022). In Damaraland mole-rats, large colony sizes contributed to increased spatial navigation skills (du Toit et al., 2012), but this has yet to be experimentally investigated in naked mole-rats.

The subterranean habitats of Bathyergidae have resulted in evolutionary adaptations of sensory capabilities and related anatomical and neuroanatomical systems which must be considered when developing and interpreting behavioral tests. Traditionally, behavioral paradigms within a laboratory setting have been designed for more typical animal models (e.g., mouse and rat) and have incorporated aspects suited to their visual and hearing capabilities. These designs are often not translatable to more atypical research models, such as the subterranean naked mole-rat (*Heterocephalus glaber*), for whom sensory limitations such as functional blindness play a role (Hetling et al., 2005). In particular, spatial memory testing paradigms such as the Morris water maze (Morris, 1984) and radial arm maze (Olton and Samuelson, 1976; Brown and Cook, 1986) often rely on visual cues that provide a basis for navigation while in the maze.

Designing behavioral tasks for subterranean rodents requires taking into account their divergent sensory abilities from other members of Rodentia, as well as incorporating appropriate motivators. In the current study, we developed an effective paradigm for investigating spatial learning and memory in naked mole-rats, with consideration of a range of factors that may affect navigational memory abilities among colony members including age, sex, body mass, and dominance ranking within the social hierarchy. We hypothesized that naked mole-rats would exhibit spatial learning in a customized maze design by reaching the reward chamber faster (reduced latency) and with optimized navigation (shorter total distance traveled and fewer errors committed) across training trials, and that spatial memory would be robust based on the naked mole-rat's adaptations to a subterranean habitat.

MATERIALS AND METHODS

Animals

Forty-three naked mole-rats (*H. glaber;* Rüppell, 1842) (25 female, 18 male) encompassing a range of body masses and ages were used in this study. For a preliminary study using a non-continuous maze design, eight animals were used (**Table 1**). Following improvements to the maze design and incorporation of a continuous maze, thirty-five animals were used. Six of these animals were excluded due to missing data for their dominance ranking. Eleven additional animals had no more than one missing trial data point. For these eleven animals, the missing data point was replaced with linear interpolation. One additional animal was excluded as an outlier due to data points in the first learning trial exceeding ten standard deviations of the mean, while another was excluded due to passing away before the end of data collection. Thus, 27 animals were included in the continuous maze study (**Table 1**). For the memory probe analysis, two additional animals were excluded, one as an outlier with values that exceed 10 standard deviations of the mean and the other excluded for missing multiple data points. Thus, 25 animals of the initial 27 were included in the memory probe analysis. Body masses of the animals included in the analysis ranged from 23.0 to 101.0 g (Braintree Scientific compact portable scale model CB 1,001 with 0.1 g precision), with ages ranging from 0.39 to 6.68 years at the start of testing. All of the naked mole-rats were members of one of two laboratory breeding colonies (Colony A and Colony B) maintained in-house, with varying status within the colony (queens, breeders, non-breeders). Not all members of each colony were used. Animals were excluded based on involvement in other ongoing studies. The vivarium housing for each colony was maintained at approximately 27.8–30°C and at least 40% relative humidity, without a light/dark cycle due to the subterranean habitat of the animals (Sherman et al., 1991). Animal housing consisted of a series of chambers interconnected with tubing and designed to mimic their natural burrow systems. Free access to food and ample bedding were provided (for further housing details, see Artwohl et al., 2002). All aspects of the research performed for this study complied with our protocol approved by the Institutional Animal Care and Use Committee at Southern Illinois University, Carbondale, IL, United States, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication Nos. 8023 and 1978).

Maze Design

We designed modified versions of the Hebb-Williams maze, a common paradigm for testing spatial learning and memory in rodents. Modifications to the Hebb-Williams maze were based on studies that used similar tests of spatial navigation in other atypical rodent animal models, including the subterranean tuco-tuco (Antinuchi and Schleich, 2003; Schleich, 2010; Brachetta et al., 2014) and the blind mole-rat (Kimchi and Terkel, 2001, 2004). The mazes were constructed using PVC pipes that were cut in half longitudinally and covered with a sheet of clear acrylic, creating a trough for the animals to navigate through

while allowing visibility for video recordings. The tubing had a 2″ diameter, equal to the tubing in the housing chamber tunnel systems for the naked mole-rats. This tubing width was chosen based on previous research that showed greater learning and memory performance in the blind mole-rat when using narrower pathways that provide tactile cues in mazes (Kimchi and Terkel, 2004).

The first maze design we tested was non-continuous and included dead-ends (**Figure 1A**) similar to other Hebb-Williams-based mazes (Kimchi and Terkel, 2001, 2004; Costanzo et al., 2009; du Toit et al., 2012). Preliminary testing using a cohort of eight animals was conducted for this version of the maze (**Table 1**). Any dead-ends within the maze were sealed and a clear acrylic sheet was placed on top of the maze, allowing visualization of animal behavior and performance in the mazes from above. The reward chamber consisted of a cylindrical housing chamber made of clear cast acrylic with 8″ diameter, 10″ height, and 1/4″ wall thickness. This reward chamber was heated with a heat lamp and filled with bedding from the animal's home colony. It was connected to the maze *via* a clear cast acrylic 2″ diameter connector tube with 1/4″ wall thickness (US Plastics, Item #44530). Performance was measured using latency to the reward chamber, total distance traveled before reaching the reward chamber, and number of entries into the error zones. An entry into an error zone was counted if over 50% of the animal's body passed into the zone, regardless of the direction of entry.

Naked mole-rats react strongly to blockages in tunnels by persistently digging and biting in an attempt to get past the blockage (Sherman et al., 1991). During initial maze design testing (**Figure 1A**), we found that this distraction affected completion of the maze ("Fail Rate," **Table 1**). Fail rate was calculated as the percentage of learning trials (Trial 1–15) in which animals did not complete the maze in the allotted 5 min (**Table 1**).

$$Fail\ Rate\ (\%)\ =\ Incomplete\ Trials/Total\ Trials\ \times\ 100 \quad (1)$$

TABLE 1 | Descriptive statistics (values shown are mean ± SEM, and range) for naked mole-rats used across two different spatial maze designs.

	Non-continuous maze animals	Continuous maze animals
Age (yrs)	**2.21 ± 0.11**	**2.94 ± 0.49**
Range	0.97–4.45	0.39–6.68
Body mass (g)	**58.42 ± 1.40**	**54.6 ± 4.8**
Range	39.8–85.6	23.0–101.0
Sex	**Male = 3**	**Male = 9**
	Female = 5	**Female = 18**
Colony	**A = 8, B = 0**	**A = 13, B = 14**
Fail rate	**43.4%**	**7.2%**

Fail rate is the percentage of trials that were incomplete for all training trials within each design. g, grams; yrs, years. Bold values are the mean +/- the standard error of the mean.

To address this, we redesigned the maze to a continuous configuration that lacked dead-ends (**Figure 1B**) and tested thirty-five animals in this maze configuration (**Table 1**). This allowed us to more effectively investigate whether the animals would be more motivated to complete the maze without having attention diverted from seeking the reward chamber due to dead-end distractors.

Reward Testing

In order to test the effectiveness of a range of motivators in naked mole-rats, and subsequently use the most effective motivator in the reward chamber, a separate experiment was performed prior to using the continuous maze. The same eight animals used in the testing of the first maze design were utilized to determine appropriate motivators for naked mole-rats. First, as a control, a start chamber was connected to a separate empty chamber using an L-shaped connector tube. Each animal's behavior was first assessed in this control condition (see Data Analysis below). To test the effectiveness of a range of motivators, a motivator was added to the empty chamber, making it a reward chamber (**Figure 1C**). For the control test, each animal was placed in the start chamber and observed for one trial of 2 min in duration. Time spent in each chamber and latency to enter the empty chamber were recorded. This was then compared to each animal's performance with a motivator present in the reward chamber. All animals were tested with one motivator (e.g., heat lamp) for one trial of 2 min in duration before switching to the next motivator, and motivators were tested in the following order: heat lamp, darkened chamber (shielded from outside light with black construction paper), food (sweet potato, banana baby food, and ProNutro—all items placed together in the food chamber, for all animals tested), home colony bedding, and foreign colony bedding (**Table 2**). The start and empty or reward chambers were wiped with 70% isopropyl ethanol between testing sessions with different animals. The resulting optimized motivator, a combination of home colony bedding and a heat lamp, was used for the continuous maze reward chamber.

Determination of Dominance Rank

Social hierarchy rank was determined using a dominance testing paradigm modeled after similar testing in previous studies in naked mole-rats (Clarke and Faulkes, 1997, 1998). All animals used in the continuous version of the maze were marked with their ID numbers on their backs using a permanent marker to allow for visual tracking of behaviors during dominance testing. Two researchers placed one naked mole-rat each in separate, circular chambers. Both chambers were connected *via* a clear polycarbonate tube. The animals were oriented at the open ends of the tube such that they met face to face when they moved in a forward direction (**Figure 1D**). Encounters were only rated if one animal passed over the top of another while in the tube. The test session ended after three instances of face-to-face pass-over behaviors were recorded. After each session, the chambers and tubing were cleaned with 70% isopropyl alcohol. Animal pairings were randomly generated with no pair undergoing more than one testing session together. Each

FIGURE 1 | Naked mole-rat behavioral testing configurations. **(A)** Modified Hebb-Williams maze with a non-continuous maze design that included dead ends. Gray regions indicate areas where an error was recorded upon entry. **(B)** Modified Hebb-Williams maze with a continuous design lacking dead ends and sharing more similarities with the extensive, interconnected underground tunnel systems of the naked mole-rat's habitat. **(C)** Motivator testing setup. Animals were placed in the start chamber and allowed 2 min to freely explore. Animal performance was tested using a range of motivators in the reward chamber and compared to performance when that chamber was empty. **(D)** Dominance testing setup. Animals were placed at the indicated entry points of each chamber, facing toward one another, and analyzed for how many times one passed over the other through the tubing across three trials.

animal underwent at least three testing sessions, each with a novel paired animal.

Dominance ranking was determined for the continuous maze animals using previously described methods with naked mole-rats (Clarke and Faulkes, 1997; Gilbert et al., 2020). Using a package extension for use with R Studio (R Core Team, 2021) to calculate the Elo rating (Neumann and Kulik, 2020), the results of each testing session across multiple days were entered to yield the rank of each individual animal tested. The higher the ranking, the more dominant the animal.

Experimental Paradigm

All spatial learning trials occurred within the same 4 h time block across every day of testing (8 a.m.–12 p.m.). All testing was performed in a brightly lit behavior room, maintained at 18.5–21°C and at least 40% relative humidity. Animals were brought into the room in transfer chambers and allowed to acclimate in the reward chamber of the maze for 5 min. Animals were then removed from the reward chamber and placed at the predetermined entry point of the maze, facing forward (**Figures 1A,B**). The naked mole-rat was allowed to explore

the maze until it reached the reward chamber. The trial was considered complete when 5 min elapsed or when the animal successfully navigated to the reward chamber, whichever came first. If the animal was unable to reach the reward chamber before 5 min elapsed, it was gently guided to the chamber by the experimenter following cessation of the trial and the trial counted as a failed trial. Upon entry into the reward chamber, a block was placed at the entrance to prevent reentry into the maze. The animal was allowed 5 min to rest in and explore the chamber, after which time the next trial began. Performance was recorded with a camcorder (FHD 1080P, YEHOOVJIANGER) and analyzed in real-time using ANY-maze software (Stoelting Co., Wood Dale, IL, United States).

Animals underwent spatial learning/training for three consecutive days with five trials per day for a total of fifteen trials (**Figure 2**). Between each trial, the maze was cleaned with 70% isopropyl alcohol to reduce the effect of olfactory cues on performance in subsequent trials. After each day of testing, the maze was deconstructed and washed with non-scented soap and water. Spatial memory retention was tested 1 day and 1 week following the last day of training for the non-continuous

TABLE 2 | Assessment of reward chamber motivators.

Motivators	Latency (s)	Dwell time (s)	Dwell time ratio
Empty chamber	**85.25 ± 13.39**	**13.64 ± 5.56**	**0.21 ± 0.35**
Range	39.56–120.00	0.00–38.50	−1.67 to 1.00
Heat lamp	**43.52 ± 12.39**	**31.26 ± 6.49**	**−1.05 ± 0.54**
Range	12.70–120.00	0.00–58.90	−3.17 to 1.00
	$t = 3.207$	$t = -2.159$	$t = 1.890$
	$(p = 0.075)$	$(p = 0.339)$	$(p = 0.503)$
Darkened chamber	**60.14 ± 16.92**	**15.20 ± 6.31**	**0.57 ± 0.19**
Range	13.50–120.00	0.00–47.70	−0.43 to 1.00
	$t = 1.861$	$t = -0.536$	$t = -2.141$
	$(p = 0.525)$	$(p = 1.00)$	$(p = 0.348)$
Food	**46.25 ± 16.38**	**26.80 ± 7.76**	**−1.72 ± 1.11**
Range	11.09–120.00	0.00–61.00	−8.32 to 1.00
	$t = 2.804$	$t = -2.006$	$t = 1.875$
	$(p = 0.132)$	$(p = 0.424)$	$(p = 0.515)$
Home colony bedding	**26.06 ± 5.50**	**60.97 ± 4.73**	**−15.48 ± 11.04**
Range	13.00–54.00	51.00–82.62	−81.62 to −1.90
	$t = 5.904$	$t = -9.623$	$t = 1.453$
	$(p = 0.003)^*$	$(p < 0.001)^*$	$(p = 0.948)$
Foreign colony bedding	**27.63 ± 5.79**	**67.98 ± 4.23**	**−15.04 ± 5.04**
Range	8.00–48.00	44.55–75.26	−47.18 to −5.10
	$t = 5.352$	$t = -7.904$	$t = 3.038$
	$(p = 0.005)^*$	$(p < 0.001)^*$	$(p = 0.095)$

Evaluations of effective motivators were compared to performance with an empty chamber lacking any motivator. Values shown are the mean ± SEM; range; and t-values resulting from a paired-sample t-test comparing each motivator to the empty chamber. P-values are shown in parentheses. *Indicates significance at p < 0.05, corrected for multiple comparisons. s, seconds. Bold values are the mean +/- the standard error of the mean.

FIGURE 2 | Spatial learning and memory testing timeline. For both the non-continuous maze and the continuous maze, animals underwent 3 days of behavioral training consisting of five trials per day. One day, 1 week, and 1 month following the last day of training, animals underwent a single memory probe trial. *One month memory probe trial was performed for the continuous maze but was not performed for the non-continuous maze due to the lack of a learning curve (see **Figure 6**).

maze and 1 day, 1 week, and 1 month for the continuous maze (**Figure 2**). On memory probe days, the animal freely explored the maze for one trial of 5 min or until it reached the reward chamber, whichever occurred first. In memory probe trials, the reward chamber contained the same motivators used in learning trials. If the animal was unable to complete the maze within the 5 min allotted, it was returned to the colony without being guided to the reward chamber.

Data Analysis

Paired sample *t*-tests were first used to examine the effectiveness of different motivators for use in the reward chamber. *P*-values

were first calculated by using raw values to compare latency to the reward chamber vs. latency to the empty chamber (**Figure 3A**) and dwell time in the reward chamber vs. dwell time in the empty chamber (**Figure 3B**). The dwell time ratio for reward (DTR_R) and control conditions with an empty chamber (DTR_E) were calculated as follows, where DT_S represents the dwell time spent in the start chamber under reward (DT_{SR}) vs. control conditions with an empty chamber (DT_{SE}), and DT represents the dwell time in the finish chamber between control (DT_E, dwell time spent in the empty chamber) vs. reward conditions (DT_R, dwell time spent in the reward chamber):

$$DTR_R = (DT_{SR} - DT_R)/DT_{SR}$$

$$DTR_E = (DT_{SE} - DT_E)/DT_{SE}$$

A paired sample *t*-test was also conducted for DTR_R vs. DTR_E (**Figure 3C**). Bonferroni corrected *p*-values were used to determine significance. A summary of results from the paired sample *t*-tests can be found in **Table 2**.

For the continuous maze, latency to the reward chamber and distance traveled were analyzed to investigate spatial learning and memory performance (analyses adapted from Vallianatou et al., 2020). For animals with one trial's worth of missing data points, the missing points were linearly interpolated by averaging the values for the trial preceding the missing point and the trial following the missing point (e.g., a Trial 4 data point was interpolated by averaging the values of the animal on Trial 3 and Trial 5). To investigate differences in the learning phase of the continuous maze, we used two different multivariate repeated
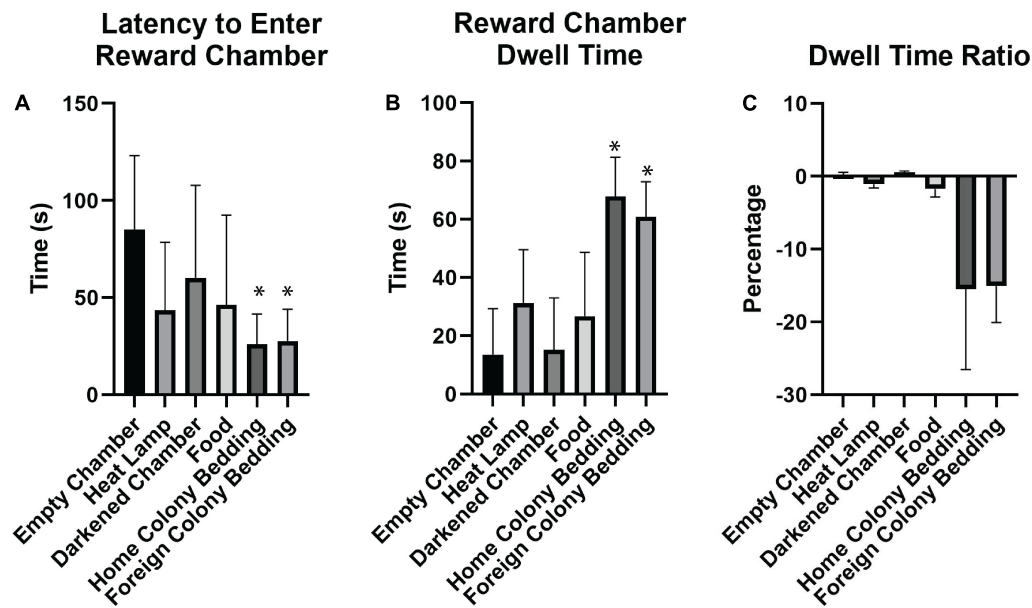
FIGURE 3 | Effectiveness of different motivators for naked mole-rats. **(A)** Of the motivators tested, home colony bedding, foreign colony bedding, and heat resulted in the shortest latencies to enter the reward chamber compared to an empty chamber. **(B)** Both home and foreign colony bedding resulted in the greatest increase in dwell time in the reward chamber compared to the start chamber. **(C)** In assessing the Dwell Time Ratio, animals spent relatively more time in the reward chamber compared to the start chamber when either home or foreign colony bedding was present. A negative percentage indicates more time spent in the reward chamber than the start chamber. *Indicates a significant ($p < 0.05$) difference from the empty chamber value. s, seconds.

TABLE 3 | Performance comparisons between both maze conformations to assess spatial memory.

	MP1 latency (%)	MP1 distance (%)	MP1 errors (%)	MP2 latency (%)	MP2 distance (%)	MP2 errors (%)	MP3 latency (%)	MP3 distance (%)
Non-continuous	**129 ± 41**	**208 ± 4.3**	**157 ± 4.5**	**110 ± 3.5**	**137 ± 10**	**142 ± 20**	N/A	N/A
Range	9–308	32–371	57–440	26–287	107–174	80–270	N/A	N/A
Continuous	**121 ± 36**	**91 ± 16**	N/A	**107 ± 31**	**116 ± 34**	N/A	**150 ± 22**	**89 ± 28**
Range	−7 to 871	−48 to 258	N/A	13–746	9–887	N/A	−961 to 328	−431 to 312

*The columns display memory probe performance as a percentage of performance on the last trial of the last training day for both maze configurations (continuous and non-continuous) at each memory probe time point. Values shown are mean ± SEM and range. The 1 month probe trial (MP3) was performed for the continuous maze but was not performed for the non-continuous maze due to the lack of a learning curve in the non-continuous maze (see **Figure 6**). Errors were not calculated for the continuous maze, due to the near-elimination of error zones in this maze configuration. MP1, memory probe 1 (tested at 1 day following training); MP2, memory probe 2 (tested at 1 week following training); MP3, memory probe 3 (tested at 1 month following training). Bold values are the mean +/− the standard error of the mean.*

measures factorial MANOVAs. These models incorporated body mass and age as covariates; sex and colony as fixed factors; and latency and distance as dependent measures. These models were used to determine any main effects or interactions on performance in a manner similar to previous studies (Costanzo et al., 2009; Oosthuizen et al., 2013; Oosthuizen, 2020). The first repeated measures MANOVA used both Day and Trial as within-subjects factors, wherein each day was subdivided into its respective 5 trials. The second model was used for ease of interpretation and modeled only Trials without distinguishing by days. In the second model, Trials were analyzed as Trial 1 through Trial 15 rather than Day 1 through 3 each with Trial 1 through Trial 5. Estimated marginal means and confidence intervals from these models were used to graph the adjusted performance values (see **Figures 5**, **6**). These models adjusted for the covariates at Body Mass = 54.60 g and Age = 2.94 yrs. The repeated measure

MANOVA was also conducted separately for each colony in order to incorporate dominance ranking as a covariate. These colony specific analyses were done because no dominance testing can be performed between animals of different colonies, as this testing results in lethal confrontations. The colony-specific models used body mass, age, and dominance ranking as covariates; sex as a fixed factor; and latency and distance as dependent measures. These analyses were also performed in two separate ways, one with two repeated measures Day and Trial and the second with Trials as the only within-subject factor. Colony A estimated marginal means were computed using: Mass = 54.63 g, Age = 2.35 years, Rank = 957.40. Colony B estimated marginal means were computed using: Mass = 54.56 g, Age = 3.49 years, Rank = 1002.96. All aforementioned statistical analyses were also conducted as separate repeated measures ANOVAs with speed as the dependent variable to complement the latency

and distance analyses. These models were conducted separately because speed is equal to distance divided by latency. Bonferroni correction was utilized for *post hoc* pairwise comparison for all Day and Trial effects.

Performance percentages on memory probe trials were computed for both maze configurations (non-continuous and continuous), comparing performance (latency and distance traveled) from individual memory probe days to the last training day. Latency and distance traveled were analyzed for both continuous and non-continuous maze designs, whereas errors were analyzed only for the non-continuous maze design. These were calculated such that the value from the memory probe trial (1, 2, or 3) was divided by the last training day trial (Trial 5 on training Day 3; **Figure 2** and **Table 3**). A Repeated Measures MANOVA using latency performance and distance performance as dependent measures; body mass and age as covariates; sex and colony as fixed factors; and memory probe trial (1, 2, or 3) as the within-subjects factor. This analysis was conducted for each colony separately and combined. This set of statistical analyses for memory probe trials were also conducted as repeated measures ANOVAs with speed as the dependent variable in lieu of latency and distance.

RESULTS

Testing the Relative Effectiveness of a Range of Behavioral Motivators in Naked Mole-Rats

We tested different motivators in order to optimize subsequent spatial maze testing performance (**Figure 1C**). Using paired sample *t*-tests for the raw values of latency, dwell time, and dwell time ratio compared to the empty chamber, similar motivators proved to be significant (**Table 2** and **Figure 3**). Latency was enhanced with the presence of home colony bedding [$t(7) = 5.904$, $p = 0.003$] or foreign colony bedding [$t(7) = 5.352$, $p = 0.005$], with heat approaching significance [$t(7) = 3.21$, $p = 0.075$]. Dwell Time Ratio was not significant for any motivator tested, but Dwell Time was significantly greater when home colony bedding [$t(7) = -9.623$, $p < 0.001$] or foreign colony bedding [$t(7) = -7.904$, $p < 0.001$] were present in the reward chamber. Based on these sets of analyses, heat and the presence of either bedding type (foreign or home bedding) were the most effective motivators when presented independently. Thus, both heat and home bedding were chosen for the reward chamber in subsequent continuous maze testing. Foreign colony bedding, though also effective, was not selected based on the potential risk of an experimental animal being attacked upon reintroduction to the home colony due to smelling like an intruder (Braude et al., 2021).

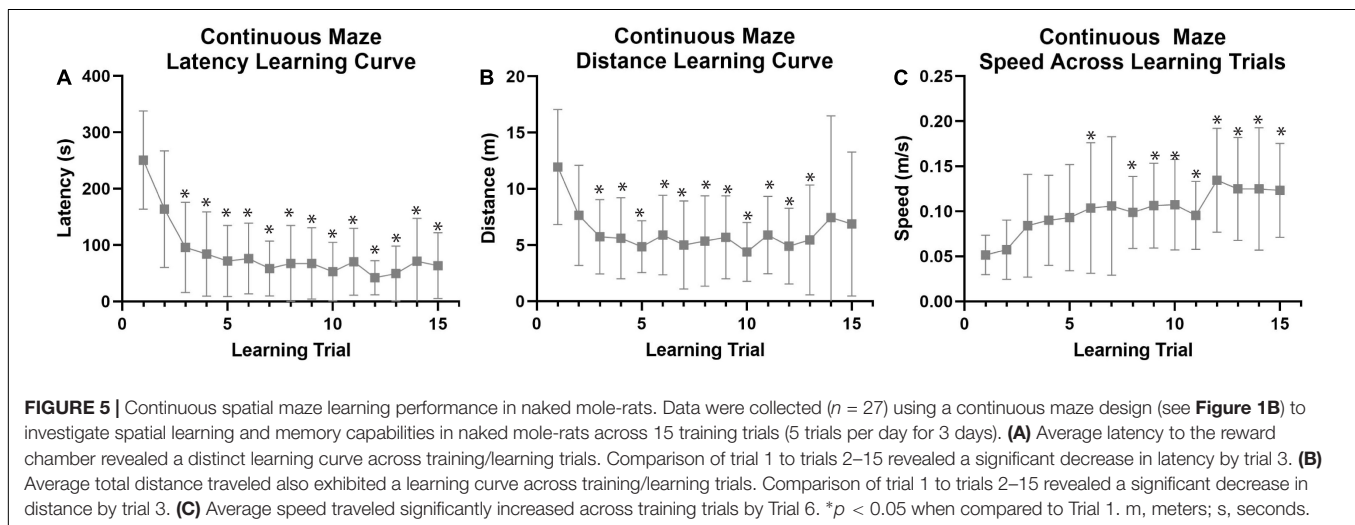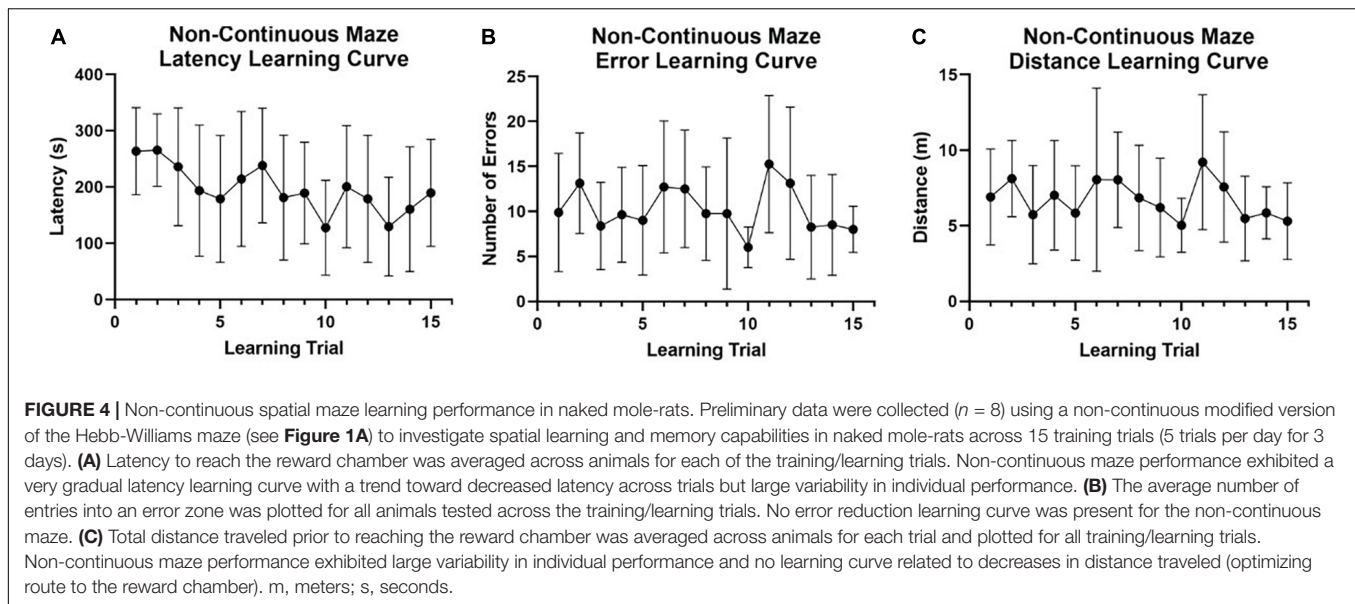### Spatial Learning in Naked Mole-Rats

For the preliminary data set collected using a non-continuous maze design ($n = 8$; **Figure 1A**), no learning curves were evident across training trials for latency to the reward chamber (**Figure 4A**), total number of errors (wrong turns) made

(**Figure 4B**), or total distance traveled (**Figure 4C**). Fail rate was also high (**Table 1**). The poor performance of naked mole-rats in this non-continuous maze design, along with the observation that the non-continuous maze design may have created a confound (i.e., dead-ends proving to be strong distractors that obscured the true potential for naked mole-rats to exhibit spatial learning, and memory improvements), led us to redesign the maze in a continuous layout with no dead-ends.

Both colonies (Colony A and Colony B) were analyzed together for maximum power with the continuous maze design ($n = 27$; **Figure 1B**), and using a repeated measures MANOVA that did not model the effect of testing on separate days for simplicity of interpretation. This analysis revealed a distinct learning curve across the 15 training trials (**Figure 5A**) that was absent in the non-continuous maze. There was a multivariate effect of trial number on performance [$F_{(28, 588)} = 3.074$, $p < 0.001$, **Figure 5A** and **Supplementary Table 1**]. The multivariate effect of Trial was driven by main effects on both latency and distance. Repeated pairwise comparisons of Trial 1 with Trials 2–15 revealed that latency (Bonferroni corrected $p < 0.05$, **Figure 5A** and **Supplementary Table 1**) and distance (Bonferroni corrected $p < 0.05$, **Figure 5B** and **Supplementary Table 1**) decreased significantly by the 3rd trial, whereas speed increased significantly by the 6th trial (Bonferroni corrected $p < 0.05$, **Figure 5C** and **Supplementary Table 3**). In addition, female animals had a significantly larger increase in speed across learning trials than males [$F_{(14, 294)} = 2.811$, $p = 0.001$, **Supplementary Table 3**], though there were no significant differences between sexes for latency and distances across trials.

When utilizing a model that did incorporate separate testing days into the model similar results were obtained. There was a multivariate effect of Trial [$F_{(8, 168)} = 3.732$, $p < 0.001$, **Supplementary Table 2**] driven by significant main effects of trial on both latency [$F_{(4, 84)} = 5.494$, $p = 0.001$, **Supplementary Table 2**] and distance [$F_{(4, 84)} = 2.809$, $p = 0.031$, **Supplementary Table 2**], but not speed [$F_{(4, 84)} = 2.396$, $p = 0.051$, **Supplementary Table 4**]. Bonferroni-corrected pairwise comparisons showed that animals traveled significantly less during Trials 2, 3, and 5 of any given day vs. Trial 1 (-1.888, -2.4843, -1.910, and -2.574 m respective changes; $p < 0.05$; **Figure 6C** and **Supplementary Table 2**). Similarly, latency significantly decreased from Trial 1 to Trials 2, 3, and 5 (-43.515, -61.82, and -72.84 s respective changes; $p < 0.05$; **Figure 6A** and **Supplementary Table 2**).

There was no multivariate effect of Day, independent of trial number. There was also no univariate main effect of Day on latency, speed, or distance traveled to reach the reward chamber. However, pairwise comparisons demonstrated that distance decreased significantly between Day 1 and 2 of training (-1.768 m, Bonferroni corrected $p = 0.002$; **Figure 6D** and **Supplementary Table 2**). There was no significant decrease between distance traveled Day 1 and 3 of training. The average latency across trials on Day 1 of the learning period was significantly longer than latencies on Day 2 (70.376 s, $p < 0.05$; **Figure 6B** and

FIGURE 4 | Non-continuous spatial maze learning performance in naked mole-rats. Preliminary data were collected (n = 8) using a non-continuous modified version of the Hebb-Williams maze (see **Figure 1A**) to investigate spatial learning and memory capabilities in naked mole-rats across 15 training trials (5 trials per day for 3 days). **(A)** Latency to reach the reward chamber was averaged across animals for each of the training/learning trials. Non-continuous maze performance exhibited a very gradual latency learning curve with a trend toward decreased latency across trials but large variability in individual performance. **(B)** The average number of entries into an error zone was plotted for all animals tested across the training/learning trials. No error reduction learning curve was present for the non-continuous maze. **(C)** Total distance traveled prior to reaching the reward chamber was averaged across animals for each trial and plotted for all training/learning trials. Non-continuous maze performance exhibited large variability in individual performance and no learning curve related to decreases in distance traveled (optimizing route to the reward chamber). m, meters; s, seconds.

FIGURE 5 | Continuous spatial maze learning performance in naked mole-rats. Data were collected (n = 27) using a continuous maze design (see **Figure 1B**) to investigate spatial learning and memory capabilities in naked mole-rats across 15 training trials (5 trials per day for 3 days). **(A)** Average latency to the reward chamber revealed a distinct learning curve across training/learning trials. Comparison of trial 1 to trials 2–15 revealed a significant decrease in latency by trial 3. **(B)** Average total distance traveled also exhibited a learning curve across training/learning trials. Comparison of trial 1 to trials 2–15 revealed a significant decrease in distance by trial 3. **(C)** Average speed traveled significantly increased across training trials by Trial 6. *p < 0.05 when compared to Trial 1. m, meters; s, seconds.

Supplementary Table 2) and Day 3 (76.556 s, p < 0.05; **Figure 6B** and **Supplementary Table 2**).

There was a significant multivariate Day by Trial interaction [$F_{(16, 36)}$ = 3.534, p < 0.001, **Supplementary Table 2**] driven by significant interactions for both latency [$F_{(8, 168)}$ = 6.006, p < 0.001, **Supplementary Table 2**] and distance [$F_{(8, 168)}$ = 4.084, p < 0.001, **Supplementary Table 2**]. There was also a significant interaction of Day and Trial on speed [$F_{(8, 168)}$ = 2.40, p = 0.018, **Supplementary Table 4**]. Both latency and distance demonstrated the greatest performance improvements across trials on Day 1 of training with performance improvements plateauing across trials in Days 2 and 3, showing that most learning occurred on Day 1.

There was no multivariate interaction between Day and Body Mass, however, there was a univariate effect on latency [$F_{(2, 42)}$ = 4.137, p = 0.023, **Supplementary Table 2**], but

not distance. Larger animals had higher latencies to reach the reward chamber in early days of training with these differences decreasing across days despite all animals traveling comparable distances.

No multivariate interaction of Trial by Age was present. However, there was a univariate effect of Trial by Age on distance [$F_{(4, 84)}$ = 2.580, p = 0.043, **Supplementary Table 2**] indicating better performance for younger animals in Trials 2 and 3, but for older animals in Trials 4 and 5. There was also a significant multivariate 3-way Day by Trial by Body Mass interaction [$F_{(16, 336)}$ = 1.988, p = 0.013, **Supplementary Table 2**] driven by significant interactions on both latency [$F_{(8, 168)}$ = 3.245, p = 0.002, **Supplementary Table 2**] and distance [$F_{(8, 168)}$ = 2.398, p = 0.018, **Supplementary Table 2**]. This effect indicated that the shape of the learning curve across days and trials was impacted by body mass with smaller animals showing faster
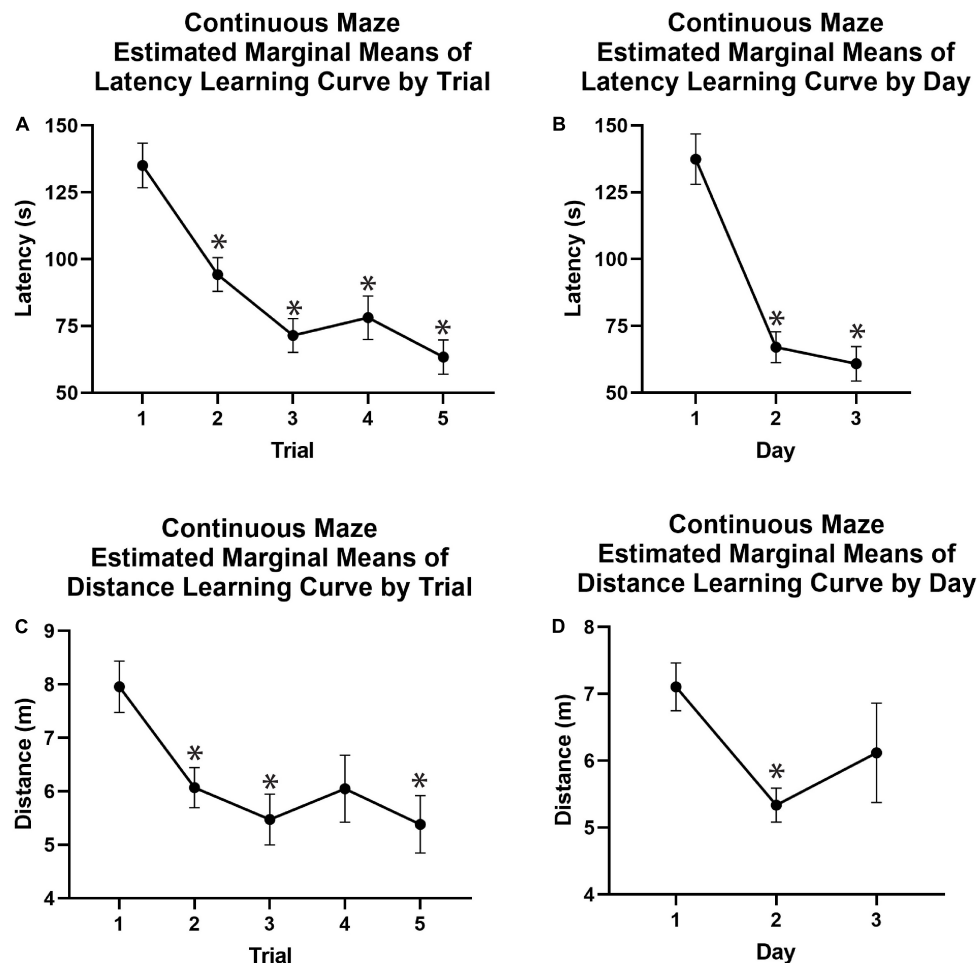
FIGURE 6 | Continuous spatial maze learning curves using estimated marginal means. **(A)** Latency to the reward chamber was significantly reduced on average in Trials 2, 3, 4, and 5 as compared to Trial 1 across Days 1–3, demonstrating a main effect of trial independent of day for latency. **(B)** The mean latency for each learning day showed that animals reached the reward chamber in significantly less time on Days 2 and 3 compared to Day 1. **(C)** Animals exhibited significant learning effects when comparing distance traveled on Trials 2, 3, and 5 to that of Trial 1 across Days 1–3, demonstrating a main effect of Trial independent of Day for distance. **(D)** Animals traveled significantly less distance on Day 2 compared to Day 1. Covariates appearing in the model were evaluated at the following values: Mass = 54.60, Age = 2.94.*$p < 0.05$. m, meters; s, seconds.

learning initially, with more variable performance in later training trials.

There was no Day by Trial by Sex multivariate interaction on performance, nor univariate effects on latency and distance. However, there was a significant 3-way univariate interaction on speed. Female animals increased speed throughout learning trials significantly more than males [$F_{(8, 168)} = 3.443$, $p = 0.001$, **Supplementary Table 4**].

No other multivariate or univariate effects or interactions (Day by Age, Day by Sex, or Day by Colony, Trial by Mass, Trial by Sex, and Trial by Colony) were significant for latency to reach the reward chamber, distance traveled, or speed.

In order to incorporate dominance ranking (a colony specific measure), into the GLM, the data set was split by colony for analysis. There were no significant effects of dominance rank on latency or distance across trials and days, nor were there any interactions of dominance rank with any of the other

variables in the model (for detailed statistics see **Supplementary Tables 1, 2**). Speed traveled varied by dominance rank for Colony B with subordinate animals traveling faster across trials than dominant animals (for detailed statistics see **Supplementary Tables 3, 4**).

These findings indicate that naked mole-rats exhibited significant spatial learning in the continuous maze. The bulk of the performance improvements occurred within the first 3 trials, indicating rapid spatial learning. Both latency and distance calculations were informative in assessing spatial learning gains in this particular maze configuration in naked mole-rats, suggesting that naked mole-rats were able to rapidly learn to navigate the continuous maze design.

Spatial Memory in Naked Mole-Rats

Mean performance was analyzed across memory probe trials for both the non-continuous maze and the continuous maze

FIGURE 7 | Continuous spatial maze memory performance in naked mole-rats. Animals were tested for spatial memory retention at 1 day, 1 week, and 1 month (memory probe 1, 2, and 3, respectively) following training. Dashed lines indicate the point of equivalence for performance on the last training day. **(A)** Latency across memory probe trials, with memory probe trial latency performance shown as a percentage of the last trial on the last training day in the learning phase of testing. No significant differences were found, indicating spatial memory retention, although this could be skewed by the high variability present. **(B)** Total distance traveled across memory probe trials, with distance traveled during the indicated memory probe trial shown as a percentage of the distance traveled on the last trial of the last training day. No significant impairments in performance were noted across the three time points.

(**Table 3**). There were no significant differences between the last trial of the last learning day vs. memory probe trials 1, 2, or 3 in assessing latency (**Figure 7A**) or distance traveled (**Figure 7B**), nor any effects of age, sex, ranking, or mass. This indicates that spatial memory was retained for at least 1 month in naked mole-rats.

DISCUSSION

The present study created a customized and effective maze design for testing spatial learning and memory in naked mole-rats. We predicted that animals would exhibit spatial learning by reaching the reward chamber faster (reduced latency) and more adeptly (shorter total distance traveled, and/or fewer errors committed, indicating optimized maze navigation) across training trials. This would result in steep learning curves and abrupt plateaus upon reaching peak performance. In order to effectively create a maze to evaluate these measures of learning and memory, we made targeted alterations to the Hebb-Williams maze design for use in naked mole-rats based on their unique sensory systems.

Naked mole-rats are functionally blind, with low visual acuity but some remnants of light sensitivity. The olivary pretectal nucleus, a region implicated in light detection, is small but maintains its typical morphology (Crish et al., 2006). Paired with conserved pupillary constriction responses to light, in addition to behavioral responses to light detection (e.g., freezing behaviors with sudden changes in brightness), naked mole-rats retain the ability to detect changes in light intensity (Hetling et al., 2005) but are otherwise visually impaired. Such reductions in

visual capabilities are thought to conserve sensory resources in favor of other modalities (e.g., tactile) that are more useful in underground tunnel systems which lack many meaningful visual cues that would facilitate behaviors such as navigation. Because traditional protocols for spatial mazes utilize visual cues, adjustments were necessary to accommodate the visual deficits of naked mole-rats.

The naked mole-rat's subterranean habitat is also thought to have driven a reduction in auditory capabilities. Naked mole-rats are most sensitive to frequencies between 65 and 12.8 kHz (Heffner and Heffner, 1993; Okanoya et al., 2018). Naked mole-rats demonstrate poor sound localization in addition to having smaller auditory brainstem nuclei related to binaural, high-frequency sound processing (Heffner and Heffner, 1993). Their cochleas are characterized by less extensive coiling (Mason et al., 2016). These features, along with differential variations in hair cell density within the cochlea (Barone et al., 2019), are all possible contributions to the demonstrated poor sound localization abilities in this species (Heffner and Heffner, 1993). Due to the relatively reduced auditory capabilities of naked mole-rats, auditory stimuli were not incorporated into the spatial maze design to help guide the animals to the reward chamber.

In contrast to auditory and visual systems, the somatosensory system of naked mole-rats is well-developed and includes an array of tactile hairs distributed on the entire body and face (Crish et al., 2003; Henry et al., 2006). With approximately 40 tactile hairs on each side of the body, naked mole-rats display robust orienting responses to stimulation of individual hairs (Crish et al., 2003). These sensory hairs are also present in some members of Bathyergidae (Bennett and Jarvis, 2004; Bennett et al., 2009; Walcher et al., 2018), providing sensory information that compensates for visual impairments in subterranean rodents (Jarvis and Bennett, 2017). Based on these sensory specializations, we constructed a spatial maze with tubing that was narrow enough to stimulate tactile hairs and provide spatial cues. Previous studies in the blind mole-rat demonstrated enhanced learning and memory performance when using similarly narrow passageways that provided tactile cues in spatial mazes (Kimchi and Terkel, 2004).

During the preliminary data collection phase of this study, we initially implemented a modified Hebb-Williams maze design that was non-continuous (**Figure 1A**). However, this maze design proved to be ineffective in eliciting the true spatial learning and memory capabilities of naked mole-rats, and no learning curves were evident when testing animals in the non-continuous maze (**Figure 6**). This may be due to the confounding distraction of tunnel dead-ends. Naked mole-rats exhibited a strong preference to attempt to dig or bite through the dead-ends of a tunnel rather than seek the reward chamber, which highly affected measures of spatial learning and memory (e.g., latency) in addition to affecting completion of the maze within the allotted time (i.e., Fail Rate, **Table 1**). These behavioral observations are supported by previous studies that have shown that naked mole-rats are highly motivated to remove any blockages in their tunnel systems (Brett, 1991).

Based on the results of the non-continuous maze, we investigated motivators that would work well paired with the

coinciding desires to bite and dig in a tunnel setting. Typically, spatial mazes rely on food rewards to motivate animals to complete the maze (Olton and Samuelson, 1976; Brown and Cook, 1986; du Toit et al., 2012). However, the colony structure of naked mole-rats makes it difficult to food deprive animals without permanently isolating them from their colony, and this in turn is likely to adversely affect behavioral performance. Thus, it was necessary to devise alternative methods of motivation. Naked mole-rats have been classified as poikilotherms—poor regulators of their own body temperature (Yahav and Buffenstein, 1992), but this varies with their activity state and thus they may be more accurately described as heterothermic (Kovalzon et al., 2020, 2021). Therefore, a heat lamp was added to the reward chamber as a motivator (**Table 2** and **Figure 3**). As burrowing rodents, the animals were quick to enter the reward chamber when bedding was present and they immediately began to dig in it and to kick it around the chamber. With both home and foreign colony bedding proving to be effective motivators (**Table 2** and **Figure 3**), we incorporated a combination of home colony bedding and a heat lamp in the reward chamber for subsequent maze testing, supporting previous research showing the desirability of home bedding (O'Riain and Jarvis, 1997; Deacon, 2012; Toor et al., 2015). Foreign colony bedding—though effective at increasing time spent in the reward chamber and decreasing latency to the reward chamber—was determined to be suboptimal and was not used in subsequent maze testing. This decision was based on the territorial nature of naked mole-rats (O'Riain and Jarvis, 1997; Braude et al., 2021) and the risk of an experimental animal being attacked upon reintroduction to the home colony due to smelling like an intruder.

In our re-designed, continuous version of a Hebb-Williams maze, there were no dead-ends that the naked mole-rats could have interpreted as a blockage in their tunnel systems (**Figure 1B**), resulting in a reduced fail rate (**Table 1**). In contrast to the non-continuous maze (**Figure 4A**), learning curves were evident in the continuous maze (**Figure 5A**). When using a repeated measures MANOVA, both latency and distance across learning trials displayed significant learning curves across trials and days (**Figures 5A,B**). Thus, the current study demonstrated spatial learning in naked mole-rats using a customized, continuous maze design, with latency to the reward chamber and distance traveled as measurements of learning. Smaller animals demonstrated faster learning (**Supplementary Tables 1**, **2**). There was no multivariate effect of Day by Trial by Age. The overall effects of age on performance may be diminished due to the comparatively small age range tested in the current study (approximately 7 years) compared to the over 30 year life span of captive naked mole-rats (Buffenstein and Craft, 2021; Buffenstein et al., 2022). In a Day by Trial by Sex interaction, neither latency nor distance were significantly different between sexes. One possible explanation for the lack of effects of sex on spatial learning and memory is that these differences would be present in species with sex differences in home ranges (Costanzo et al., 2009), of which our naked mole-rats do not exhibit due to shared burrow systems for the males and females. There was no significant effect of dominance ranking (**Supplementary Tables 1**, **2**) despite

previous publications showing an effect of social hierarchy on spatial memory (Costanzo et al., 2009).

Using repeated measures MANOVA analyses to analyze memory retention of spatial learning gains, significant memory performance declines were not evident. This indicates good memory retention for at least 1 month (**Table 3** and **Figure 7**), however, it is important to note that the motivators were present in the reward chamber during memory probe trials and the animals could have been following sensory cues rather than explicitly utilizing spatial memory. These results are supported by previous studies showing that other species of mole-rats didn't experience decreases in spatial memory performance/retention until 1 month following training – outperforming more distant relatives within Rodentia, the rat and the prairie vole (Kimchi and Terkel, 2001).

CONCLUSION AND FUTURE DIRECTIONS

The results of this study provide novel insight into the considerations that need to be taken when establishing behavioral tests for atypical animal models. In conducting behavioral testing of members of Bathyergidae, it is important to create tasks suited to their evolutionary adaptations. This includes modifying traditional maze setups to account for sensory capabilities and subterranean specializations. In addition, atypical motivators (e.g., home bedding) and removal of competing and confounding motivators (e.g., tunnel dead-ends) may need to be incorporated in order to optimize a species' performance in a behavioral task and to elicit a true representation of the species' spatial learning and memory capabilities. For naked mole-rats specifically, the use of a heat lamp paired with home bedding in the reward chamber effectively replaced typical motivators (food or liquid rewards following food deprivation) which are not ideal for animals within a social colony structure where isolation could prove detrimental. Because *H. glaber* has many behavioral and anatomical distinctions from other bathyergids, the methods used in the current study would likely require certain modifications in order to optimize spatial learning and memory testing for other members of Bathyergidae.

An apparent floor effect was evident in the steep learning curve associated with continuous maze testing in naked mole-rats, but latency and distance proved to be effective measures of spatial learning (**Figure 5**). In addition, the acquired spatial learning was retained for at least 1 month (**Figure 7**). In future studies, the continuous maze design that we developed would benefit from increased complexity in order to better test the extent of spatial learning and memory. Further time points (e.g., 2 months, 6 months, 1 year) and comparing memory probe trial performance with and without motivators present in the reward chamber may help to elucidate the true extent of spatial memory retention in naked mole-rats, in addition to determining factors that may affect memory loss.

This study provides a basis for future behavioral tests to be developed to aid translational research such as the use of naked mole-rats in neurodegenerative research (Markey et al., 2015)

or examination of spatial learning and memory impairments following tooth loss (Okamoto et al., 2010; Chen et al., 2018; Lin et al., 2020), in addition to basic science research focused on evolutionary adaptations specific to burrowing rodents (Edrey et al., 2011; Smith et al., 2015; Grimes et al., 2017). Given the unique adaptations of sensory systems in bathyergids, future investigation of their behavioral use of specialized senses such as magnetic orientation (Oliveriusová et al., 2012) could yield valuable information about how these systems contribute to learning and memory performance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Southern Illinois University, Carbondale, IL, United States.

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AUTHOR CONTRIBUTIONS

NH and DS contributed to the experimental design. NH and LA collected data for the study. NH and KS analyzed the data. NH, KS, and DS interpreted the data. NH prepared the manuscript figures. NH and DS drafted the manuscript with revisions made by NH, LA, KS, and DS. All authors contributed to the article and approved the submitted version.

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Capture Order Across Social Bathyergids Indicates Similarities in Division of Labour and Spatial Organisation

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The social mole-rats of the family Bathyergidae show elaborate social organisation that may include division of labour between breeders and non-breeders as well as across non-breeders within their groups. However, comparative behavioural data across the taxa are rare and contrasts and similarities between species are poorly understood. Field studies of social bathyergids usually involve capturing all group members until the entire group is captured. Because each animal is only captured once and traps are typically placed in close proximity to active foraging areas, the order in which animals are captured provides an indication of the foraging activity of different individuals and of the spatial organisation of the group within the burrow system. Here, we compare the association of capture order with breeding status, sex, and body mass in four species and subspecies of social bathyergids, which vary in group size and represent all three social genera within the family Bathyergidae. We show that in naked and Damaraland mole-rats (*Heterocephalus glaber* and *Fukomys damarensis*), male and female breeders are captured later than non-breeders, whereas in two different subspecies of the genus *Cryptomys* only female breeders are captured later than non-breeders. The effect sizes vary largely and are 10 times larger in naked mole-rats as compared to *Fukomys* and 3–4 times larger than in *Cryptomys*. Among non-breeders, sex effects are notably absent in all species and body mass predicted capture order in both naked and Damaraland mole-rats. In naked mole-rats, larger non-breeders were captured earlier than smaller ones, whereas in Damaraland mole-rats intermediate-sized non-breeders were captured first. Our data suggest that there are similarities in behavioural structure and spatial organisation across all social bathyergid species, though the most pronounced differences within groups are found in naked mole-rats.

Keywords: cooperative breeding, division of labour, helping behaviour, cooperation, Bathyergidae, social evolution, eusociality, cooperative behaviour

INTRODUCTION

The African mole-rats (family Bathyergidae) currently consist of 30 species with varying degrees of sociality, from solitary living to cooperatively breeding groups (Jarvis and Bennett, 1993; Faulkes et al., 1997; Bennett and Faulkes, 2000; Visser et al., 2019; Faulkes and Bennett, 2021). Group living occurs in three genera (*Heterocephalus*, *Fukomys*, and *Cryptomys*) and has probably evolved twice independently, once in *Heterocephalus* and once in the ancestral state of the *Fukomys/Cryptomys* complex (Jarvis and Bennett, 1993; Faulkes et al., 1997; Faulkes and Bennett, 2016). Although there are large differences in the mean and the maximum group sizes across these three genera, there are also important similarities in the social organisation of mole-rats (Bennett and Faulkes, 2000). Mole-rat groups mostly consist of a monogamous pair and their offspring that delay dispersal and remain philopatric for extended periods, generating groups with high reproductive skew and high average relatedness between individuals (Burland et al., 2002; Bishop et al., 2004; Patzenhauerová et al., 2013). The main cooperative activity of all group members is to contribute to excavating a large underground burrow system that provides access to their main food source, which are plant tubers and roots (Brett, 1991; Bennett and Faulkes, 2000; Šumbera et al., 2012; Thomas et al., 2016). The extensive involvement of non-breeders in these activities and the possibility that, like in social insects, some individuals may specialise in certain activities have generated substantial research interest in division of labour in social mole-rat groups. However, data from wild populations are rare, and it remains unclear to what extent different species show similarities in their behavioural organisation (Jarvis, 1981; Bennett, 1990; Braude, 1991; Lacey and Sherman, 1991; Lacey and Sherman, 1997; Scantlebury et al., 2006; Zöttl et al., 2016a; Siegmann et al., 2021).

Studies of social mole-rats in captivity highlight that investment in cooperative burrowing often differs between breeders and non-breeders and across non-breeders of different sizes. In captive naked mole-rats (*Heterocephalus glaber*), both male and female breeders may be substantially less involved in burrowing than non-breeders (Jarvis, 1981; Jarvis et al., 1991). Some studies have also suggested that smaller non-breeding individuals spend more time excavating than larger non-breeders (Jarvis, 1981; Faulkes et al., 1991; Lacey and Sherman, 1991; Jacobs and Jarvis, 1996) though other studies identified large variation around these patterns and suggest that body size may not be a particularly good predictor of burrowing activity (Jarvis et al., 1991; Lacey and Sherman, 1991, 1997; Mooney et al., 2015; Gilbert et al., 2020; Siegmann et al., 2021). In the second social species that has been studied in detail in captivity, the Damaraland mole-rat (*Fukomys damarensis*), breeders show substantial contributions to burrowing behaviour, especially in relatively small groups, whereas in larger groups the relative time investment of breeders in burrowing behaviour is lower than that of non-breeders (Houslay et al., 2020). Some studies suggested that small non-breeding individuals invest more time in burrowing behaviour than larger non-breeders (Bennett, 1990; Jacobs et al., 1991; Gaylard et al., 1998) though later studies

on a large number of known-age individuals showed that, as individuals grow, they increase their investment in burrowing behaviour until they reach the age of 1–1.5 years when rates of burrowing start to decline (Zöttl et al., 2016a; Thorley et al., 2018). This decline coincides with the age of dispersal in wild groups and produces a pattern where individuals of intermediate body sizes may be the individuals with the highest time investment in cooperative burrowing (Zöttl et al., 2016a; Thorley et al., 2018; Torrents-Ticó et al., 2018a,b). Substantially less research has focussed on the cooperative behaviour of other species in the genera *Fukomys* and *Cryptomys*; those studies that have done so find that while age effects on general activity are often present, there are rarely consistent differences related to breeder status or body mass (Bennett, 1989; Burda, 1989, 1990; Moolman et al., 1998; Schielke et al., 2012; Van Daele et al., 2019).

Behavioural studies of social mole-rats in the wild are rare, and it is possible that the effects of breeding status and body mass on cooperative behaviour may be more pronounced than in captivity. For example, in Damaraland mole-rats, the difference in foraging investment between breeders and non-breeders was larger in a wild population than in a captive population (Francioli et al., 2020; Houslay et al., 2020). Additionally, radio tracking of giant mole-rats and Ansell's mole-rats (*Fukomys mechowii* and *Fukomys anselli*) suggested that space use and activity patterns differed between breeders and non-breeders and possibly also across non-breeders of different sizes (Lövy et al., 2013; Šklíba et al., 2014, 2016). By contrast, a recent study on Natal mole-rats (*Cryptomys hottentotus natalensis*) did not detect any differences in foraging activity of breeders and non-breeders, or across non-breeders of different sizes (Finn et al., 2022). However, it remains difficult to compare the results from these studies quantitatively because they employ different biologging methodologies–either radio-tracking or RFID-based monitoring systems (radio-frequency identification)–leading to different types of data and often largely contrasting sample sizes. Additionally, the species of different genera often differ in mean group size and the comparability of data across different species of mole-rats would be greatly improved if the data were collected with similar methods and normalised across group sizes to control for differences in mean group size between species.

One source of information on the behavioural organisation of social mole-rats in the wild is the order in which individuals are captured from groups ("capture order"; Jarvis, 1985; Bennett, 1989; Brett, 1991; Bennett and Faulkes, 2000). When capturing social mole-rats, Hickman traps baited with sweet potato are placed in underground tunnels close to active foraging areas (Hickman, 1979). This means that within groups, individuals that are more active in these foraging areas will have a higher probability of being captured early on, whereas individuals that visit these areas less often will likely be captured later. As a consequence, capture order can give an indication of division of labour and spatial organisation within groups of social mole-rats and has the advantage of using a standardised method across species. Previous field studies of naked and Damaraland mole-rats suggested that reproductive females are often captured among the last animals within groups (Brett, 1991; Jacobs et al., 1991). Across non-breeders, body mass may affect capture order

in naked mole-rats, though the evidence is mixed with some studies finding a negative correlation between body mass and capture order in a majority of groups (5 out of 6 groups, Brett, 1991) and other studies only finding such a relationship in some of the groups (4 out of 12, Jarvis, 1985). Among Damaraland mole-rats and common mole-rats (*Cryptomys hottentotus*), such correlations have rarely been found (Bennett, 1989; Jacobs et al., 1991; Bennett et al., 1994; Voigt et al., 2019).

Here, we use capture order data from field studies of four species and subspecies of bathyergid mole-rats (naked mole-rats, Damaraland mole-rats, Natal mole-rats [*Cryptomys hottentotus natalensis*], and Mahali mole-rats [*Cryptomys hottentotus mahali*]), representing all three major social taxa *Heterocephalus*, *Fukomys*, and *Cryptomys*, as an index of variation of cooperative behaviour across these species. To ensure comparability between the species we use the same trapping techniques in all four species and use a z-transformation to control for differences in mean group size across the species (Cheadle et al., 2003; Curtis et al., 2016; Verhulst, 2020). This transformation produces a normalised capture index where capture rank is represented in standard deviations from the mean capture rank within each group ensuring that model estimates of effect sizes from species with different group and sample sizes are directly comparable. We first assess whether breeding males and breeding females are captured later than non-breeders and secondly analyse the association between capture order and body mass and sex in all four species. Finally, we compare the effect sizes of breeder status and body mass across all four species to assess their similarities in division of labour and spatial organisation.

MATERIALS AND METHODS

Naked Mole-Rat Field Study

We captured naked mole-rats (*Heterocephalus glaber*) in Meru National Park, Kenya within a 5 km radius of the Bawatherongi campsite (0.163389°, 38.212167°) between May 1 and August 20, 1989. Each group was trapped only once that year using a modification of the Hickman trap (Hickman, 1979). Active burrows were identified by the fresh volcano shaped pile of soil kicked up by the mole-rats. The tunnels beneath the volcanoes were excavated for as little as 10 cm and no more than 1 m. The traps were placed at opened tunnels, covered with soil, and shaded. Subsequently, we visually monitored the traps and removed the animals immediately upon capture. Trapping began at sunrise and ended at sunset due to the high density of lions within the park. Up to 6 traps were set in a cluster of volcanoes. Mole-rats were numbered with a permanent marker and kept in a plastic basin full of soil in the shade until the end of the trapping day. Sweet potato was placed in the tunnel openings overnight to prevent untrapped mole-rats from blocking the tunnels and to encourage them to continue visiting the location. All animals were housed in 1 × 0.5 m rectangular metal boxes in the laboratory until the entire group was captured. Complete capture was determined by the observation that no sweet potato was eaten overnight, no mole-rats were captured, and tunnels remained unblocked for 3 days. The animals were weighed, sexed, and their

breeding status was determined by visual inspection (Braude et al., 2001). Breeding females were identified by their elongated bodies, perforate vagina, and prominent nipples. Breeding males were identified by their prominent genitalia and very thin bodies. Animals were then permanently marked by toe-clipping and were released back into the burrow from which they were captured.

In total, the dataset consisted of 11 groups, including 676 individuals (23 breeders and 653 non-breeders). Protocols were approved by and conducted under the auspices of the Research Division of the Office of the President of Kenya, Permit #15C/116.

Damaraland Mole-Rat Field Study

We captured Damaraland mole-rats (*Fukomys damarensis*) in the area surrounding the Kuruman River Reserve (−26.978560°, 21.832459°) in the Kalahari Desert of South Africa between September 2013 and May 2020 (Finn et al., 2018; Thorley et al., 2021). The groups were trapped every 6–12-months using modified Hickman traps that were baited with sweet potato (Hickman, 1979). When foraging and expanding their tunnel systems, mole-rats create characteristic mounds of sand and we accessed their burrows by digging a trench between mounds and exposing the tunnel. Subsequently, we trapped all individuals, one by one, until each individual of the group was captured and all traps remained untouched for at least 24 h. During capturing, the traps were checked every 2–3 h throughout the day. After capture, the mole-rats were kept in a closed box and transported to the laboratory where they were sexed, weighed to the nearest gram and their breeding status was determined. For each individual we also recorded the capture time and derived a capture order as a rank-index where the first individual captured in a group received the index "1," the second the number "2," etc. Individuals that were captured at the same time, both received a tied rank index number (e.g., 7.5 if they were captured as 7th and 8th individual). The presence of ties may slightly reduce existing variance in capture ranks but is unlikely to bias patterns of capture order. While in the laboratory the captured animals were housed in custom built tunnel systems made out of PVC tubes with Perspex windows and provided with nesting material and sweet potato. Breeding females are commonly the largest females in their group and can be identified from their perforated vagina and prominent teats, whereas breeding males were identified using longitudinal capture records and body mass data. All captured individuals were marked with passive integrated transponder (PIT; Trovan Unique, DorsetID, Netherlands) tags on the first capture to facilitate individual identification throughout the study.

In total, the dataset consisted of 91 groups, 1,209 individuals, and 2,588 individual capture events (507 breeders and 2,081 non-breeders).

The fieldwork protocols were reviewed and approved by the Animal ethics committee of the University of Pretoria (EC032-13, EC006-15, EC050-16, EC059-18) and subsequently approved by Northern Cape Nature Conservation.

Natal Mole-Rat Field Study

We captured Natal mole-rats (*Cryptomys hottentotus natalensis*) during field work at Glengarry Holiday Farm in KwaZulu-Natal, South Africa (−29.322530°, 29.712982°) from August 2019 to

July 2021. The groups were captured with the same methods as described for Damaraland mole-rats, except that traps were checked from sunrise through to 10 p.m., and the door to the trap closed overnight during winter if the air temperature was near freezing. The groups were recaptured at 6-month intervals. Group membership was initially determined by capture location, a lack of aggression between individuals, and later confirmed by recapture of individuals in the same group during subsequent captures. For each individual we also recorded the capture time and derived a capture order as a rank-index where the first individual captured in a group received the index "1," the second the number "2," etc. Individuals that were captured at the same time, both received a tied rank index number (e.g., 7.5 if they were captured as 7th and 8th individual). The captured mole-rats were housed with family members at ambient temperature (15–25°C) in large boxes provided with wood shavings as bedding and fed sweet potato *ad libitum*. All animals were sexed, weighed to the nearest gram, and assigned a reproductive status at capture. Reproductive females were identified by a perforate vagina and prominent teats (Bennett and Faulkes, 2000). Male reproductive status was determined by body mass, presence of testes, and prolonged presence in the group. All individuals greater > 20 g were implanted with a subcutaneous PIT tag (Trovan Unique, DorsetID, Netherlands) to uniquely identify them. After 48 h without any signs of individuals present in the burrow system (i.e., no blocked tunnels, fresh mounds, triggered traps, or bait eaten), the group was assumed to be completely captured and the group was released back into their burrow.

In total, the dataset consisted of 47 groups, 385 individuals, and 577 individual capture events (125 breeders and 452 non-breeders). The field protocols were approved by the animal ethics committee of the University of Pretoria (EC001-19) and the Department of Forestry and Fisheries (DALRRD section 20 12/11/1/8, 1468). Permission to trap the mole-rats was granted by Ezemvelo Nature Conservation (Permit OP27-2020, OP1545-2021).

Mahali Mole-Rat Field Study

We captured Mahali mole-rat (*Cryptomys hottentotus mahali*) groups between October 2016 and September 2017 at Patryshoek, Pretoria (−25.663333°, 28.039583°), South Africa. The mole-rats were captured using Hickman traps, baited with a small piece of sweet potato (Hickman, 1979). The traps were placed at the entrance of excavated burrows, in close proximity to mole-rat mounds. We captured the entire group, and a group was considered completely captured when no trap activity (i.e., tunnel blocking or animal capture) was observed within five consecutive days. For a different study purpose, the animals were permanently removed and subsequently sacrificed (Hart et al., 2021). On capture, the body mass of each animal was recorded to the nearest 0.01 g. The breeding males were distinguishable from non-breeding males by their large descended inguinal testes and yellow staining around the mouth. Furthermore, the breeding males were usually, but not always, the largest male in each group (Bennett and Faulkes, 2000; Hart et al., 2021). The breeding females possessed prominent axillary teats and a perforated vagina, which was absent in the non-breeding females.

During the dissection process, the breeding status of females was confirmed by the presence of foetuses or placental scars on the uterine horns of breeding females.

In total, the dataset consisted of 32 groups including 265 individuals (65 breeders and 200 non-breeder). The field protocols were approved by the Animal Ethics Committee of the University of Pretoria (No. EC044-16) and the Department of Forestry and Fisheries DALRRD section 20 12/11/1/8 (MG). Permission to trap the mole-rats was granted by the Gauteng Department of Nature Conservation (Permit No. CPF6-0127).

Data Management and Statistical Analyses

For all four species we removed groups where the group size was smaller than three individuals and groups where no reproductive females were captured. We also removed juvenile individuals that are known to contribute very little to cooperative behaviour in the groups (Lacey and Sherman, 1991; Zöttl et al., 2016a) with cut-off weights that correspond roughly to 20% of adult body mass (naked mole-rat 15 g, Damaraland mole-rat 50 g, Natal mole-rat, and Mahali mole-rat 40 g).

To facilitate direct comparability of the effect sizes and alleviate the differences arising from contrasts in mean group size across species, we z-transformed the capture index for each group capture event using the "scale" function in R (hereafter referred to as z-score). Transforming data to a z-score is achieved by first subtracting the mean capture index for a given group capture from each individual capture index. This results in negative values for early captured individuals and positive values for late captured individuals. Subsequently, we divided the mean centred data by the standard deviation of the capture index per group. The result of this transformation is that each individual's z-score now represents the capture order expressed in standard deviations (SD) distance from the mean group size. This z-score is directly comparable between species that differ in group size and similar transformations are commonly used in meta-analyses or for comparing results that are biassed by analytical batch effects (Cheadle et al., 2003; Curtis et al., 2016; Verhulst, 2020).

To estimate the effects of breeding status and sex on capture order we fitted a linear mixed effect model with the capture order z-score as the response variable, and sex and breeding status as fixed factors, including the interaction between sex and breeding status. Because some individuals were captured repeatedly over the study population in Damaraland mole-rats and Natal mole-rats, we also included a random effect of animal identity for these species. In contrast, in naked mole-rats and in Mahali mole-rats no repeated captures of the same individuals were recorded, so we instead fitted the equivalent linear model (omitting the random effect).

To assess whether body mass among non-breeding individuals is associated with capture order, we excluded all breeders from the data set and carried out the z-transformations for capture order index and body mass. Because males and females in three of the four species show pronounced sexual dimorphism in body mass, we applied the same z-transformation for females and males within each species (Schielzeth, 2010). Subsequently, we

fitted a linear mixed effect model with the capture order index (z-score) as the response variable, sex as fixed factor, body mass (z-score) and squared body mass (z-score, allowing non-linear relationships) as covariates. Again, in models for Damaraland mole-rats and Natal mole-rats we included the animal identity as a random effect, whereas for naked and Mahali mole-rats we fitted the equivalent linear model, omitting any random effects.

All analyses were carried out in R (R Core Team, 2019).

RESULTS

Breeding Status and Sex-Related Differences

In both naked mole-rats and Damaraland mole-rats breeders were captured later than non-breeders. Though the results are qualitatively similar, the effect size of breeders being captured later was about 10 times higher in naked mole-rats than in Damaraland mole-rats (**Tables 1A,B** and **Figure 1**). It is important to note that because the response variable was z-transformed within each group, differences in group size cannot explain differences in effect size. Similarly, breeding females of Natal mole-rats and Mahali mole-rats were captured later than non-breeders (**Tables 1C,D** and **Figure 1**). However, in contrast to naked and Damaraland mole-rats, breeding males of these species clustered with non-breeders and were captured significantly earlier than breeding females (**Tables 1C,D** and **Figure 1**). In these two species, the effect sizes of the breeding females being captured later were about 3–4 times higher than in Damaraland mole-rats and about 3–4 times lower than in naked mole-rats (**Figure 1**).

Body Mass Effects Across Non-breeders

Across non-breeders, in naked mole-rats we found that larger individuals were captured earlier than smaller group members and there was no indication of a convincing quadratic effect of body mass on capture order (**Table 1A** and **Figure 2A**). In contrast, in Damaraland mole-rats, body mass has a quadratic effect on capture order, indicating that intermediate sizes of non-breeders were captured first, and smaller and larger non-breeders both were captured later (**Table 1B** and **Figure 2B**). Also, with respect to this result, it is noteworthy that the effect size in naked mole-rats was larger than the body mass effect in Damaraland mole-rats. We did not find an effect of body mass on capture order in Natal and Mahali mole-rats. Because the sample sizes in these two species were substantially smaller than in Damaraland mole-rats, effects of similar size and uncertainty as in Damaraland mole-rats would not have been statistically significant in these species (**Tables 1C,D** and **Figures 2C,D**).

DISCUSSION

Because of the potential similarities to the social organisation of eusocial insects, the social organisation of the African mole-rats has been a widely and controversially debated topic over the last 40 years (Jarvis, 1981; Bennett, 1990; Bennett and Faulkes,
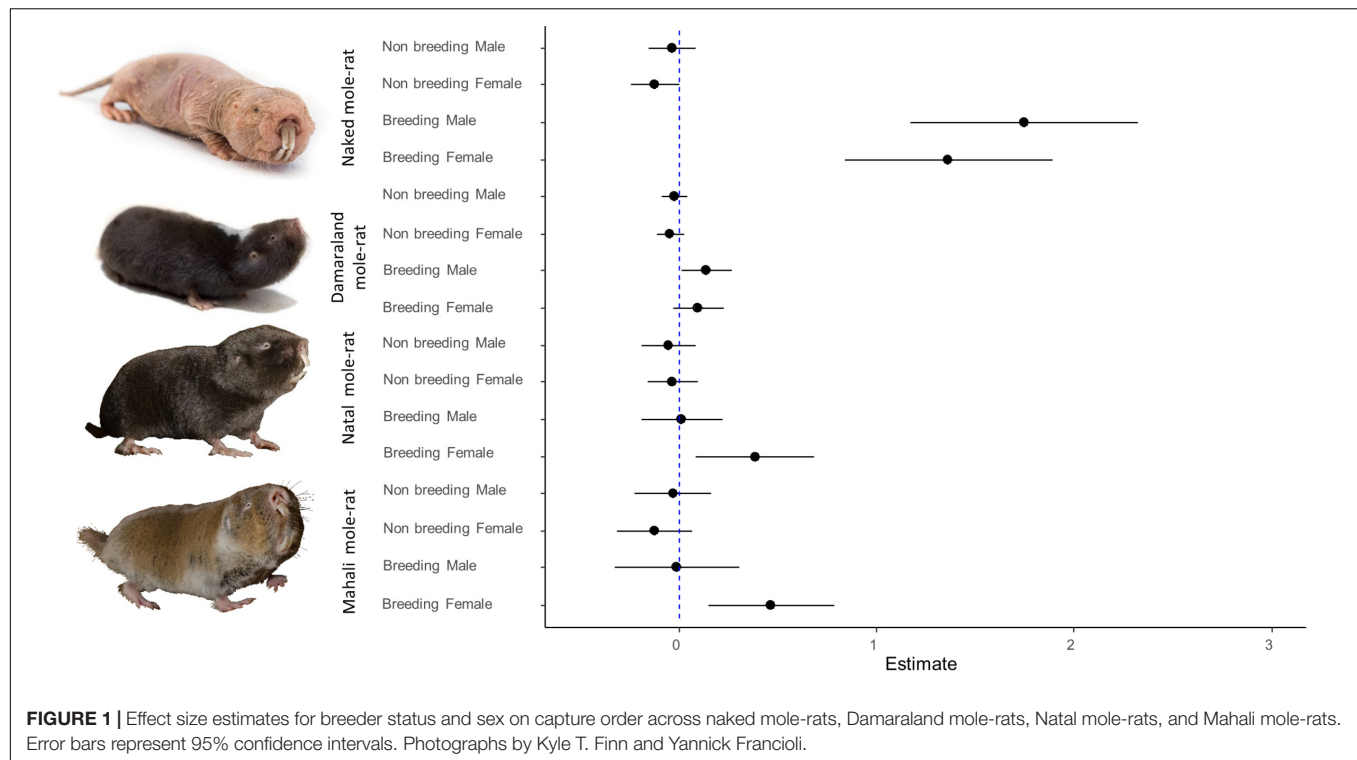
TABLE 1 | The effects of breeder status, sex, and body mass on capture order in four species of social *bathyergid* mole-rats.

	Estimate	SE	*t*-value	*p*-value
(A) Naked mole-rats				
Effects of breeder status and sex ($R^2 = 0.12$)				
Females (Intercept)	−0.12	0.06	−1.91	0.06
Males	0.09	0.09	1.02	0.31
Reproductive status	1.49	0.28	5.38	<0.001
Sex x Reproductive status	0.29	0.41	0.72	0.47
Size effects across non-breeders ($R^2 = 0.07$)				
Females (Intercept)	−0.05	0.06	−0.76	0.45
Male	0.09	0.09	1.05	0.29
Body mass (z-score)	−5.33	0.96	−5.57	<0.001
Body mass (z-score)^2	1.49	0.96	1.56	0.12
(B) Damaraland mole-rats				
Effects of breeder status and sex (marginal R^2/conditional R^2: 0.005/0.075)				
Females (Intercept)	−0.04	0.03	−1.26	0.21
Males	0.02	0.05	0.49	0.62
Reproductive status	0.14	0.07	1.99	0.048
Sex x Reproductive status	0.02	0.10	0.17	0.87
Size effects across non-breeders (marginal R^2/conditional R^2: 0.02/0.05)				
Females (Intercept)	0.00	0.03	−0.14	0.89
Male	0.01	0.05	0.26	0.80
Body mass (z-score)	−4.02	0.94	−4.29	<0.001
Body mass (z-score)^2	3.66	0.94	3.91	<0.001
(C) Natal mole-rat				
Effects of breeder status and sex (marginal R^2/conditional R^2: 0.02/0.14)				
Females (Intercept)	−0.03	0.07	−0.49	0.63
Males	−0.02	0.10	−0.19	0.85
Reproductive status	0.42	0.17	2.52	0.01
Sex x Reproductive status	−0.35	0.21	−1.70	0.09
Size effects across non-breeders (marginal R^2/conditional R^2: 0.001/0.13)				
Females (Intercept)	0.02	0.06	0.36	0.72
Male	−0.04	0.09	−0.45	0.65
Body mass (z-score)	−0.42	351.26	−0.46	0.65
Body mass (z-score)^2	−0.18	388.16	−0.20	0.84
(D) Mahali mole-rat				
Effects of breeder status and sex ($R^2 = 0.04$)				
Females (Intercept)	−0.12	0.10	−1.26	0.21
Males	0.09	0.14	0.68	0.50
Reproductive status	0.59	0.19	3.12	<0.01
Sex x Reproductive status	−0.57	0.27	−2.14	0.03
Size effects across non-breeders ($R^2 = 0.02$)				
Females (Intercept)	−0.03	0.10	−0.33	0.75
Male	0.07	0.14	0.52	0.61
Body mass (z-score)	−1.11	0.92	−1.20	0.23
Body mass (z-score)^2	1.52	0.92	1.65	0.10

Shown are the estimates, standard error of the mean (SE), t-value, and p-value for (A) naked mole-rats, (B) Damaraland mole-rats (C) Natal mole-rats, and (D) Mahali mole-rats.
x denotes interactions between fixed factors.

2000; Burda et al., 2000; Scantlebury et al., 2006; Faulkes and Bennett, 2016, 2021; Braude et al., 2021; Thorley et al., 2021; Buffenstein et al., 2022). Despite this interest, our understanding of the behavioural organisation of social mole-rats has been

FIGURE 1 | Effect size estimates for breeder status and sex on capture order across naked mole-rats, Damaraland mole-rats, Natal mole-rats, and Mahali mole-rats. Error bars represent 95% confidence intervals. Photographs by Kyle T. Finn and Yannick Francioli.
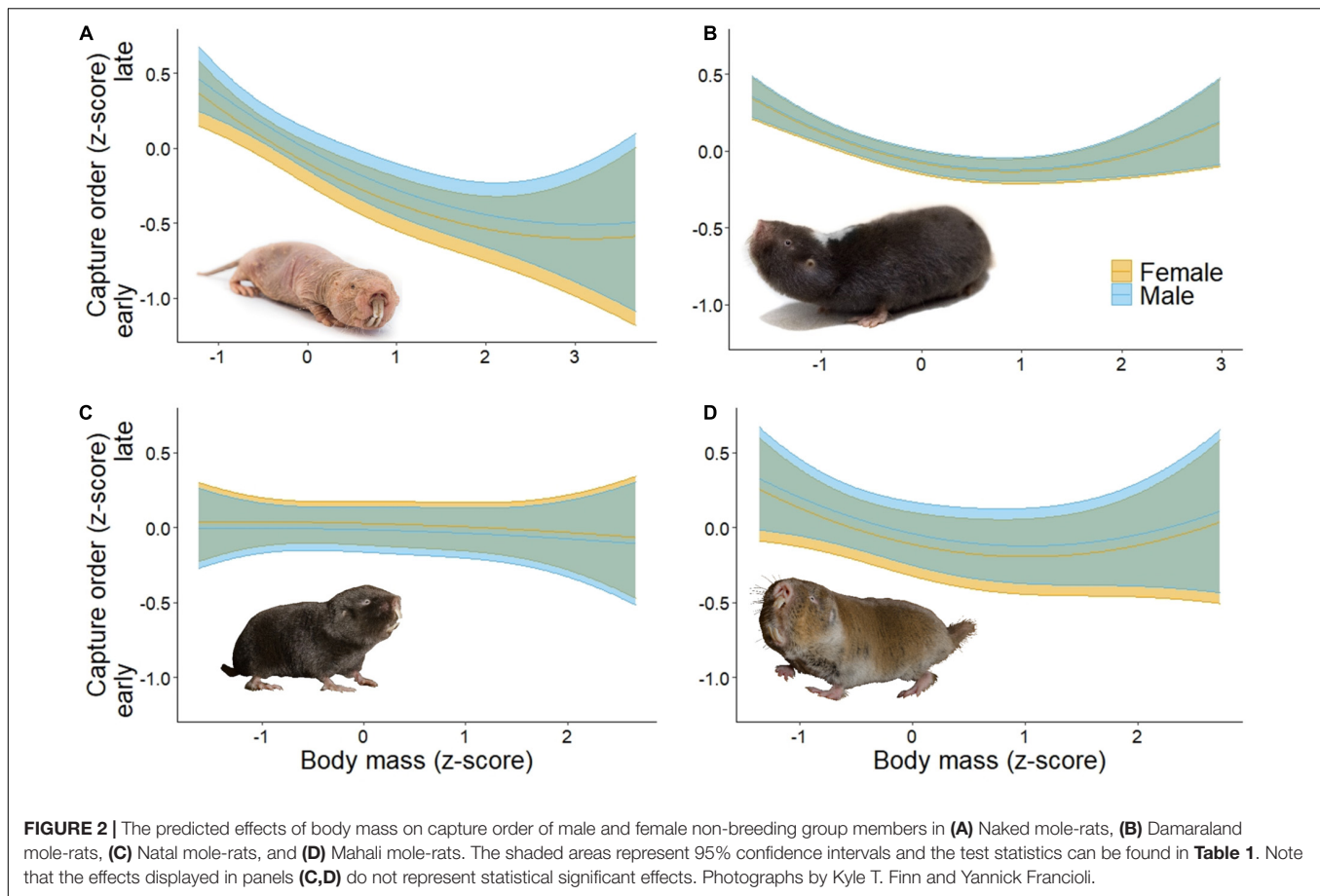
limited by the difficulty of collecting behavioural data from wild populations and by the lack of comparative data across different species of social mole-rats. We used capture order data from wild populations of four species of social bathyergid mole-rats to assess similarities in the behavioural and spatial organisation across the three social genera (*Heterocephalus*, *Fukomys* and *Cryptomys*). Our results suggest that across social mole-rats, breeding females are less active and less involved in cooperative burrowing and foraging. Although there are qualitative similarities in the social organisation of different mole-rat species, our results show that there are large quantitative differences between species. The differences between breeders and non-breeders in cooperative investment are much larger in naked mole-rats than in the Damaraland mole-rat, the Mahali mole-rat, and the Natal mole-rat. Additionally, only in naked mole-rats was capture order among non-breeders strongly associated with body mass, whereas such body mass effects were small in Damaraland mole-rats and possibly absent in both subspecies of *Cryptomys hottentotus*.

The results of our study align well with some previous studies of naked mole-rat behaviour and help to clarify some of the open questions about labour division in this species. In naked mole-rats, one of the most critical parts of cooperative burrowing is that individuals need to eject the surplus sand from their burrows through open mounds. This is energetically costly and potentially risky because of the exposure to predators (Jarvis, 1981; Braude, 1991; Brett, 1991) and previous studies suggested that large individuals may engage more in this activity than small individuals (Braude, 1991). Our data corroborate these suggestions by showing that in naked mole-rats, large

non-breeders are captured earlier than smaller ones when the traps baited with sweet potato are placed in close proximity to active volcanoes. Because burrowing and ejecting sand from tunnels is not possible in captivity, studies in artificial tunnels systems have quantified this behaviour by recording how much substrate is moved by individuals and by the amount of time individuals spend gnawing on obstructions or the tunnel walls. These studies show that sometimes smaller individuals are more active (Jarvis, 1981; Jarvis et al., 1991; Jacobs and Jarvis, 1996) and that there is large behavioural variation that cannot be explained by differences in body mass (Lacey and Sherman, 1991, 1997; Gilbert et al., 2020; Siegmann et al., 2021). Our results are inconsistent with a clear negative relationship between cooperative burrowing and body mass and largely consistent with studies emphasising that there is substantial between-individual variation in burrowing activity that is unrelated to body mass. Additionally, it is important to appreciate that capture order in naked mole-rats is probably a better indicator of sand ejecting behaviour (i.e., volcanoeing) than of general activity or digging activity in more central areas of the burrow.

Across all social African mole-rats, we have the best understanding of the cooperative behaviour in Damaraland mole-rats and our comparative data yield insights into division of labour of other social mole-rat species by extension (Bennett, 1990; Gaylard et al., 1998; Scantlebury et al., 2006; Zöttl et al., 2016a; Thorley et al., 2018). In Damaraland mole-rats digging activity increases with body mass early in life before it starts to decline around the time of dispersal when individuals also reach their maximum body mass (Zöttl et al., 2016a,b; Thorley et al., 2018) and estimates of relative foraging activity in wild groups

FIGURE 2 | The predicted effects of body mass on capture order of male and female non-breeding group members in **(A)** Naked mole-rats, **(B)** Damaraland mole-rats, **(C)** Natal mole-rats, and **(D)** Mahali mole-rats. The shaded areas represent 95% confidence intervals and the test statistics can be found in **Table 1**. Note that the effects displayed in panels **(C,D)** do not represent statistical significant effects. Photographs by Kyle T. Finn and Yannick Francioli.

show that breeders exhibit a 50% reduction of foraging activity as compared to non-breeders (Francioli et al., 2020). These patterns are also reflected in our capture order data by showing that breeders of both sexes are captured later, and that intermediate sizes of non-breeders are captured earlier than smaller and larger individuals. If the effect size of capture order in Damaraland mole-rats reflects a 50% decrease in cooperative burrowing by breeders, this would suggest by extension that in naked mole-rats breeders may only show around 5% of the burrowing investment of non-breeders (10 fold difference in effect size) and that in the species of the genus *Cryptomys* breeding females may show intermediate levels of relative burrowing investment. In contrast to Damaraland mole-rats and Naked mole-rats, breeding males in Natal and Mahali mole-rats were not captured later than non-breeding males, which indicates that their levels of cooperative burrowing may be similar to those non-breeding group members. In line with other studies (Bennett, 1989; Moolman et al., 1998; Bennett and Faulkes, 2000), in both subspecies of *Cryptomys* we did not detect any significant body mass related patterns of capture order and it is likely that if such effects existed then they would be small, as in Damaraland mole-rats.

Previous studies in different mole-rat species suggested that although some species show pronounced size dimorphism, this rarely translates into behavioural differences between the sexes (Jarvis et al., 1991; Schielke et al., 2012; Zöttl et al.,

2016a,b, 2018; Gilbert et al., 2020; Caspar et al., 2021; Siegmann et al., 2021). Also, in our study, behavioural sex differences are notably absent in all species of social mole-rats. This pattern is unusual across cooperative breeders where sex differences in helping behaviour are common and often linked to sex differences in philopatry and the relatedness (Clutton-Brock et al., 2002; Russell, 2004; Stiver et al., 2006; Downing et al., 2018) though may be expected for a breeding system as in social mole-rats where some of the key incentives for the evolution of sex differences of helping behaviours are missing. In social mole-rats mean relatedness of the sexes to the offspring is often similar because monogamous pairs are common (Burland et al., 2002; Patzenhauerová et al., 2013). Additionally, males and females only differ marginally in the duration of philopatry, disperse at similar rates and rarely inherit a breeding territory (Hazell et al., 2000; Torrents-Ticó et al., 2018a,b; Mynhardt et al., 2021; Thorley et al., 2021), which is expected to limit the opportunity for delayed direct fitness benefits to disproportionately favour one sex helping more than the other.

In conclusion, our study of capture rates across social mole-rats suggests that there are broad similarities in the division of labour across all three main social genera *Heterocephalus*, *Fukomys*, and *Cryptomys*. However, by controlling for differences in mean group size across species through normalising

the capture order data we were able to show that the effect sizes of differences between breeders and non-breeders, and across non-breeders of different sizes were many-fold larger in naked mole-rats than in any other species included in this study. Our results suggest that while some behavioural differences in burrowing activity between breeders and non-breeders and across non-breeders occur in most social mole-rat species, the naked mole-rat may show substantially more pronounced behavioural contrasts than all other species.

DATA AVAILABILITY STATEMENT

All data and code is available in a public repository (https://figshare.com/articles/dataset/Data_and_R-Code_for_Capture_order_across_social_bathyergids_indicate_similarities_in_behavioural_and_spatial_organisation/19524937).

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of the University of Pretoria (EC032-13, EC006-15, EC050-16, EC059-18, EC044-16, and EC001-19).

AUTHOR CONTRIBUTIONS

MZ conceived to the idea and analysed the data together with HB. MZ wrote the manuscript with input from HB, KF, DH, JT, NB, and SB. All authors contributed to the data collection and approved the final submission.

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Population genetics of the naked mole-rat *Heterocephalus glaber*: The role of rivers in shaping genetic structure

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We used nested clade phylogeographic analysis (NCPA) of mitochondrial DNA sequence data to examine the processes contributing to population structure in naked mole-rats. We examined sequence variation in the (1097 bp) control region D-loop of the mitochondrial genome in 303 individuals from 174 colonies of naked mole-rats (*Heterocephalus glaber*) located mainly within an 870 km^2 area in Meru National Park, Kenya. Four rivers were found to be correlated to a significant fragmentation inference in the NCPA. The largest pairwise divergence between haplotypes from populations separated by rivers was 2.74%, which was well over half of the divergence reported between the extremes of the distribution from southern Ethiopia to southern Kenya (4.6%). However, the size of the river (measured in current discharge) was not a good predictor of the amount of sequence divergence between populations separated by a river. It appears that a large-scale historical fragmentation event may have conflated fragmentation patterns on a smaller scale, when recent colonization and range expansion brought two old lineages together at a location with a relatively small river separating them.

Introduction

Like other members of the Bathyergidae, naked mole-rats (*Heterocephalus glaber*) spend most of their lives underground foraging on roots and tubers. Because of their fossorial adaptations, it has been assumed that naked mole-rats are fairly limited in their dispersal ability (Brett, 1991; Steinberg and Patton, 2000). Large group sociality has been hypothesized to contribute to the already limited dispersal

because animals were hypothesized to use cooperative breeding and care of siblings to maximize inclusive fitness. New colonies were assumed to result from fissioning of existing colonies and subsequent matings between close relatives were hypothesized to be typical. Combined with the isolation resulting from the limited dispersal of fossorial rodents, this population dynamic was expected to result in an extremely fragmented population genetic structure with high within-colony relatedness (Reeve et al., 1990).

However, dispersal and outbreeding appear to be occurring more frequently. This is based on the discovery of a disperser morph (Oriain et al., 1996), above-ground dispersal (Braude, 2000), nascent colony formation involving unrelated breeders (Braude, 2000), and preference to outbreed in the lab settings when unrelated mates are available (Ciszek, 2000). In light of these findings, we have examined the patterns of distribution of population genetic structure, in order to understand how common these processes may be in wild populations. Processes thought to have an effect on population structure include mate choice, the interaction of dispersal capability with the environment, and historical events such as fragmentation, extinction/recolonization, and range expansion. In this study, we concentrate on the latter two.

Both Darwin and Wallace recognized the role of rivers in dividing populations and isolating them for periods of time sufficient to accumulate morphological differences between the populations they separate (Wallace, 1849; Darwin, 1872). Studies in many taxa, including frogs, birds, rodents, and primates, have elaborated on the river barrier hypothesis (Sick, 1967; Capparella, 1988; Ayres and Cluttonbrock, 1992; Patton et al., 1994; Lougheed et al., 1999; Aleixo, 2004; Nicolas et al., 2011, 2019; Voelker et al., 2013; Kopuchian et al., 2020; Allen et al., 2021; de Fraga and Carvalho, 2021). In general, river size (width, flow-rate, discharge) is expected to positively correlate with indices of differentiation between populations separated by rivers.

Furthermore, rivers have already been implicated as dispersal barriers for a species of subterranean pocket gophers (Davis, 1986). There is evidence that rivers affect naked mole-rats in the same way (Ingram et al., 2015), despite a report (Hickman et al., 1983) that naked mole-rats may be able to swim if necessary. Hickman et al. (1983) actually only found that one naked mole-rat individual tested was able to tread water for 3 min before needing to be rescued from drowning.

Several studies report low variation in one population of naked mole-rats (Faulkes et al., 1990; Reeve et al., 1990; Honeycutt et al., 1991) that had been previously hypothesized to be the descendants of a small number of founders that crossed the Athi river in southern Kenya during a drought (Jarvis, 1985). Ingram et al. (2015) sampled populations of naked mole-rats north and south of the Tana and Athi Rivers and, using both mitochondrial and nuclear DNA markers, documented phylogeographic structuring associated these two river systems with serial bottleneck events with the species

range expansion. Historical founder events, fragmentation, and range expansion can leave residual patterns in the population genetic structure (Templeton et al., 1995). Rivers may cause fragmentation, isolation and genetic divergence. Alternatively, isolated populations may experience local extinction and subsequent recolonization, which leaves residual genetic patterns indicative of range expansion. While Ingram et al. (2015) documented population structuring correlated with two large river systems, these populations were sampled at a broad geographic scale, and located at the extreme southern portion of the species range, with limited genetic diversity due to historical founder events. Zemlemerova et al. (2021) also identified deep divergence in populations of naked mole-rats both within Ethiopia and across the broader species distribution. In the present study, we examine the effect of rivers as barriers to dispersal at a fine scale in populations naked mole-rat located within Meru National Park, Kenya. Based on the riverine barrier hypothesis, we hypothesized that genetic divergence between colonies on each side of a river will be positively correlated with river size.

Materials and methods

Sampling

All samples were collected along a 120 km transect in Meru National Park and Kora Reserve, Kenya under authority of the Office of the President (Permit: 15C/116). We obtained the samples for this study by trapping naked mole-rat individuals during four consecutive field seasons conducted between 2001 and 2004. Individuals were trapped using Hickman tube traps (Braude and Ciszek, 1998), tissue was stored in DMSO/EDTA/NCl buffer, initially at ambient temperatures and later at –20°C. We sampled at least two individuals from each colony, with the exception of colonies from which only a single individual was captured. To avoid bias from oversampling in some areas, this analysis only includes colonies trapped at least one kilometer apart.

River flow-rate

Rivers crossing the study site vary in discharge volume. We measured the flow rate of nine rivers (Supplementary Figure 1) using a flowmeter impeller stick manufactured by Geopacks. We assumed a roughly rectangular river profile and calculated discharge (Q) by multiplying mean velocity x mean depth × width of the river. For the largest river in Kenya, the Tana, which could be crossed only *via* a high steel bridge, we used previously published discharge estimates. The Tana was reported to have a discharge rate of 258 cumecs at Grand Falls (based on 17 years of data) and 210 cumecs at Garissa (based on 39 years of data) on average for the month of

June (Goudie et al., 1986). Grand Falls and Garissa are locations upstream and downstream of our study area, respectively. We used the month of June average of the discharge values from these two sites to have a relative sense for the scale of the Tana compared to the rivers that we measured ourselves. Although these rivers have seasonal fluctuations, June was an appropriate month for river measurements because most above-ground dispersal has been observed during mid-year.

Sequencing

We used the following primers that were developed and described in detail by Faulkes et al. (1997a) to amplify the mitochondrial control region- Primer A: 5′-GAATTCCCCGGTCTTGTAAACC-3′ and Primer D: 5′-TCTCGAGATTTTCAGTGTCTTGCTT-3′. Based on the sequences obtained in our study, we designed a new forward primer, "Control C" (5′-TATCAGGCATCTGGTTCTTAC-3′).

We extracted DNA from toes using the QIAGEN tissue extraction kit following the rodent-tail protocol. We amplified the D-loop region using primers Control A and D. PCR conditions: 200 mM Tris pH 8.4, 250 mM KCl, and 25 mM $MgCl_2$. The PCR cycling had the following profile: 94°C 2 min, then 35 cycles of 94° denaturation for 20 s, 45° annealing temperature for 20 s, and 72° extension for 2 min, followed by a final extension at 72° for 10 min.

We purified the amplified product using a VIOGENE gel purification kit and sequenced the entire region using two overlapping forward primers Control A and C. We used Big Dye v.3 with a sequence buffer (40 mM Tris-Hcl pH 9.0 and 1 mM $MgCl_2$) and 40–60 ng of amplified product. We cleaned the sequence reactions using a 96-well sephadex column plate. We dried the reactions in a vacuum centrifuge and added 5 ul of formamide before running them on an automated BaseStation sequencer from MJ Research.
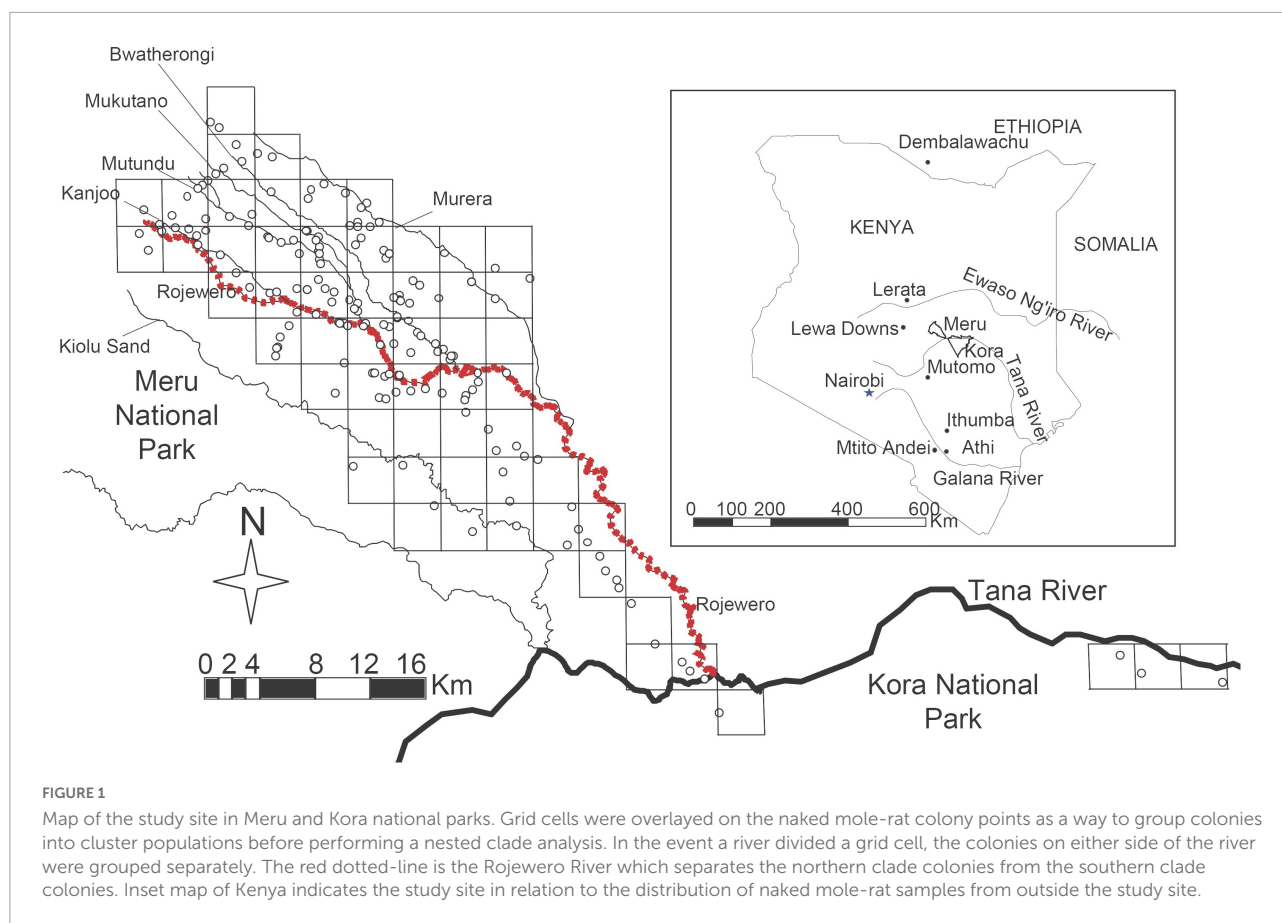
River pairwise sequence divergence

To obtain genetic-distance measurements across the rivers of the study area, we made pairwise comparisons of colonies that were separated by one river and were within a distance less than 3 km but greater than 1.5 km. We chose this distance interval for two main reasons. First, this interval bounds the maximum distance (2.4 km) an individual has been observed to disperse (Braude, 2000). Second, choosing a small interval controls for geographical distance in these genetic measures as much as possible while minimizing the loss of statistical power in sample number. Choosing to limit the distance to less than 1.5 km would have eliminated many of the rivers from the analysis because the colonies on opposite sides of the river were more frequently greater than 1.5 km apart.

Phylogenetic analysis

We used published sequences from individuals collected in Athi, Ithumba, Lerata, Ethiopia, and Mtito Andei (Faulkes et al., 1997a) to obtain a phylogeny to help root our haplotype network before performing a nested clade phylogeographic analysis (NCPA). We obtained a phylogeny from a parsimony analysis in PAUP (Swofford, 1993) with bootstrap support from 1000 replications. We added samples from two new regions: around the town of Mutomo (labeled MutomoNS1) and on the ranchland of Lewa Downs (Lewa4013 and Lewa4026). The NatV samples were collected by Nathan Mathuku, a resident of Mutomo. The map in Figure 1 shows all these locations in relation to our study site at the scale of the entire country of Kenya.

Nested clade phylogeographic analysis is a phylogeographic analysis that separates historical patterns from contemporary gene flow. NCPA makes use of information about the geographic distribution and genealogy of haplotypes to make inferences concerning the role of recurrent gene flow and historical phenomena such as fragmentation events and range expansions in the formation of current population structure (Templeton et al., 1995). We estimated haplotype trees using statistical parsimony (Templeton et al., 1992) using the program TCS v. 1.13 (Clement et al., 2000). We then used the haplotype tree to construct a series of hierarchically nested clades following the nesting rules given in Templeton et al. (1987) and Templeton and Sing (1993). Finally, we used the program GeoDis 2.0 (Posada et al., 2000) to calculate the various NCPA measures and test the null hypothesis of no association of a haplotype or clade of haplotypes in a cladogram with geographical position within a nesting clade.

Nested clade phylogeographic analysis makes phylogeographic inferences from genomes or genomic regions that show little or no recombination – the very regions upon which evolutionary history is most clearly written (Templeton et al., 1995). NCPA uses haplotype trees estimated by a Bayesian procedure known as statistical parsimony (Templeton et al., 1992). This estimation procedure allows the haplotype tree to be regarded as estimated with error or uncertainty, and this uncertainty is incorporated directly into the nested clade analysis (Templeton et al., 1992; Crandall and Templeton, 1993). The haplotype trees are converted into a nested statistical design that yields independent contrasts, thereby making corrections for multiple testing straightforward. The inferences derived from these nested clades have been subjected to the most extensive validation for any phylogeographic technique using 150 positive controls from actual data for which prior data existed for phylogeographic events or processes (Templeton, 2004). These results from analyzing real data sets indicate that NCPA is a powerful method for phylogeographic inference that is not subject to a high rate of false positives. In contrast to real data, Knowles and Maddison (2002) used computer

FIGURE 1
Map of the study site in Meru and Kora national parks. Grid cells were overlayed on the naked mole-rat colony points as a way to group colonies into cluster populations before performing a nested clade analysis. In the event a river divided a grid cell, the colonies on either side of the river were grouped separately. The red dotted-line is the Rojewero River which separates the northern clade colonies from the southern clade colonies. Inset map of Kenya indicates the study site in relation to the distribution of naked mole-rat samples from outside the study site.

simulations purporting to show a high rate of false positives. They used an out-of-date version of NCPA that explicitly excluded the situation they simulated, thereby ensuring that false positives would be abundant. When their simulation results were re-analyzed with the 2002 version of NCPA that was freely available to them, not a single type I or type II error occurred, and the simulated scenario was inferred with 100% accuracy (Templeton, 2009). Hence, the simulations of Knowles and Maddison (2002) reveal that NCPA is a remarkably powerful tool with extremely low values of both type I and type II errors. There have since been other simulations purporting to show that NCPA has a high false positive rate, with the most recent being Panchal and Beaumont (2010). All of these are based on serious misrepresentations of NCPA, and when these misrepresentations are corrected, the type I error rate is at or below the pre-set level of 0.05 (Templeton, 2009, 2015, Allen et al., 2021). Finally, NCPA does not require a prior phylogeographic model, but rather phylogeographic inferences are built up directly from the data by retaining only statistically significant inferences. As a result, NCPA can reveal phylogeographic events that had not been anticipated. In contrast, simulation approaches to phylogeography require detailed models before-hand, and inference is strictly limited to what is simulated in a set of possible models based on prior

expectations. Given that we do not have much prior information on the phylogeographic history of naked mole-rats and that actual data and simulated data indicate that NCPA is a powerful method of phylogeographic inference not subject to a high rate of false positives, NCPA is the most appropriate method for analyzing this species.

The unusual social structure of this species required us to perform an extra step that involved grouping colonies into population clusters before using NCPA. In their social structure, one female breeder bears a large number of offspring that comprise the bulk of the colony. It was necessary for us to perform this grouping because we were using the genetic perspective of a maternally inherited molecular marker and so most, if not all, of the individuals that we captured from each colony represent one individual, the female breeder who bore them.

Our initial strategy treated the colonies as population units. However, this treatment yielded ambiguous results when using the key for NCPA because some inferences require information regarding whether clades contain overlapping or non-overlapping distributions of haplotypes. Here, the term "overlapping distributions" was intended to refer to different haplotypes found in the same population. However, since most of our populations were monomorphic we would have had

difficulty interpreting overlap. Therefore, we chose to group colonies together into clusters and use these clusters as our population unit. To group colonies into clusters, we overlayed a grid on our populations and grouped populations falling within the same grid cell (**Figure 1**). We performed the placement of this grid using a program in ArcGIS (ESRI, 2003) that uses a layer of points (in our case the colony GPS coordinates) to create a surface composed of uniform grid cells of a size specified by the user (Rathert, 2003). We automated the placement of the grid cells by allowing ArcGIS to establish a random start point to avoid introducing bias from human judgment. We chose the size of the grid cell as 3 km × 3 km loosely based on the maximum distance an individual was recorded to disperse (2.4 km) (Braude, 2000). We used the number of different mitochondrial haplotypes within a colony as individuals in these new population units. Therefore, we treated most colonies as single individuals, except for the few cases in which we captured both female and male breeders and found they had different haplotypes. Also, we treated each of the two colonies that were found to be internally heterogeneous (heteroplasmic) as 2 individuals, each individual bearing 1 of the 2 haplotypes.

Ideally population genetic structure is best characterized using a large number of markers distributed throughout the nuclear and mitochondrial genomes, however, our study was restricted to the perspective of a single DNA marker that is maternally inherited (mtDNA). Although not ideal, this genetic marker was highly informative for our particular application to characterize historical processes of a species that has a social structure that centers around dominant female breeders.

Because rivers are uninhabitable territory, we did not group colonies that were not located on the same side of a river. However, we ignored two rivers in this grouping process. The first river, Ithumbanchego, was a small seasonal stream bed that was dry. The second was the Mulika river that was also very small and ran within a kilometer distance parallel to the Bwatherongi, a similar-size stream that was included in the analysis. Only one colony would have been grouped differently (creating a new population with just one effective individual) if we were to have used the river, and it did not affect the results of our analysis. We averaged the GPS coordinates of all the colonies within each grid cell cluster to obtain one set of coordinates for each new population. Our clustering process reduced our original population count within our study area from 168 colony populations to 75 cluster populations.

In order to estimate the time of the event inferred by NCPA we used the dating method described by Templeton (2003). The equation for this estimation requires calculation of the sequence divergence of the more recent derived clade from that of the nearest older clade, as well as the sequence diversity within each clade. We used t = (Dxy–0.5*(Dx + Dy))*mutation rate, where Dxy is the average divergence between the recently derived clade from the nearest older clade, and Dx and Dy are the average diversity within the recent and older clade, respectively. In order

to calculate confidence intervals for the time estimate a gamma function is used to account for genetic drift in modeling the distribution of time (Kimura, 1970). This method assumes a neutral rate of mutation and the accuracy of this mutation rate is critical for the proper dating of an event.

Results and discussion

We found 54 unique haplotypes among the 174 colonies that we sequenced. Most of the colonies were monomorphic, containing a single haplotype among the individuals sequenced. Two colonies contained heteroplasmic individuals. Heteroplasmy has been described before in naked mole-rats by Honeycutt et al. (1991). We were able to detect consistently the relative frequency of both genomes in the sequence data, as confirmation of their existence (i.e., we conducted multiple independent extractions of DNA from the tissue sample of the putative heteroplasmic individuals and confirmed the same sequence result). In both cases of heteroplasmy, the haplotypes were only different by one basepair mutation.

River pairwise sequence divergence

According to one of the predictions based on the riverine barrier hypothesis, the amount of genetic divergence between populations separated by a river should be greater that the divergence between populations on the same bank (Capparella, 1988). Further, this divergence should positively correlate with the river size which can be measured in many ways such as discharge, flow-rate, and width (Ayres and Cluttonbrock, 1992). This prediction assumes that the rivers observed today have been relatively constant over time and colonies have come from one source and been present within all the river regions from that initial moment to the present. The prediction about river size also assumes that there is no threshold effect. For example, if animals cannot disperse across a river that is greater than a particular size, all rivers above that size will have the same effect on gene flow. These assumptions may differ from reality, but these predictions serve as a useful starting point to test whether rivers have played a significant role in isolating populations based on the observed genetic patterns.

There exists a positive relationship between discharge and average pairwise sequence divergence (p-value = 0.0006, slope = 0.0049, r^2 = 0.8809). The Tana and Kiolu Rivers were not included in the analysis because of insufficient sample sizes (n = 1). This significant positive correlation is largely due to the high sequence divergence across the Rojewero River.

We performed a series of one-way ANOVAs to test the effect of rivers on average pairwise sequence divergence. The Rojewero is significantly greater than the other rivers in terms of average pairwise sequence divergence between haplotypes sampled on

either side of it (again excluding the Kiolu and Tana due to inadequate sample sizes) (p-value < 0.00001).

We found that not all the rivers fit a simple model that predicts that average pairwise sequence divergence is directly proportional to their discharge. Although we did not have the sample sizes to test the largest river (the Tana) in the same way as all the others, even if we ignored geographical distance and used pairwise differences of all four of the colonies on the south of the Tana compared to the total number of colonies north of the Tana (south Rojewero), the average pairwise sequence divergence was still significantly less. The values for the Rojewero and Tana average sequence divergence were 0.01991 and 0.0059, respectively (p-value < 0.00001). This was surprising because the discharges were so significantly different. The Tana is the largest river in Kenya with a discharge average (234 cumecs) that is almost two orders of magnitude greater than the second largest river in our study, the Rojewero (3.7 cumecs).

However, if we were to ignore for a moment the two largest rivers, Tana and Rojewero, and also exclude the values contributed by the presumed migrant colony discussed previously for the Ithumbanchego average sequence divergence, we were able to detect a positive correlation between river discharge and sequence divergence for the remaining rivers. This correlation is mainly due to the Murera and Mutundu, the 2 s largest rivers after the Rojewero, because they both had significantly larger sequence divergence as compared to the other smaller rivers. Therefore, we have found some correlative evidence for the isolating effect of rivers as well as the possibility that at least for the two largest rivers, a different type of historical event may have played a role in our study population. This pattern does indicate a threshold effect. The Rojewero and Tana seem to be above a threshold effect on gene flow, but the smaller rivers are not.

Phylogenetic analysis

Faulkes et al. (2004) presented evidence that naked mole-rats spread north to south and the Ethiopian haplotype was basal. Recent work by Zemlemerova et al. (2021) has shown two ancient lineages of naked mole-rats distributed in Eastern Ethiopia and Southern Ethiopia. The lineage that spread into Kenya and a sublineage in the Southern Ethiopian clade ("Borena National Park Megadu block") are sister taxa (Zemlemerova et al., 2021). By rooting our phylogeny with the Ethiopian haplotype (**Figure 2**), we obtain similar results as also found by Faulkes et al. (1997a). There is a large genetic break between northern and southern haplotypes, and interestingly the Rojewero river, less than 20 m in width, appears to be the barrier separating them. Representatives of the sister haplogroups of the southern clade are located in the extreme southeastern edge of the mole-rat distribution at Ithumba and Athi. From these populations there may have been

more recent colonization of areas to the north and southwest (Mtito Andei). In the north, the sister haplogroups appear in two colonies from Lerata (Lerata 2 and 3). Faulkes et al. (1997a) also detected what could be a similar contact zone between north and south lineages on a similar small scale (less than 100 m apart). Surprisingly, the Lerata1 and 4 colonies appear to have a closer phylogenetic connection with the southern haplotypes from Athi and Ithumba. The bootstrap support was quite high (96 between Lerata 1 and 4, and 99 between Ithumba to the Lerata/Athi group). Unfortunately, there was no mention of whether any geographical barriers were separating the colonies Lerata 2 and 3 from Lerata 1 and 4. These Lerata colonies are around 100 km north of Meru National Park, but also are close to a west-to-east river, the Ewaso Ng'iro. Only considering our samples from central Kenya may have led to a conclusion that there has been an ancient fragmentation event between colonies north of the Tana from those in the south. However, considering the possibility of a similar contact zone at a northern location in Lerata suggests that a west-to-east fragmentation should be considered as well. Other possibilities at Lerata may include ancient polymorphism in the Lerata population, a recent translocation, or mislabeled sample locations. Further, if the Lerata 1 and 4 colonies were found to be spurious, our results would support a relatively ancient fragmentation occurred that separated sister taxa from southern Ethiopia from a southern population in Kenya (Mtito Andei as representative; "Mtito1," Zemlemerova et al., 2021); and over time, a more recently derived northern Kenyan clade may have fragmented from the southern Kenyan clade (Lerata 2 and 3 as representative haplotypes, Zemlemerova et al., 2021). More thorough sampling across the distribution of this species is required to resolve the nature of this fragmentation; however, our results do strongly suggest that fragmentation rather than isolation-by-distance gene flow has produced the genetic divergence exhibited between our study's northern and southern haplotypes.

Nested clade phylogeographic analysis

The north clade and south clade were separated by a minimum of 25 mutational steps and so we analyzed these networks separately. Both clades had a few ambiguities in the network (northern clade, **Figure 3A**; southern clade, **Figure 4A**). We constructed multiple network alternatives that considered all possible connections to test how the different configurations affected the inferences obtained by the NCPA key (**Supplementary Material**); however, we present results based on the most parsimonious nesting for both northern (**Figure 3B**) and southern (**Figure 4B**) clades, and given the geographic distributions of the haplotypes we observed (**Figures 5A**, **6A** for northern and southern clades, respectively).
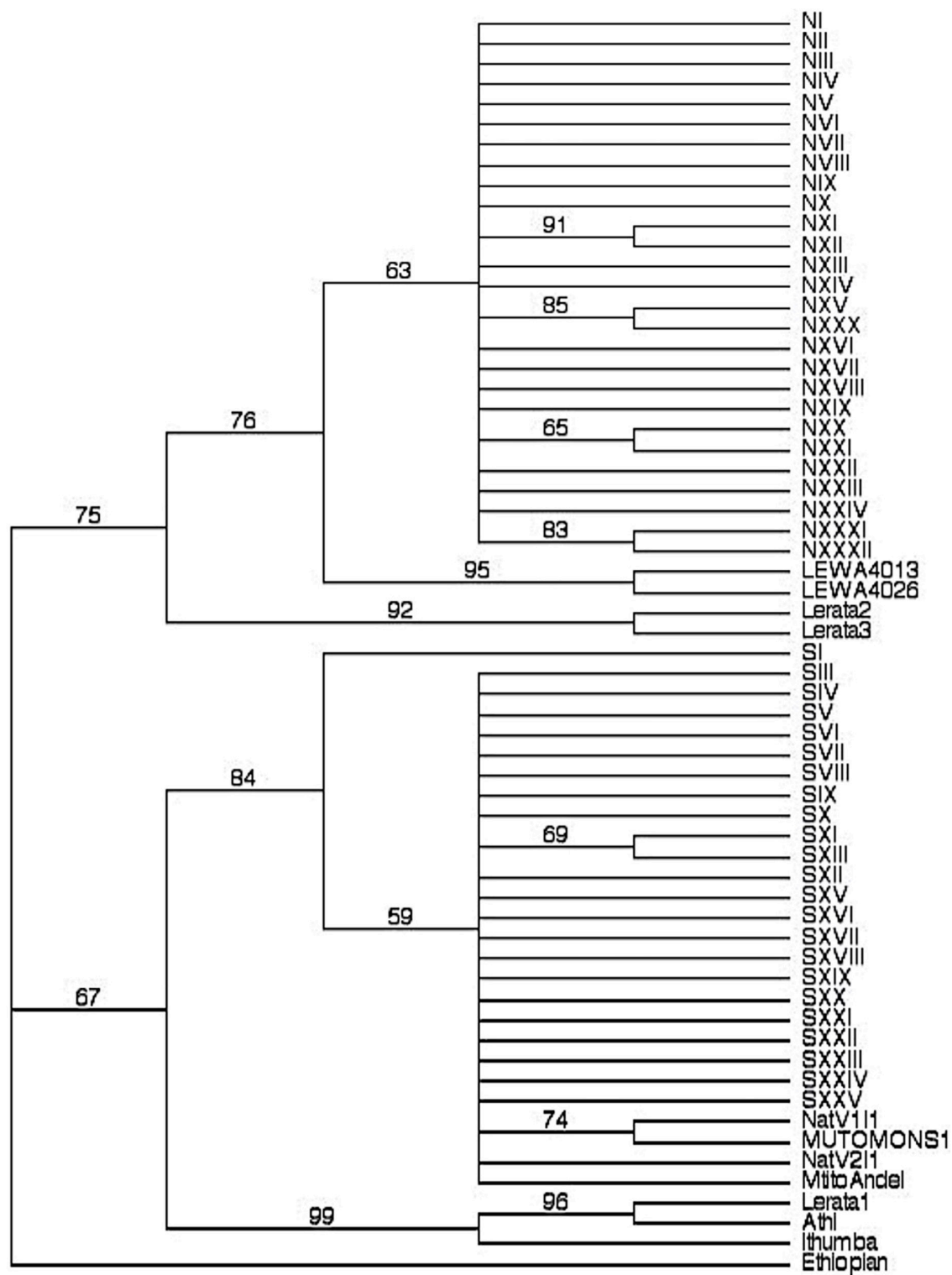
FIGURE 2

Phylogeny with bootstrap values from 1000 replications. The haplotypes found in our study site are labeled as N or S (referring to its membership in either the northern or southern haplotype network) with a roman numeral that was given to identify each unique haplotype. The haplotypes included from Faulkes et al. (1997a) are named Lerata, Mtito Andei, Ethiopian, Ithumba, and Athi. Faulkes et al. (1997a) sampled four colonies from Lerata, and found 3 haplotypes (Colonies 1 and 4 shared the same one). Both the NatV1 and V2 haplotypes were located in the area of Mutomo. We rooted this phylogeny with the same haplotype from the Ethiopian colony used as an outgroup by Faulkes et al. (1997a, 2004).
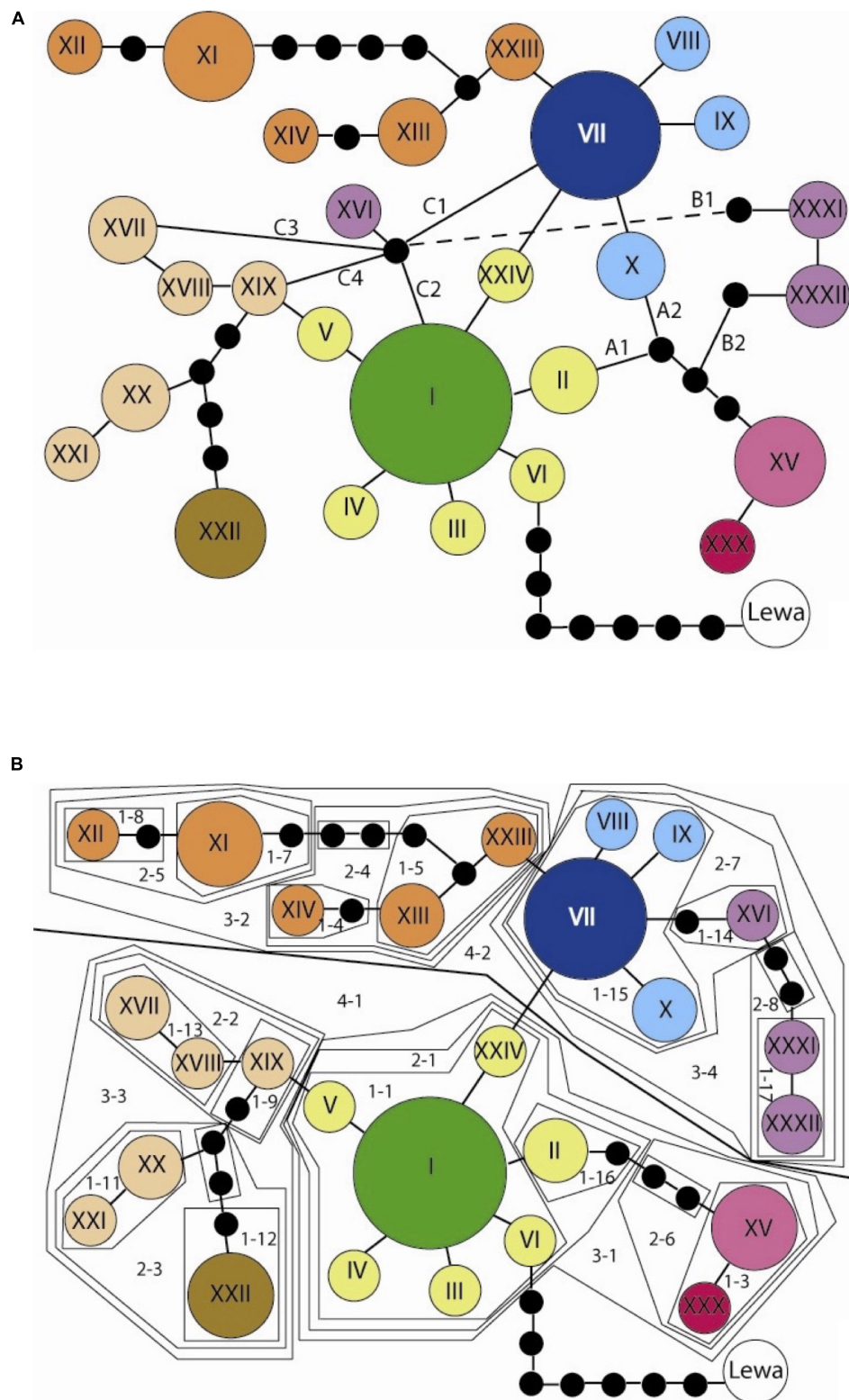
FIGURE 3

(A) Northern haplotype network with all the parsimonious connections. The black dots indicate unobserved haplotypes and each line represents one mutational step. The colors correspond to those used on the distribution map. The letters A, B, and C label the multiple possible connections that are referred to in the text and can produce 16 possible combinations of this network. The Lewa haplotype was found outside the study site at Lewa Downs and was used to root the network. (B) Northern network configuration 5 (A1B1C1). This configuration depicts what we considered to be the most reasonable or likely connections based on the criteria established in Crandall and Templeton (1993). The nested clades are numbered, and to distinguish the nesting in this configuration from the alternative configurations the clade numbers should be considered to have the subletter "a."
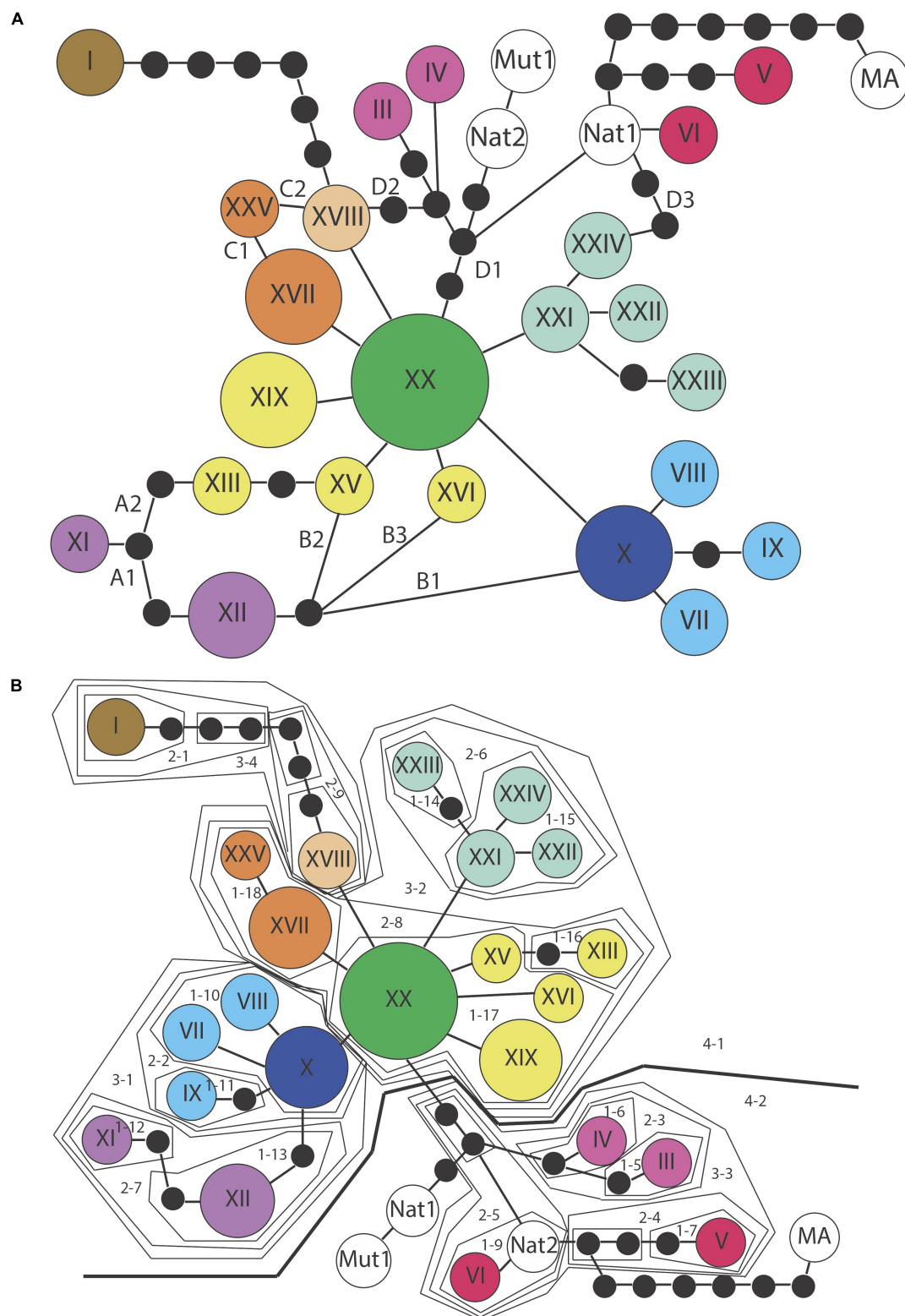
FIGURE 4
(A) Southern haplotype network showing all possible parsimonious connections. **(B)** Southern haplotype network most reasonable configuration 1. The combination of connections shown in **A** for this configuration (A1B1C1(D1)) were chosen based on three criteria described in Crandall and Templeton (1993) for resolving ambiguities and rooting the phylogeny.

(A) Northern haplotype distributions. The roman numerals identify unique haplotypes and the colors correspond to those in the haplotype network in **Figure 3**. The light blue areas without roman numerals represent swamps. The Rojewero is labeled. Notice the three colonies that were found on the south side of the Rojewero: two from the yellow II haplotype and one from the dark blue VII haplotype. Bridges are located close to these "migrant" colonies. None of the colonies from the southern network are shown. These networks are separated by 25 bp steps. **(B)** Map showing the significant inferences of historical events of the northern NCPA. Arrows indicate the following inferences from Table 1: 1: restricted dispersal, 2: allopatric fragmentation, 3: range expansion, 4: allopatric fragmentation, 5: allopatric fragmentation, "?": restricted dispersal. The "?" signifies the lack of robustness for this event, 6: long-distance colonization/subsequent fragmentation or fragmentation/range expansion.

Northern clade network inferences

Nested clade phylogeographic analysis inferences with the most support (**Table 1**) are identified with arrows indicating the geographical areas where the event occurred (**Figure 5B**). We see evidence that restricted dispersal occurs within the larger areas between rivers. This is most clearly seen within the 1–1 clade (**Figure 5B**, arrows at numeral one). In addition, the following rivers have shown potential for being significant barriers to dispersal: the Kanjoo, Mutundu, and Murer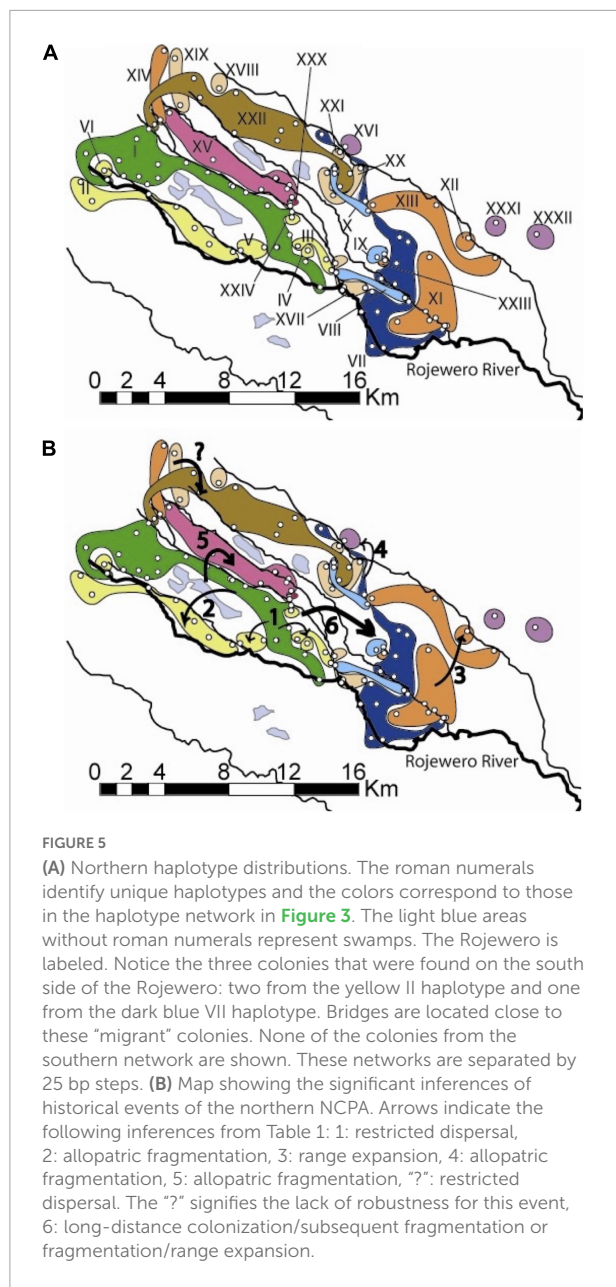a. First, the Kanjoo was supported as a significant barrier through the high probability alternative configurations 1 through 3,

which yield an allopatric fragmentation in the 2–1 clades with haplotype II originating from I (**Figure 5B**, arrow 2). Although this inference was significant in only half of the configurations (**Figure 3A**), this half consists of the configuration A1B1* (where * could be C1, C2, or C4) that collectively have a probability of 0.8892 compared to a probability of 0.1108 for the combined three non-significant networks. Second, the Mutundu River was supported as a significant barrier in all six networks, which yield allopatric fragmentation in the nested 3–1/3–4 clades involving haplotype XV originating from the 2-step clades of interior haplotype I or VII (**Figure 5B**, arrow 5). In addition, the Mutundu may also be responsible for yielding the inferences of fragmentation and long-distance colonization of the level 3 and 4 clades that included networks 2, 3, 4, and 5 with a collective probability of 0.6523 (**Figure 5B**, arrow 6). Third, the Murera River showed a very faint level of significance as a barrier due to the small number of samples collected on the eastern side. More thorough sampling may resolve the placement of the Murera colonies and whether the Murera River is correlated to a fragmentation event or not. However, the A1B1C1 high probability configuration and the lower probability A2B1C2 configuration did yield a significant inference of allopatric fragmentation involving the Murera haplotype XVI originating from the interior VII haplotype (**Figure 5B**, arrow 4), but the bulk of the probability mass over all network configurations was non-significant for fragmentation.

Finally, a small range expansion between the site of the XI haplotype and its tip XII haplotype is inferred from all six networks (**Figure 5B**, arrow 3). However, the remaining inferences, such as the possible contiguous range expansion in the north (**Figure 5B**, arrow with "?") and the contiguous range expansion from the eastern haplotypes colored in orange to the far northwestern haplotype in orange are still in question.

Significant inferences and alternative clades for the northern portion of our study area are discussed in more detail the **Supplementary Material**.

Southern clade

We found inferences of restricted dispersal in the southern clade of our study site concentrated around the middle of the region where the bulk of the distribution of the main haplotype XX is located (**Table 2** and **Figure 6A**). From the interior XX haplotype, the 2–6 clade was inferred as a restricted dispersal/IBD event (**Figure 6B**, arrow 1). Also we see either fragmentation or IBD within the 1–10a clade (**Figure 6B**, arrow 2), which cannot be resolved without more sampling. Then we observe evidence that there may be contiguous range expansion (**Figure 6B**, arrow 3) and long-distance colonization (**Figure 6B**, arrow 4), respectively, to the southeast and northwest from the interior XX haplotype. Anecdotal support for an inference of long-distance colonization comes from our sampling data.

TABLE 1   Chain of inferences for configuration 5 (A1B1C1) of northern haplotype network.

Figure 5B Ref. Number for arrows	Clade	Chain of inference	Result
1	1–1a	1-2-3-4-NO	Restricted gene-flow with Isolation by distance
—	1–15a	1-2-11-17-NO	Inconclusive outcome
2	2–1a	1-2-3-4-9-10-YES	Allopatric fragmentation
—	2–4a	1-19-20-2-11-12-NO	Contiguous range expansion
3	2–5a	1-19-20-2-11-12-NO	Contiguous range expansion
4	2–7b	1-19-NO	Allopatric fragmentation
5	3–1e	1-19-NO	Allopatric fragmentation
—	3–2a	1-2-3-4-NO	Restricted gene-flow with Isolation by distance
—?	3–3a	1-2-3-4-NO	Restricted gene-flow with Isolation by distance
—	3–4a	1-19-20-NO	Inadequate sampling
	4–1a	1-19-20-2-11-12-NO	Contiguous range expansion. 3-3a from 3 to 1.
6	Total clade 4a	1-2-11-12-13-YES	Long distance colonization possibly coupled with subsequent fragmentation or past frag. Followed by range expansion

TABLE 2   Chain of inference for the Southern Haplotype Network configuration 1 (A1B1C1(D1)) that was the most reasonable.

Figure 6B Ref. number	Clade	Chain of inference	Result
2	1–10	1-2-3-4-9-10-NO	Sampling inadequate to discriminate fragmentation and IBD (VIII from X)
3	1–17	1-2-11-12-NO	Contiguous range expansion (XIX from XX)
5	1–17	1-19-NO	Masked allopatric fragmentation (XVI from XX)
4	2–8	1-2-11-12-13-YES	Long distance colonization possibly coupled with subsequent fragmentation or past frag. Followed by range expansion (XVII from XX)
6	2–8	1-19-NO	Masked allopatric fragmentation (XIII from XV)
	3–1	1-19-20-2-11-17-NO	Inconclusive outcome
1	3–2	1-2-3-4-NO	Restricted gene-flow with Isolation by distance (XXI from XX)
7	Total Cladogram	1-19-NO	Allopatric fragmentation (South Tana populations to North Tana)

The "Figure 6B ref. number" indicates the event associated with the arrows pictured in that figure.

We returned to areas in the south within the course of the season and to the northwest several times over 3 years without having success in capturing or finding signs of colony activity (i.e., colony volcanoes). In the south we hypothesized that excessive drying and hard soils may contribute to their absence and further to the northwest this absence may be due to the swampy conditions. However, we also have to consider that these absences may be a result of their location beyond a frontier population. The fact that very few colonies have crossed sides of the Rojewero supports this idea that the frontier is a new one. Finally, there is weak evidence based on single colonies that both the Kiolu River (Figure 6B, arrow 5) and Rojewero (Figure 6B, arrow 6) have experienced allopatric fragmentation events. However, we find strong evidence for a single fragmentation event that has split the north Tana and south Tana populations (Figure 6B, arrow 7). The Tana "split" is a much more recent fragmentation than the one presumed to have occurred between the populations north and south of the Rojewero. Based on the rooted phylogeny of these populations, the haplotypes in the southern network in our study were derived from the haplotypes found in southern Kenya at Ithumba and Athi. The northern network is sister to the Lewa and Lerata haplotypes.

Significant inferences and alternative clades for the southern portion of our study area are discussed in more detail the Supplementary Material.

Time estimation

The mutation rate in the mitochondrial control region is variable in mammals ranging from 3% in horses, 7–11% in primates, and 15% divergence in cape buffalo per million years (Slade et al., 1998; Van Hooft et al., 2002). To estimate the age of the two major fragmentation events in our study occurring between the northern and southern banks of the Tana and the opposite banks of the Rojewero, we used the extremes of this range of mutation rates to obtain a relative sense for the difference in time scale of the
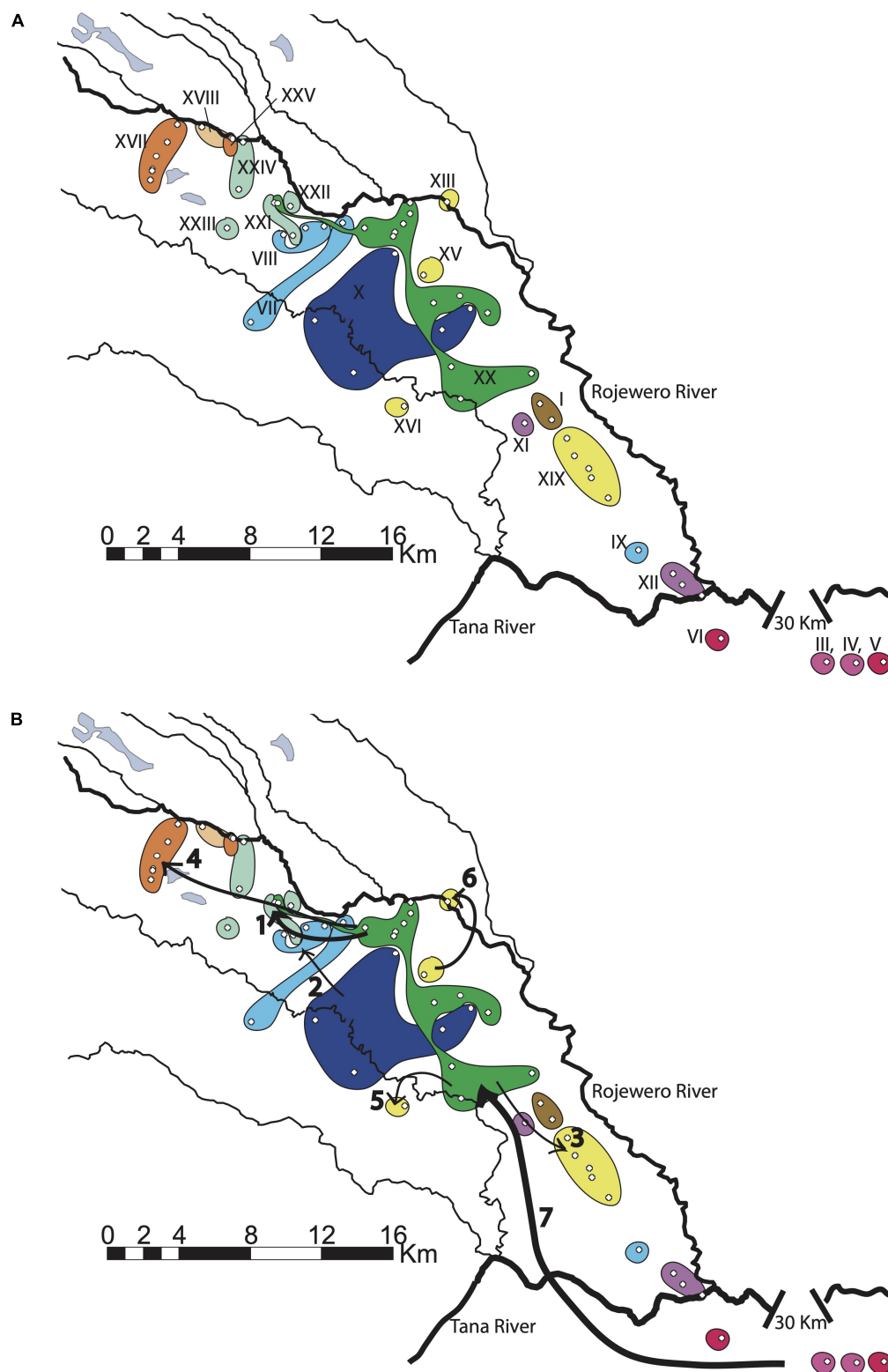
FIGURE 6
(A) Southern haplotype distributions with labels. **(B)** Significant historical inferences of the southern NCPA. Size of the arrows indicates the level of the nested clade, the larger the arrow the higher the nesting level. Arrows indicate the following inferences: 1: restricted gene-flow, 2: cannot discriminate fragmentation from restricted gene flow, 3: contiguous range-expansion, 4: long-distance colonization/fragmentation or fragmentation/range expansion, 5: allopatric fragmentation, 6: allopatric fragmentation, 7: allopatric fragmentation.

two events. Using the 3% sequence divergence per million years, we estimated that the fragmentation between the sides of the Rojewero occurred 0.695 MYA (95% C.I. = 0.217–1.45 MYA). In contrast we estimated the fragmentation event across the Tana River to have occurred 0.067 MYA (95% C.I. = 0.016–0.154). Alternatively using the 15% rate, we find these dates to be 0.139 MYA (95% C.I. = 0.043–0.289 MYA) and 0.013 MYA (95% C.I. = 0.003–0.031 MYA), respectively. The 95% confidence intervals of these date estimations are non-overlapping under both mutation rates, which is strong support that the fragmentation between the clades separated by the Rojewero is significantly older than the fragmentation across the Tana.

Riverine barrier effects on naked mole-rat population structure

We found support for the riverine barrier hypothesis (RBH) among one group of rivers in our study site. However, regarding the two largest rivers in this study, we found evidence for a more complicated history involving colonization and range expansion that did not fit a prediction of the RBH.

Our analyses testing associations of river size measures with pairwise sequence divergence as well as results from nested clade phylogeographical analysis (NCPA) did yield some support for the RBH. The river size measure, average discharge, had a consistent positive association with average pairwise sequence divergence which is concordant with one of the predictions RBH. Out of the other three river size measures, average river width also was found to have a significant positive association with sequence divergence, although only for the small rivers (i.e., excluding the Rojewero River). This result is similar to what Ayres and Cluttonbrock (1992) found in that both river width and discharge were discovered to fit an RBH prediction (these measures were negatively correlated with an index of similarity for primate fauna found on both banks of Amazonian rivers). Results from our NCPA on both the northern and southern area of our study site also supported the RBH by yielding six associations of fragmentation events with the presence of rivers. For the NCPA of our northern study area, fragmentation events were associated with the following three rivers: Kanjoo, Mutundu, and Murera. However, the Kanjoo and Murera fragmentation inferences were not robust in the context of the ambiguities in the haplotype network, although the Kanjoo was supported by the three most probable haplotype networks (a collective probability of 0.8892). In the case of the Murera, this inference had been based primarily on one colony, and additional sampling was recommended by the NCPA inference key to fill in gaps in sampling. The amount of pairwise sequence divergence between populations separated by a river may be partly responsible for the robustness of NCPA fragmentation inferences, because both the Mutundu

and Murera had significantly larger sequence divergence as compared to Mukutano, Kanjoo, Bwatherongi, and Mulika rivers. In the NCPA of our southern study area, we found significant inferences of allopatric fragmentation associated with the Kiolu Sand, Rojewero, and Tana rivers. In this case, because the southern network was analyzed separately from the northern network, the inference involving the Rojewero in an allopatric fragmentation does not pertain to the enormous split separating the two networks but instead is based on a single colony on the north bank of the Rojewero that is assumed to have crossed recently. Likewise, the Kiolu had a single colony isolated on the south bank. In both these cases, additional sampling is required to strengthen the inference. The Tana River yielded a very robust inference of allopatric fragmentation.

Potential riverine barrier effects can be outweighed by historical events

Many studies have had mixed results fitting observed genetic patterns to RBH predictions. In other rodent species there was a lack of strong evidence linking rivers as barriers using the historical perspective afforded by sequence data (Patton et al., 1994, 1996; Matocq et al., 2000; Roratto et al., 2015; Mizerovská et al., 2019). In other taxa the potential isolating effect of rivers was found to be superseded by other factors, such as the presence of ancient ridges [(frog species) Gascon et al., 2000; Lougheed et al., 1999] and movement of a glacier [(shrew species) Lugon-Moulin et al., 1999]. Similar in the way these studies failed to support the RBH, we also found genetic results that do not fit an RBH prediction. The Tana and Rojewero are the two largest rivers in our study site in terms of discharge and they also differ significantly in size from each other. The Tana is the largest river in Kenya with a discharge average (234 cumecs) and is almost two orders of magnitude greater than the Rojewero (3.7 cumecs). However, these rivers violate the RBH prediction that river size should be positively associated with pairwise sequence divergence, because the smaller Rojewero River yielded a significantly greater sequence divergence than the Tana (0.01991 vs. 0.0059, respectively, p-value < 0.00001). According to date estimators and the 95% C.I. based on a log likelihood test, the fragmentation correlated with the Rojewero is a significantly older event than the fragmentation correlated with the Tana. However, the fact that the older fragmentation is associated with the presence of the smaller Rojewero river does not necessarily mean that the Rojewero river has been the barrier solely responsible for that fragmentation event. On the contrary, results from a phylogeny and haplotype network we constructed which combines both haplotypes from our study and previously published haplotypes from Faulkes et al. (1997a) supports our hypothesis that the naked mole-rat population

inhabiting the region bordered by the Rojewero to the north and the Tana to the south has most likely been part of a relatively recent migration originating from populations south of the Tana River. This evidence is based on the fact that haplotypes from the southern portion of the naked mole-rat species distribution (Ithumba and Athi) are connected to the basal most nodes in our phylogeny, and southern haplotypes (Mutomo and Mtito Andei) are sister with our southern Tana haplotypes, indicating that our southern Rojewero population may more recently derived. NCPA show range expansion and long-distance dispersal into the area just south of the Rojewero and anecdotal evidence indicates this area is a frontier of new colonies (Much of this area is still devoid of naked mole-rat colonies despite what seems to be suitable habitat). Therefore, from all these lines of evidence, we hypothesize that the Rojewero is a meeting point of relatively old northern and southern lineages of the Kenyan lineage of naked mole-rats.

The large fragmentation event was first detected by Honeycutt et al. (1991) using restriction fragment analysis of mitochondrial DNA from colonies collected at three sites (Samburu, Mbuvu, and Mtito Andei). Samburu is geographically very close to our Lewa Downs colonies and similarly Mbuvu is close to our Mutomo samples. Honeycutt et al. (1991) showed that average nucleotide sequence divergence between Samburu and Mtito Andei and Samburu and Mbuvu was much higher (5.4%) than the divergence between Mtito Andei and Mbuvu (0.4%). This result is very similar to our findings in which Mtito Andei and Mutomo were different by 0.4–0.8%, while the sequence divergence between Mtito Andei and Lewa Downs was 3% and Mutomo to Lewa, 2.7–2.8%. So although the discovery of this large genetic break is not novel, resolving the break to a fine separation along the banks of the relatively small Rojewero is a significant finding.

Potential for glacial cycles that fragmented naked mole-rat populations

Unfortunately, until we have an accurate mutation rate for our species, we cannot conclude much from the dating estimates by way of associating fragmentation events with paleoclimactic changes or catastrophic events. However, it should be noted that East Africa has experienced four glacial cycles within the past 400,000 years (DeMenocal, 1995). These cycles have been associated with changes in the ranges of savannah and woodland areas that have in turn affected the displacement of the mammals adapted to these habitats (Vrba, 1992). Naked mole-rats occupy the space of their tunnels more like a plant occupies space and movement is mostly by the reproductive/dispersive phase of the life cycle. Hence they are even more impacted by glaciation or other climatic shifts that the ungulates and primate

discussed by Vrba (1992). Further, Zemlemerova et al. (2021) performed a calibration method and dated a greater divergence (Middle Pleistocene; ca. 1.4–0.8 Mya) between naked mole-rat lineages from East Ethiopia and South Ethiopia/North Kenya; their dataset provided context for the timing of divergence in our dataset since their contained representatives of the northern and southern lineages (Lewa and Mtito Andei). This context makes it likely that the timing of the divergence of the northern and southern lineages in our study occurred more recently than the Middle Pleistocene, which is supported by the estimate that resulted using 3% sequence divergence per million years (ca. 0.695 MYA). In general, the Pleistocene climatic cycles have been suggested to play a large role shaping genetic structure of East African rodents (e.g., Aghová et al., 2017, 2019).

Naked mole-rat population structure mysteries remain

Despite the lines of evidence that we summarized supporting our hypotheses, a couple of mysteries remain. First, the haplotypes from Lerata prevent us from concluding that there is one contact zone centered at the Rojewero River where relatively ancient northern and southern lineages meet. Lerata is north of our study site and contains both northern and southern haplotypes. Lerata colonies 2 and 3 and Lerata colonies 1 and 4 are more closely related to the northern and southern haplotype groups, respectively. It is possible a west-to-east fragmentation may have occurred or alternatively one of the following may be true: Lerata haplotypes may include an ancient polymorphism in the Lerata population or there was a recent translocation or contamination of samples. Second, the haplotype from Mtito Andei located at the extreme southern end of the naked mole-rat distribution is more similar to the haplotypes in our study (South of Tana) than to haplotypes from colonies geographically closer to it at Ithumba and Athi. We simply do not have sufficient sampling to resolve these mysteries. The high degree of genetic structure exhibited by this species requires fine scale sampling (perhaps just slightly coarser than the present study) across the entire species distribution. Additional support for our conclusions may be found by studying other rodent species living in the same areas. Gerbils (*Gerbillus harwoodi*), spiny mice (*Acomys wilsoni*), and elephant shrews (*Galegeeska rufescens*) are all present in sufficient density for a population genetic survey (Alibhai and Key, 1985). Alternatively, a species of tenebrionid beetle, *Paoligena heterocephaloides*, which cohabitates and may even have a mutualistic relationship with naked mole-rats, would be another option to explore for correspondence in population structure. These beetles have wings, but have never been found outside of naked mole-rat burrows (Penrith, 1982).

Concluding remarks

There are multiple forces at work shaping population structure of naked mole-rats. Faulkes et al. (1997b) has highlighted isolation by distance from the limited dispersal of naked mole-rats as one of the main reasons for the increased genetic distance with geographical distance. A closer look has shown us that at fine scales riverine barriers and historical events may play a profound role in this effect. Our study uncovered a complexity of population genetic patterns that we used to infer range expansion, fragmentation, and restricted dispersal across the range of this species. This study underscores the importance of using a phylogenetic component to elucidate the likely historical events giving rise to current patterns of genetic variation before estimating population-genetic statistics such as relatedness. Without the historical perspective conclusions regarding the other processes such as mate choice that are involved in shaping the pattern of population structure can be misleading.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Research Office of the President of Kenya, 15C/116.

Author contributions

JH contributed to the collection of samples, molecular analysis, data analysis, and writing of the manuscript. SB contributed to the collection of samples and writing of the manuscript. CI contributed to writing of the manuscript. AT contributed to data analysis and writing of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer HB declared a past co-authorship with one of the author, SB to the handling editor.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.857660/full#supplementary-material

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Functional anatomy and disparity of the postcranial skeleton of African mole-rats (Bathyergidae)

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The burrowing adaptations of the appendicular system of African mole-rats (Bathyergidae) have been comparatively less investigated than their cranial adaptations. Because bathyergids exhibit different digging modes (scratch-digging and chisel-tooth digging) and social systems (from solitary to highly social), they are a unique group to assess the effects of distinct biomechanical regimes and social organization on morphology. We investigated the morphological diversity and intraspecific variation of the appendicular system of a large dataset of mole-rats (n = 244) including seven species and all six bathyergid genera. Seventeen morpho-functional indices from stylopodial (femur, humerus) and zeugopodial (ulna, tibia-fibula) elements were analyzed with multivariate analysis. We hypothesized that scratch-diggers (i.e., *Bathyergus*) would exhibit a more specialized skeletal phenotype favoring powerful forelimb digging as compared to the chisel-tooth diggers, and that among chisel-tooth diggers, the social taxa will exhibit decreased limb bone specializations as compared to solitary taxa due to colony members sharing the costs of digging. Our results show that most bathyergids have highly specialized fossorial traits, although such specializations were not more developed in *Bathyergus* (or solitary species), as predicted. Most chisel tooth-diggers are equally, or more specialized than scratch-diggers. *Heterocephalus glaber* contrasted significantly from other bathyergids, presenting a surprisingly less specialized fossorial morphology. Our data suggests that despite our expectations, chisel-tooth diggers have a suite of appendicular adaptations that have allowed them to maximize different aspects of burrowing, including shoulder and neck support for forward force production, transport and removal of soils out of the burrow, and bidirectional

locomotion. It is probably that both postcranial and cranial adaptations in bathyergids have played an important role in the successful colonization of a wide range of habitats and soil conditions within their present distribution.

"Africa has produced many mammal curiosities, and the Bathyergidae take a high rank among these"

JR **Ellerman**, 1956

Introduction

African mole-rats (Bathyergidae) are highly specialized subterranean rodents that spend most of their lives underground and build extensive and complex burrow systems (Jarvis et al., 1998; Bennett and Faulkes, 2000; Le Comber et al., 2002). Among bathyergids, only one genus (*Bathyergus*) is a scratch-digger that predominantly uses its long fore-claws to break up mostly sandy soils, while the rest of the bathyergids (*Heliophobius, Georychus, Cryptomys, Fukomys,* and *Heterocephalus*) are chisel-tooth diggers that primarily use their highly procumbent incisors to break up soils of different degrees of hardness, varying from sandy to highly compacted (Jarvis and Sale, 1971; Bennett and Faulkes, 2000). Additionally, all the chisel tooth-digging genera have very short claws in the forefeet and hindfeet (**Figure 1**), thus suggesting a more relegated function of claws for breaking up soils. Bathyergids also have a wide spectrum of social organizations ranging from solitary to highly social (Jarvis and Bennett, 1993; Jarvis et al., 1994; Burda et al., 2002), as well as a wide range of body sizes, from ∼35 g in *Heterocephalus glaber* up to 2 kg in *Bathyergus suillus*. Such combination of features makes African mole-rats a unique group of mammals to assess the effects of digging mode and social behavior on the morphology of the burrowing apparatus.

Although many aspects of their ecology, physiology, behavior, and evolutionary history have been well-documented (e.g., Bennett and Faulkes, 2000; Šumbera., 2019; Visser et al., 2019; Oosthuizen and Bennett, 2022), a comparative assessment of their postcranial morphology and development including all genera is still lacking. The fossil record of this group is underrepresented, and most fossil taxa are known basically on their cranial and dental material only (Lavocat, 1973; Winkler et al., 2010; Bento Da Costa and Senut, 2022). Several studies of extant bathyergids have focused on the cranial and dental anatomy of a few species (e.g., Berkovitz and Faulkes, 2001;

Hart et al., 2007; Barčiová et al., 2009; Van Daele et al., 2009; Gomes Rodrigues et al., 2011; McIntosh and Cox, 2016a; Caspar et al., 2021). Only more recent assessments have incorporated a comparative approach including a larger number of species (Gomes Rodrigues and Šumbera, 2015; Gomes Rodrigues et al., 2016; Mason et al., 2016; McIntosh and Cox, 2016b; Fournier et al., 2021). In general, these studies have found clear differences between chisel-tooth diggers and scratch-diggers, with the former having a more specialized dental and craniomandibular morphology, including more procumbent incisors, shorter snout, relatively wider and taller skulls with enlarged zygomatic arches, strongly hystricognathous mandible, and increased jaw and condyle lengths relative to their size, all features that facilitates higher bite forces and wider gapes to maximize breaking up soils (Gomes Rodrigues et al., 2016; McIntosh and Cox, 2016a,b).

Regarding their limb anatomy, several studies on rodents and mammals have included a few bathyergid species in their analyses, although these are usually represented by small sample sizes or are still unpublished (e.g., Carleton, 1941; Cuthbert, 1975; Hildebrand, 1978, 1985; De Graaff, 1979; Stein, 2000; Samuels and Van Valkenburgh, 2008; Thomas, 2013; Prochel et al., 2014; Wilson and Geiger, 2015). Recently, Sahd et al. (2019) assessed the effects of hind-foot drumming in the musculoskeletal system of *B. suillus, Georychus capensis,* and *Cryptomys hottentotus natalensis,* and Doubell et al. (2020) compared the forelimb musculoskeletal anatomy of *B. suillus* and *H. glaber*. Also, Montoya-Sanhueza and Chinsamy (2018) and Montoya-Sanhueza et al. (2019) focused on the postnatal development of the long bones and the patterns of mineral mobilization (bone formation and resorption) of the femur of *B. suillus*, respectively. More recently, Montoya-Sanhueza et al. (2022a,b) assessed the development of bone superstructures associated with fossoriality, and the proximal morphology of the femur of a large sample including all bathyergid genera. The latter studies represent the first comparative assessments of the limb anatomy including all bathyergid genera.

The present study aims to determine the patterns of intraspecific and interspecific variation of the appendicular digging apparatus of Bathyergidae. We quantified the morphological diversity (disparity) of the humerus, ulna, femur and tibia-fibula of all six bathyergid genera including
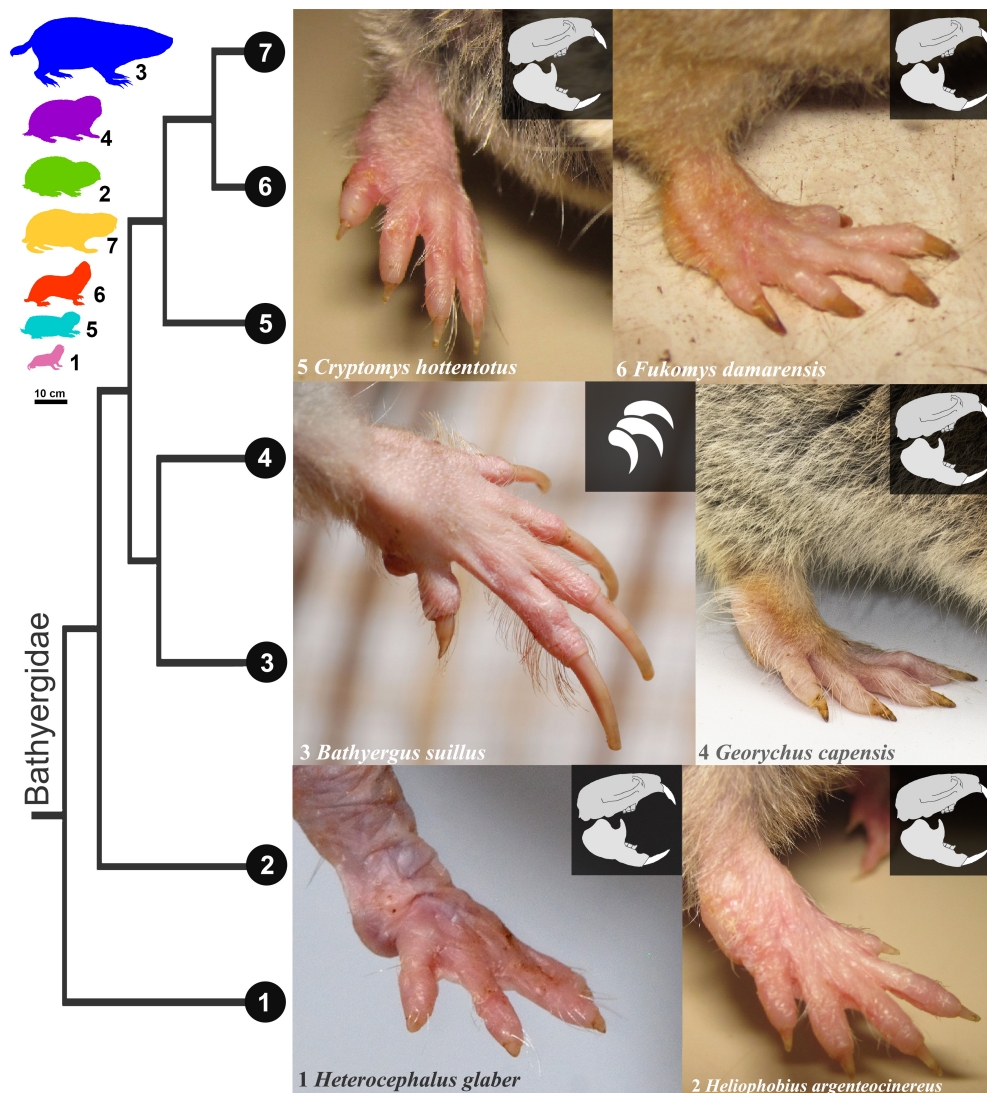
FIGURE 1

Morphology of the manus of six African mole-rats species and a phylogeny of the Bathyergidae (based on Uhrová et al., 2022). Note the long claws of the scratch-digger *Bathyergus suillus* (claw symbol) and the reduced claw size of the rest of the chisel-tooth digger genera (skull symbol), particularly the poor development of claws in *Heterocephalus glaber*. Mole-rat silhouettes represent approximate body size differences between species. *Fukomys mechowii* (#7) is not portrayed in the figure, since exhibits similar claw morphology as other *Fukomys* species. Photography of species 1, 2, 3, 5, and 6 by Germán Montoya-Sanhueza© and species 4 by Maria Oosthuizen©.

seven species (**Table 1**). We assessed the relationship between morphology and behavior (i.e., digging mode and social organization) in this family, and predicted that the scratch-digging taxa (i.e., *Bathyergus*) would exhibit a more specialized skeletal phenotype favoring powerful parasagittal motion of forearms and downward thrust of the forefeet to break up the soil as compared to the chisel-tooth digging taxa, because their limbs are directly involved in breaking the soils during the initial phase of burrowing. Thus, *Bathyergus* should exhibit a more distally located deltoid tuberosity to increase the lever arm for powerful retraction and flexion of the humerus, as well as an enlarged olecranon process to increase the power

stroke of the arm during elbow extension (Vassallo, 1998). Because the hindlimb skeleton is assumed to perform relatively similar functions in both scratch-diggers and chisel-tooth diggers, involving body stabilization and soil transport/removal (Gambaryan and Gasc, 1993; Stein, 2000; Moore Crisp et al., 2019), we expect similar levels of specialization among species.

We also examined if social organization has an effect on morphological variation. It is known that *H. glaber* forms organized sequences of "cooperative" digging, where colony members work together in a relay forming chains (Jarvis and Sale, 1971; Tucker, 1981), although this behavior is probably also present in other social bathyergids (Šumbera., 2019). It

TABLE 1   Main characteristics of the seven bathyergid species analyzed in this study.

Common name	Species	Sample size	Digging mode	Social system
Naked mole-rat	*Heterocephalus glaber*	65	Chisel-tooth digging	Highly social
Silvery mole-rat	*Heliophobius argenteocinereus*	29	Chisel-tooth digging	Solitary
Cape dune mole-rat	*Bathyergus suillus*	39	Scratch-digging	Solitary
Cape mole-rat	*Georychus capensis*	33	Chisel-tooth digging	Solitary
Common mole-rat	*Cryptomys hottentotus*	34	Chisel-tooth digging	Social
Giant mole-rat	*Fukomys mechowii*	12	Chisel-tooth digging	Highly social
Damaraland mole-rat	*Fukomys damarensis*	32	Chisel-tooth digging	Highly social

has been documented that increased group size in mole-rats lowers the costs of foraging in the social *C. hottentotus* (Spinks and Plagányi, 1999), that solitary species such as *Heliophobius argenteocinereus* have a more effective working metabolism as compared to *Fukomys mechowii* (Zelová et al., 2010), and that the increased number of non-breeding subordinates in *Fukomys damarensis* is associated with reductions in the workload of breeders (Houslay et al., 2020). These data suggest that social species may share the effort of digging activities, probably reducing the selection of an extremely specialized burrowing apparatus. Consequently, we also expect solitary species to exhibit increased mechanical advantage in their limb bones as compared to social species.

Materials and methods

Specimens and skeletal maturity

A total of 244 specimens comprising seven species of all six bathyergid genera were analyzed (**Table 1** and **Figure 1**). The sample comprises skeletally mature specimens of both sexes, as well as individuals of unknown sex. Body mass (BM) was obtained for almost all individuals. In this study, skeletal maturity is defined as individuals having full alveolar eruption of all upper or lower molars. Patterns of molar eruption were obtained using dental information from multiple sources (Hamilton, 1928; Taylor et al., 1985; Bennett et al., 1990; Jarvis and Sherman, 2002; Hart et al., 2007; Gomes Rodrigues et al., 2011; Gomes Rodrigues and Šumbera, 2015; Berkovitz and Shellis, 2018; Montoya-Sanhueza et al., 2021a,b). The skeletal maturity of specimens lacking craniodental material was based on gross morphological features of the body and limbs, such as having an adult body size and/or well-developed limbs showing fully developed secondary centers of ossification at epiphyses and a fused distal epiphysis of the humerus (e.g., Klein, 1991). The majority of the specimens were wild-caught, although some individuals of *F. damarensis* and *F. mechowii*, and all individuals of *H. glaber* were born in captivity. The colonies of *H. glaber* were housed in tunnels made of glass without substrate to dig in. Additional details of captivity conditions for *H. glaber*

are described elsewhere (Montoya-Sanhueza et al., 2021a). All material analyzed here is housed in the Department of Biological Sciences at the University of Cape Town (UCT), South Africa.

Functional anatomy and morpho-functional indices

Stylopodial (femur and humerus) and zeugopodial (ulna and tibia-fibula) elements from either the right or left side of the individual were dissected and skeletonized. The general anatomy of each bone was described and compared among species following the anatomical nomenclature of previous studies (e.g., Holliger, 1916; Greene, 1935; Salton and Sargis, 2008, 2009; Böhmer et al., 2020; **Figure 2A**). A detailed description of the main anatomical characteristics of the long bones of bathyergids is presented in the **Supplementary Figures 1**, **2**. A total of 19 linear measurements including length and diameter of limb bones were obtained (**Figure 2B**), mostly based on Echeverría et al. (2014) and Montoya-Sanhueza et al. (2019), and the references therein. All measurements were recorded to the nearest 0.01 mm using a digital caliper, and are fully described in **Supplementary material**. Seventeen morpho-functional indices (**Table 2**) that reflect the main aspects of the bone shape at the diaphyseal, proximal and distal portions of the bone were calculated from linear measurements following previous studies (Howell, 1965; Hildebrand, 1985; Rose, 1989; Vizcaíno et al., 1999, 2016; Elissamburu and Vizcaíno, 2004; Salton and Sargis, 2008; Samuels and Van Valkenburgh, 2008; Echeverría et al., 2014; Wilson and Geiger, 2015; Montoya-Sanhueza et al., 2019; and references therein). These morpho-functional indices represent an easy and straightforward approximation to estimate mechanical advantage and function of the main muscles related to limb function, specifically for scratch-digging (e.g., Hildebrand, 1985; Samuels and Van Valkenburgh, 2008). In particular, we follow the study of Montoya-Sanhueza et al. (2019), which reviewed and modified some previous functional indices to appropriately assess the functional implications of parasagittal scratch-digging with the forelimb and strength of the hindlimb. In total, four indices represent the morphology of the humerus (RDT, HRI,
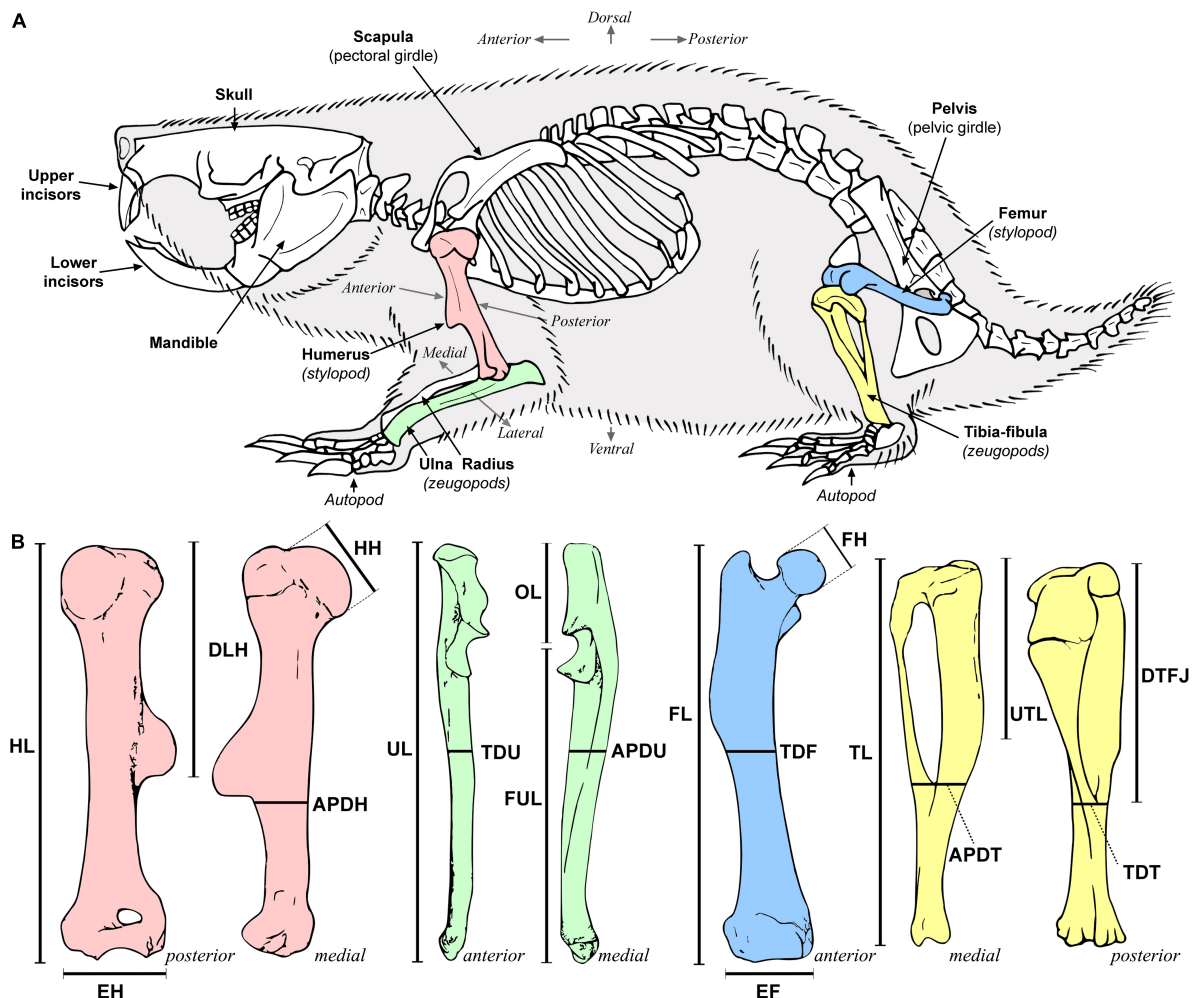
FIGURE 2
Skeletal anatomy and appendicular morphology of mole-rats. **(A)** Anatomical and directional terminology used to describe the relative position of structures in the skeleton. Bones analyzed are in colors: humerus (red), ulna (green), femur (blue), and tibia-fibula (yellow). **(B)** Linear measurements obtained in this study. Total lengths of humerus (HL), ulna (UL), femur (FL), and tibia-fibula (TL); transversal diameters of ulna (TDU), femur (TDF), and tibia-fibula (TDT); anteroposterior diameters of humerus (APDH), ulna (APDU), and tibia-fibula (APDT); deltoid tuberosity length (DLH); humeral (HH) and femoral head (FH) diameters; humeral (EH) and femoral (EF) epicondylar widths; olecranon length (OL); functional length of the ulna (FUL); length of the distal tibio-fibular junction (DTFJ); tibial tuberosity (UTL). Linear measurements are fully described in **Supplementary Methods**. The skeletal anatomy, body silhouette and long bones are based on *Bathyergus suillus* (extracted and modified from Montoya-Sanhueza et al., 2019).

EIH, HH), four of the ulna (IFA, URI, URI*, BI), three of the femur (FRI, EIF, FHI) and five of the tibia-fibula (TSI, TRI, TRI*, TJI, CI) (**Table 2**). The BI, CI and an additional index, the intermembral index (IMI) represent proportions between bone elements, thus indicating locomotor advantage (Howell, 1965). Since *H. glaber* lacks some relevant bone superstructures in their long bones (i.e., lack of a projected deltoid tuberosity and non-fused tibia and fibula, Montoya-Sanhueza et al., 2022a), the indices RDT, TRI, TRI* and TJI were not calculated for this taxon. The calculation and functional significance of all indices are presented in **Table 2**. Additionally, because sex differences are known to affect the growth trajectories and morphology of small mammals, sexual

dimorphism in body mass and morpho-functional indices was also assessed.

Ecomorphological groups

To assess the effects of a specific subterranean lifestyle on bone phenotype, three ecomorphological groups that account for the main behavioral and functional features of bathyergids were established based on a combination of their digging mode and social organization, and are classified as follow: (i) solitary scratch-diggers, (ii) solitary chisel-tooth diggers, and (iii) social chisel-tooth diggers. Note that the hypothetical group of "social

scratch-diggers" was not defined in the present study since there are no extant bathyergid species reported with such combination of features.

Statistical analysis

Multivariate analysis

A series of multivariate analysis of variance (MANOVA) coupled with Tamhane *post hoc* tests were carried out to assess interspecific differences in morpho-functional indices. Forelimb (n = 244) and hindlimb (n = 217) data sets were not equal in completeness, so these were assessed separately. This allowed emphasizing differences between limb regions. Because some morpho-functional indices were not calculated for *H. glaber* (see above), two MANOVAs were performed for each limb region, one including all species but excluding some indices, and a second including all indices, but excluding *H. glaber*. This allowed the testing of differences in bone morphology and fossorial specializations with and without the influence of *H. glaber*. Homoscedasticity of the sample was tested by log_{10} transformation, although without improvement, so the data were analyzed without transformation. Consequently, because all MANOVAs showed unequal variances, both Wilks' lambda and Pillai trace statistics are provided, since the latter test is more robust under violations of homoscedastic covariance (Quinn and Keough, 2002). Graphs (bar charts) were prepared for all indices and show mean (central point), standard error (s.e., shorter whiskers) and standard deviation (s.d., longer whiskers). Morphological data are presented as mean and standard deviation (mean ± s.d.). A significance level of 0.05 was used for all analyses.

Ordination analysis

Principal Component Analysis (PCA) and Discriminant Analysis (LDA) were performed to identify the major components of variation among the seven species and the three ecomorphological groups, respectively. The PCA used separated datasets for forelimbs (n = 244) and hindlimbs (n = 217), and specimens with an incomplete dataset of indices (either for the forelimb and hindlimb) were still included, but performing the "iterative imputation" method as an estimation for the missing values. For the LDA, forelimb and hindlimb indices were combined, and only specimens (n = 216) with a complete set of measurements (i.e., for humerus, ulna, femur, and tibia-fibula) were analyzed. The LDA produces linear combinations of variables (canonical variates) that best separate *a priori* defined groups, based on maximizing differences between groups and reducing their within-group differences. MANOVAs were coupled to the LDA to assess

differences among ecomorphological groups. We also estimated whether specimens could be classified into a defined group. As in the multivariate analysis, separated PCAs and DAs were performed (see above), a dataset including all species, and a dataset including all indices. All datasets included individuals of known and unknown sex. The algorithm to assess the correlation matrix for the PCA was the "variance-covariance," since all the linear measurements used to build the indices were originally measured in the same unit (mm). Variables were analyzed to highlight "between group" (species and ecomorphological) differences.

Sex differences

Sex differences in both body mass (BM) and morpho-functional indices were assessed by non-parametric two-tailed (Wilcoxon) Mann-Whitney U-test, and two-way PERMANOVA for all species (n = 190) and indices, coupled with Bonferroni correction (excluding *H. glaber*), respectively. One-way PERMANOVA was used to test sex differences in *H. glaber* (n = 59). A significance level of 0.05 was used for all analyses, and p-values were obtained using 9,999 permutations. In total, 139 females and 110 males were analyzed.

All analyses and plots were performed in PAST version 2.17c (Hammer et al., 2001) and IBM SPSS version 25 (IBM Corp, 2017).

Results

A qualitative description of the forelimb and hindlimb anatomy of bathyergids is presented in the **Supplementary Results**. Also refer to Montoya-Sanhueza et al. (2022a,b).

Multivariate analysis of the forelimb

The main characteristics of the forelimb are presented in **Figure 3A**. Descriptive statistics of the morpho-functional indices are presented in **Table 3**, and corresponding whisker plots in **Figure 3B**. The MANOVA including all species (and excluding RDT) showed significant differences among species [Wilks' λ = 0.013; $F_{(42,1086)}$ = 38.961; p < 0.001] (**Table 4**). The variables that better explain morphological differences can be identified by high "partial eta squared" (PES) values (> 0.50), which accounts for the proportion of variance explaining interspecific differences. All indices, except URI showed high PES values, ranging between 0.504 (IFA) and 0.697 (BI) (**Table 4** and **Supplementary Table 1**). In the humerus, HRI showed the highest PES values (0.566), thus contributing most to species differentiation, while BI (0.697) and URI* (0.590) showed the highest PES in the ulna. In this analysis, *H. glaber*

TABLE 2 Morpho-functional indices, calculation, and functional significance.

Index		Calculation	Functional significance
RDT	Relative position of the deltoid tuberosity	Humeral head-distal origin of deltoid process length divided by humeral length (DLH/HL)	An estimator of in-lever arm of the deltoid and pectoral muscles. Higher values (more distally located DT) indicate stronger flexion of the arm.
HRI	Humerus robustness index	Anteroposterior diameter at diaphysis divided by humeral length (APDH/HL)	Indicates robustness of the humerus and its ability to resist bending and torsional stresses, mainly in the anteroposterior axis.
EIH	Humeral epicondylar index	Epicondylar width of the humerus divided by humeral length (EH/HL)	Indicates the relative area available for the origin of the forearm flexors, pronators, and supinators.
HHI	Humeral head index	Maximum diameter (anteroposterior) of humeral head divided by humeral length (HH/HL)	Represents the relative size of the articular surface of the glenohumeral joint, and gives an idea of the area available for stabilization and shock absorption of the shoulder needed for digging, as well as extensiveness for anteroposterior movements of the humerus.
IFA	Index of fossorial ability	Olecranon length divided by the functional ulnar length (OL/FUL)	This index reflects the mechanical advantage of the triceps and epitrochlearis muscles during elbow extension.
URI	Robustness of the ulna (ML)	Mediolateral (transverse) diameter of the ulna at the diaphyseal midpoint divided by functional ulnar length (TDU/FUL)	Reflects the resistance of the ulna to bending in the mediolateral axis.
URI*	Robustness of the ulna (AP)	Anteroposterior diameter of the ulna at the diaphyseal midpoint divided by functional ulnar length (APDU/FUL)	Reflects the resistance of the ulna to bending in the anteroposterior axis, as well as indicates the relative surface available for the insertion of muscles involved in pronation and supination of the forearm, and flexion of the manus and digits.
BI	Brachial index	Functional ulnar length divided by humeral length (FUL/HL)	Indicates the relative proportions of proximal (humerus) and middle (ulna) elements of the forearm, and gives an indication of the extent to which the forelimb is apt for fast movements.
FRI	Femur robustness index	Transverse diameter of the femur at the diaphyseal midpoint divided by the functional femoral length (TDF/FL)	Indicates robustness of the femur and its ability to resist bending and torsional stresses in the mediolateral axis.
EIF	Femoral epicondylar index	Epicondylar width of the femur divided by femoral length (EF/FL)	Indicates the relative area available for the origin of the gastrocnemius, extensor and flexor muscles used mainly for flexion of the knee and plantar-flexion of the pes.
FHI	Femoral head index	Maximum diameter of femoral head divided by femoral length (FH/FL)	Indicates the dimension of the femoral head, and gives an idea of the range of motion of the hip joint.
TSI	Tibial crest index	Proximal tibia length (distance from the proximal articular surface of the tibia to the distal point of the tibial tuberosity), divided by the tibial length (UTL/TL)	Reflects the strength of the leg and the relative width available for the insertion of the gracilis, semitendinosus and semimembranosus muscles and the foot flexors. Also hamstrings and biceps femoris muscles acting across the knee and hip joints.
TRI	Robustness of the tibia-fibula (ML)	Mediolateral (transverse) diameter of the tibia-fibula divided by the tibial length (TDT/TL)	Indicates robustness of the tibia and its ability to resist bending and torsional stresses in the mediolateral axis.
TRI*	Robustness of the tibia-fibula (AP)	Anteroposterior diameter of the tibia-fibula divided by the tibial length (APDT/TL)	Indicates robustness of the tibia and its ability to resist bending and torsional stresses in the anteroposterior axis.
TJI	Tibio-fibular junction index	Length of the distal tibio-fibular junction divided by the tibial length (DTFJ/TL)	Indicates the extension of the tibio-fibular fusion along the distal diaphysis, and hence the resistance to bending and torsional loads during biomechanically demanding activities against the substrate. Lower values indicate a larger fusion area, and therefore a stronger diaphysis.
CI	Crural index	Tibial length divided by femoral length (TL/FL)	Indicates the relative proportions of proximal (femur) and middle (tibia) elements of the hindleg. Indicates how well the hindlimbs are apt for speed.
IMI	Intermembral index	Forelimb length divided by the hindlimb length [(HL + FUL)/(FL + TL)]	Indicates the relative symmetry between the foreleg and hindleg.

Measurements are illustrated in **Figure 2**, and described in **Supplementary Methods**. Indices are based on Echeverría et al. (2014) and Montoya-Sanhueza et al. (2019), and references therein. Anteroposterior (AP), mediolateral (ML).
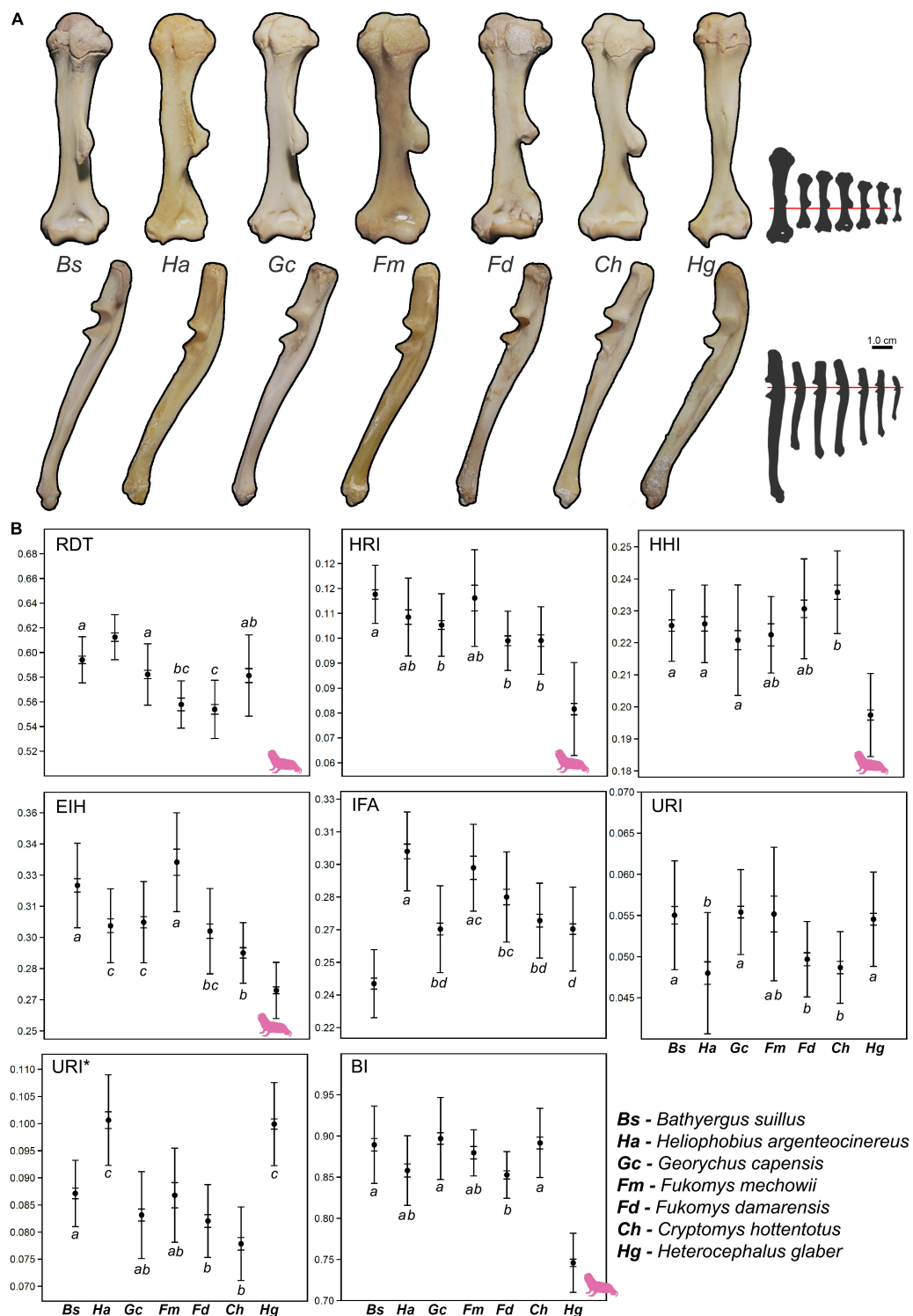
FIGURE 3

Quantification of the forelimb morphology (humerus and ulna). **(A)** Humeral (top row) and ulnar (bottom row) morphology of all species. Bones scaled to same size to emphasize differences in bone shape. Small bone silhouettes show the real relative size of bones between species. The humeri are aligned to the distal origin of the deltoid tuberosity (except in *Heterocephalus glaber*), while the ulnae are aligned to the center of the trochlear notch. **(B)** Whisker plots showing differences among species. Mean (central point), s.e (short whiskers) and s.d. (long whiskers). Species that share the same letter represent homogeneous subsets, as identified by *post hoc* (Tamhane) tests. The pink silhouette represents *H. glaber* and indicates the indices for which this species differs significantly from other bathyergids, as well as the indices not measured for this species (i.e. lack of morphological specialization). Taxa are primarily ordered from solitary to social species, and secondarily from the largest to the smallest species. Abbreviation of indices in **Table 2**.

TABLE 3 | Descriptive statistics (mean and s.d.) of forelimb and hindlimb morpho-functional indices.

Index	*B. suillus*	*He. argenteocinereus*	*G. capensis*	*F. mechowii*	*F. damarensis*	*C. hottentotus*	*H. glaber*	Bathyergidae**	Bathyergidae
Forelimb	(*n* = 39)	(*n* = 29)	(*n* = 33)	(*n* = 12)	(*n* = 32)	(*n* = 34)	(*n* = 65)	(*n* = 179)	(*n* = 244)
RDT	0.594	0.612	0.587	0.559	0.554	0.581	–	0.581	–
s.d.	0.019	0.018	0.020	0.017	0.024	0.033	–	0.022	–
HRI	0.118	0.111	0.111	0.114	0.105	0.104	0.084	0.111	0.107
s.d.	0.009	0.012	0.009	0.013	0.009	0.010	0.014	0.005	0.011
HHI	0.225	0.226	0.220	0.223	0.231	0.236	0.197	0.227	0.223
s.d.	0.011	0.012	0.017	0.012	0.015	0.013	0.013	0.006	0.012
EIH	0.325	0.306	0.308	0.329	0.302	0.293	0.274	0.310	0.305
s.d.	0.020	0.018	0.020	0.016	0.021	0.015	0.014	0.014	0.019
IFA	0.245	0.306	0.276	0.303	0.288	0.274	0.270	0.282	0.280
s.d.	0.016	0.018	0.019	0.018	0.018	0.017	0.019	0.022	0.021
URI	0.055	0.048	0.055	0.053	0.050	0.049	0.055	0.052	0.052
s.d.	0.007	0.007	0.006	0.006	0.005	0.004	0.006	0.003	0.003
URI*	0.087	0.101	0.083	0.084	0.082	0.078	0.100	0.086	0.088
s.d.	0.006	0.008	0.009	0.006	0.007	0.007	0.008	0.008	0.009
BI	0.889	0.858	0.889	0.881	0.852	0.892	0.746	0.877	0.858
s.d.	0.047	0.042	0.046	0.030	0.028	0.042	0.036	0.017	0.052
Hindlimb	(*n* = 39)	(*n* = 29)	(*n* = 23)	(*n* = 11)	(*n* = 18)	(*n* = 33)	(*n* = 64)	(*n* = 152)	(*n* = 217)
FRI	0.119	0.127	0.129	0.134	0.120	0.124	0.110	0.125	0.123
s.d.	0.009	0.014	0.013	0.013	0.013	0.017	0.010	0.006	0.008
EIf	0.239	0.262	0.257	0.300	0.279	0.264	0.240	0.267	0.263
s.d.	0.016	0.014	0.013	0.021	0.022	0.018	0.012	0.021	0.021
FHI	0.136	0.144	0.139	0.146	0.144	0.132	0.138	0.140	0.140
s.d.	0.010	0.007	0.007	0.011	0.008	0.007	0.012	0.006	0.005
TSI	0.491	0.483	0.456	0.468	0.445	0.425	0.388	0.461	0.451
s.d.	0.020	0.032	0.028	0.023	0.042	0.032	0.027	0.025	0.036
TRI	0.094	0.081	0.094	0.080	0.073	0.079	–	0.083	–
s.d.	0.006	0.008	0.010	0.007	0.008	0.009	–	0.009	–
TRI*	0.127	0.149	0.144	0.153	0.130	0.128	–	0.139	–
s.d.	0.012	0.019	0.009	0.018	0.014	0.013	–	0.012	–
TJI	0.599	0.531	0.517	0.547	0.533	0.529	–	0.543	–
s.d.	0.018	0.036	0.025	0.025	0.023	0.025	–	0.029	–
CI	0.927	0.987	0.979	1.035	1.059	1.067	1.028	1.009	1.012
s.d.	0.027	0.039	0.027	0.034	0.028	0.033	0.034	0.054	0.050
IMI	0.862	0.875	0.884	0.910	0.851	0.844	0.856	0.871	0.869
s.d.	0.030	0.024	0.026	0.030	0.023	0.019	0.019	0.024	0.023

Family mean values excluding *Heterocephalus glaber* are indicated with a double asterisk (**). See abbreviation of species and indices in Table 1, 2.

showed the statistically significant lowest values of HRI and BI in comparison to the rest of the bathyergids (**Table 3** and **Figure 3B**).

The MANOVA including all indices (and excluding *H. glaber*) also showed significant differences among species [Wilks' λ = 0.029; $F_{(40, 726)}$ = 22.823; $p < 0.001$]. High PES values were obtained only for IFA (0.588), although URI* (0.499) and RDT (0.409) also showed relatively high values (**Table 4** and **Supplementary Table 1**). The rest of the variables (HRI, HHI, EIH, URI, and BI) showed low values (< 0.35), indicating that such variables do not contribute considerably to

species differentiation when the RDT index is included and *H. glaber* is absent (**Table 4**).

Pair-wise *post-hoc* tests showed significant differences between *H. glaber* and all other species for almost all indices, except for ulnar variables such as IFA, URI and URI* (**Figure 3B**). Thus, despite the functional length of the ulna (BI) of *H. glaber*, it appears relatively shorter as compared to other bathyergids, its general shape does not differ greatly from other species (**Figure 3B**). Only *B. suillus* showed a marked difference with the rest of the bathyergids, specifically the significantly lowest IFA values within the family (**Table 3** and **Figure 3B**).

TABLE 4 MANOVAs on morpho-functional indices of the forelimb and hindlimb, including all species and all indices.

	Test	Value	*F*	Hypothesis Df	Error Df	*P*	Partial eta squared (PES)	Indices (PES) > 0.50
Forelimb *(all spp)*	Pillai's Trace	2.469	23.580	42	1,416	<0.001	0.412	All except URI
	Wilks' Lambda	0.013	38.961	42	1086.94	<0.001	0.512	
Forelimb *(all indices)*	Pillai's Trace	2.251	17.396	40	850	<0.001	0.450	IFA
	Wilks' Lambda	0.029	22.823	40	726.37	<0.001	0.508	
Hindlimb *(all spp)*	Pillai's Trace	1.942	16.745	36	1,260	<0.001	0.324	EIF, TSI, CI
	Wilks' Lambda	0.041	26.799	36	902.98	<0.001	0.413	
Hindlimb *(all indices)*	Pillai's Trace	2.304	13.575	45	715	<0.001	0.461	EIF, TJI, CI
	Wilks' Lambda	0.023	18.534	45	624.88	<0.001	0.532	

Indices with high partial eta square (PES) values (> 0.50) are also presented (see all PES values in Supplementary Table 1).

This analysis showed that there are clear differences in the variable contribution of morpho-functional indices when *H. glaber* is included in the assessment. For example, the brachial index (BI) showed one of the highest PES values (0.697) when all species are analyzed, although showed one of the lowest values (0.139) when *H. glaber* is excluded (Supplementary Table 1). This indicates that some indices in *H. glaber* have a high explanatory power for species differentiation.

Multivariate analysis of the hindlimb

Since *H. glaber* does not exhibit a distal fusion of the tibia-fibula (Figure 4A), the TJI, TRI, and TRI* indices were not calculated for this species. Descriptive statistics of the morpho-functional indices are presented in Table 3, and corresponding whisker plots in Figure 4B. The MANOVA including all species showed significant differences among species [Wilks' λ = 0.041; $F_{(36, 902)}$ = 26.799; p < 0.001] (Table 4, Figure 4B, and Supplementary Table 1). The highest PES values were found in EIF, TSI, and CI, while the rest of the indices showed quite low values (<0.33) (Table 4 and Supplementary Table 1). The highest PES were in the tibia-fibula, the CI and TSI (0.686 and 0.667, respectively), for which *H. glaber* presented the significantly lowest TSI values among bathyergids (Figure 4B).

The MANOVA including all indices (and excluding *H. glaber*) also showed significant differences among species [Wilks' λ = 0.023; $F_{(45, 625)}$ = 18.534; p < 0.001]. The indices with the highest PES values were CI (0.754), TJI (0.599), and EIF (0.507) (Table 4), so that the tibio-fibular indices contributed most to species differentiation. Contrary to the previous analysis, the TSI now showed lower values, thus indicating that for the tibio-fibula, the TSI does not contribute considerably to species differentiation. The high number of indices with high PES values in the hindlimb suggests more marked differences in the femur

and tibia of bathyergids in comparison to their forelimb bones (Supplementary Table 1). Tamhane *post hoc* tests showed only one significant difference between *H. glaber* and the other bathyergids, presenting the lowest TSI values, while *B. suillus* showed the highest and lowest values for TJI and CI, respectively, among bathyergids (Figure 4B).

In general, the MANOVAs (including all species) showed that the forelimb indices, particularly those associated with the humerus explain a greater proportion of the species differentiation as compared to the hindlimb indices when *H. glaber* is included in the analyzes.

Ordination analysis of the forelimb

The PCA that included all species (PCA$_{F1}$) generated six components, with most of the variation contained in the three first (98.28%), although most of this variation is explained by PC1, which contains 81.75% of the total variance (Table 5 and Figures 5A–C). The indices that contributed most to the variation in PC1, following the criterion defined in previous studies, such as a high correlation between variables (r^2 > 0.60, Wilson and Geiger, 2015), were BI (r^2 = 0.72) and HRI (r^2 = 0.62) (Supplementary Table 2). Marked differences were observed in the morphospace occupied by *H. glaber* and the rest of the bathyergids along PC1. Most individuals and species were distributed in the positive side of PC1, showing both high BI and EIH (Figure 5A), while *H. glaber* occupied a large area in the negative side of PC1 associated with a robust ulna (high URI and URI*). The PC2 represented only 11.56% of the total variation. The index that contributed most to the variation in this axis was the IFA (r^2 = 0.82) (Supplementary Table 2). Most specimens were equally distributed along the positive and negative sides of this axis, although the solitary species *B. suillus* and *He. argenteocinereus* tended to be associated with the extremes of the negative and positive sides, respectively. *Bathyergus suillus* tended to show
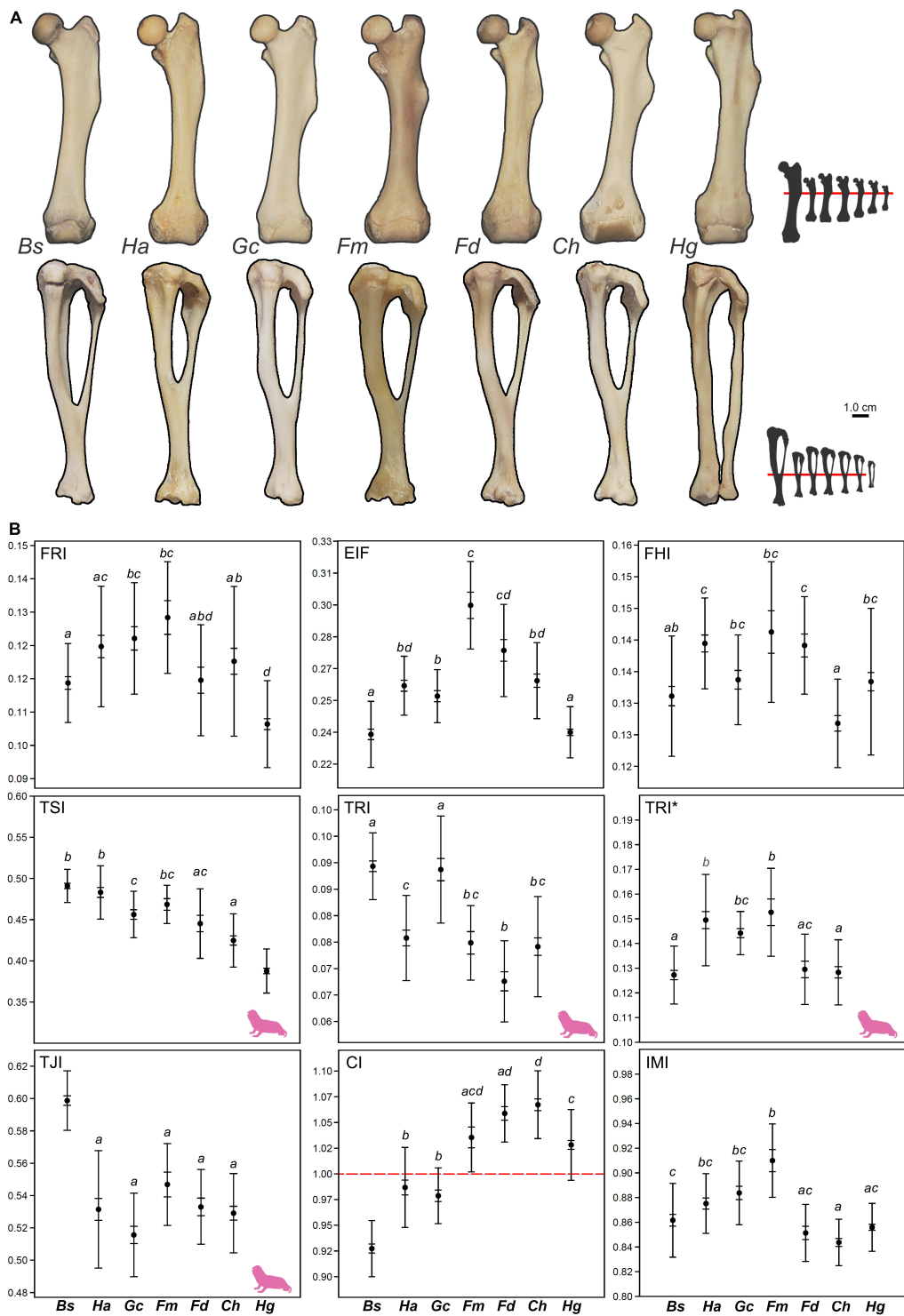
FIGURE 4

Quantification of the hindlimb morphology (femur and tibia-fibula). **(A)** Femoral (top row) and tibio-fibular (bottom row) morphology of all species. Bones scaled to same size to emphasize differences in bone proportions. Small bones silhouettes show the real relative size of bones between species. The femora are aligned to the distal origin of the third trochanter, while the tibia-fibula are aligned to the distal tibio-fibular junction (except in *Heterocephalus glaber*). **(B)** Whisker plots showing differences among species. The red line in the crural index (CI) indicates symmetry (= 1) between femur and tibia-fibula. Mean (central point), s.e (short whiskers) and s.d. (long whiskers). Species that share the same letter represent homogeneous subsets, as identified by *post hoc* (Tamhane) tests. The pink silhouette represents *H. glaber* and indicates the indices for which this species differs significantly from other bathyergids, as well as the indices not measured for this species (i.e. lack of morphological specialization). Taxa ordered as in **Figure 3**. See abbreviation of indices in **Table 2**, and of species names in **Figure 3**.

TABLE 5 Coefficients, eigenvalues, and proportion of variance for each principal component (PC) obtained for the analysis of forelimbs and hindlimbs, including all species (PCA$_{F1}$ and PCA$_{H1}$) and all indices (PCA$_{F2}$ and PCA$_{H2}$).

Indices	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Forelimb—All species (PCA$_{F1}$)						
HRI	0.185	0.028	0.277	0.294	0.136	0.765
HHI	0.184	0.068	−0.336	0.094	0.859	0.068
EIH	0.258	0.076	0.834	−0.274	0.284	−0.262
IFA	0.013	0.985	−0.070	−0.090	−0.113	0.057
URI	−0.010	−0.085	0.129	−0.149	−0.253	0.518
URI*	−0.106	0.098	0.238	0.888	−0.070	−0.236
BI	0.924	−0.044	−0.192	0.101	−0.287	−0.116
Eigenvalue	0.003	0.000	0.000	0.000	0.000	0.000
% Variance	81.758	11.560	4.959	0.938	0.758	0.027
Forelimb—All indices (PCA$_{F2}$)						
RDT	0.326	0.899	−0.010	0.059	0.026	
HRI	0.094	0.048	0.283	−0.097	0.027	
HHI	−0.020	−0.043	−0.290	−0.018	0.858	
EIH	0.100	−0.110	0.846	−0.219	0.290	
IFA	−0.772	0.224	0.240	0.531	0.041	
URI	0.072	−0.047	0.108	−0.022	−0.399	
URI*	−0.067	0.305	0.172	−0.180	0.101	
BI	0.519	−0.175	0.145	0.790	0.086	
Eigenvalue	0.001	0.000	0.000	0.000	0.000	
% Variance	43.773	31.050	15.510	8.745	0.923	
Hindlimb—All species (PCA$_{H1}$)						
FRI	−0.029	0.196	0.038	−0.697	0.280	0.629
EIF	0.136	0.569	0.025	0.230	−0.675	0.384
FHI	−0.005	0.101	0.066	0.672	0.574	0.453
TSI	−0.493	0.560	−0.578	−0.017	0.194	−0.267
CI	0.848	0.314	−0.215	−0.076	0.261	−0.248
IMI	−0.134	0.463	0.783	−0.064	0.174	−0.346
Eigenvalue	0.003	0.001	0.000	0.000	0.000	0.000
% Variance	67.706	24.902	6.727	0.491	0.134	0.040
Hindlimb—All indices (PCA$_{H2}$)						
FRI	0.016	0.161	−0.044	0.134	0.385	
EIF	0.223	0.408	0.318	−0.028	−0.488	
FHI	0.007	0.135	0.050	−0.277	−0.446	
TSI	−0.335	0.274	0.223	−0.689	0.193	
TRI	−0.319	−0.232	0.847	0.252	0.146	
TRI*	−0.070	0.735	0.066	0.435	−0.002	
TJI	0.847	−0.030	0.314	−0.083	0.258	
CI	−0.003	0.347	−0.099	−0.130	0.538	
IMI	−0.112	−0.007	−0.119	0.393	0.000	
Eigenvalue	0.004	0.001	0.001	0.000	0.000	
% Variance	70.527	17.618	9.536	2.014	0.305	

relatively low IFA values, while *He. argenteocinereus* high values.

The second PCA including all indices, and excluding *H. glaber* (PCA$_{F2}$) generated five components, with most of the variance contained in the four first components (99.08%) (**Table 5** and **Figures 5D,E**). Species distribute almost evenly in the center of the two first components (74.82%). The main contributor to PC1 (43.77%) was IFA (r^2 = 0.76) (**Supplementary Table 2**). Most species share both positive and negative values indicating
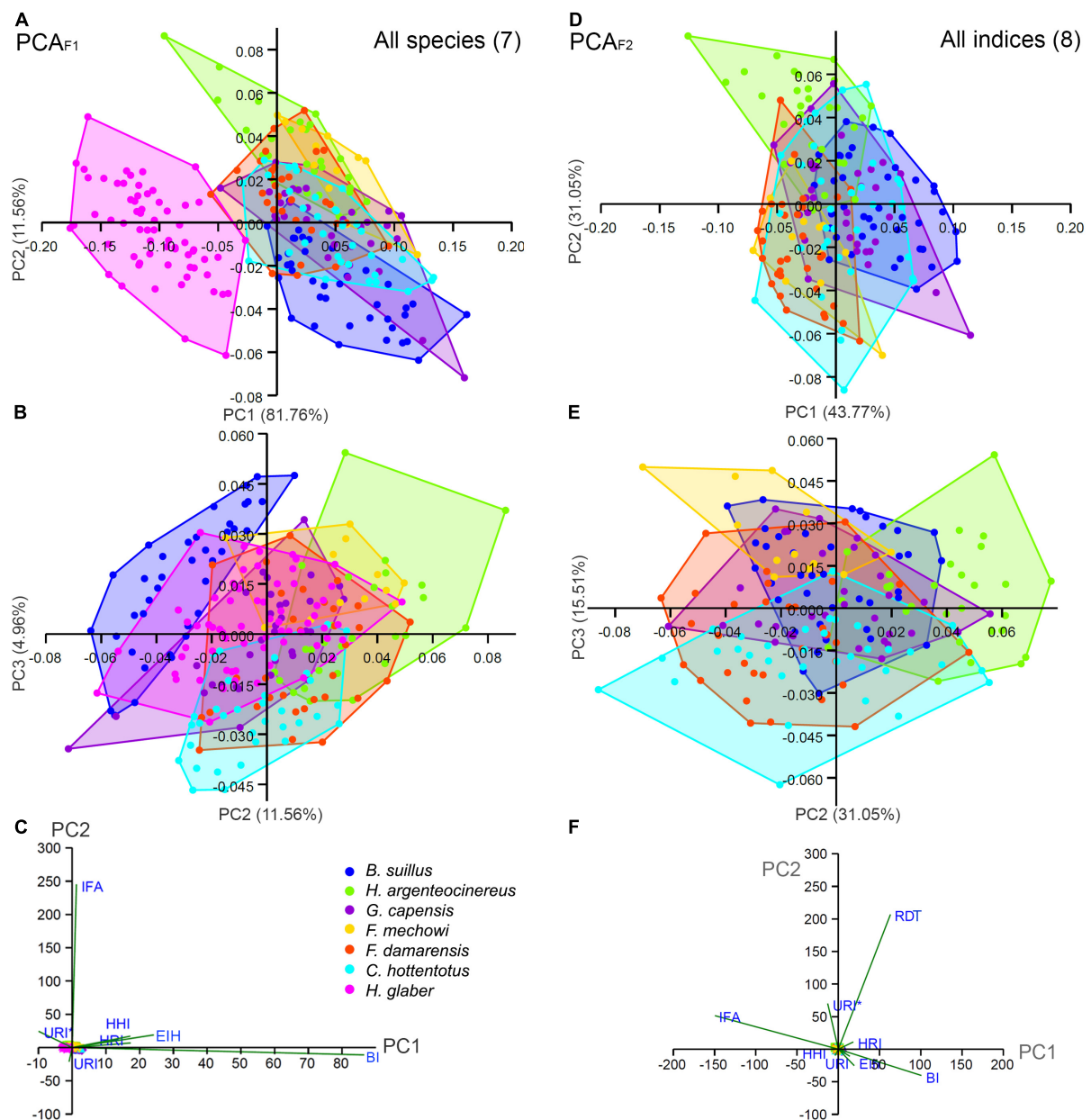
FIGURE 5

Ordination analysis for the morpho-functional indices of the forelimb including all species (PCA_F1) and all indices (PCA_F2). **(A)** Principal components (PC1 and PC2) of PCA_F1. **(B)** PC2 and PC3. **(C)** Biplots for PC1 and PC2. **(D)** PC1 and PC2 of PCA_F2. **(E)** PC2 and PC3. **(F)** Biplots for PC1 and PC2.

intermediate IFA values and only *B. suillus* positioned mostly in the positive side of PC1, thus indicating relatively lower values for IFA. *Fukomys* spp. and *He. argenteocinereus* tended to have higher IFA values. The main contributors to PC2 (31.05%) were RDT ($r^2 = 0.68$) and URI* ($r^2 = 0.67$) (**Supplementary Table 2**), which were mostly associated with the positive side of the axis. Only *He. argenteocinereus* and *F. mechowii* distributed either in the positive and

negative sides of the axis, respectively, whereas all the other species showed an intermediate distribution in the axis.

Ordination analysis of the hindlimb

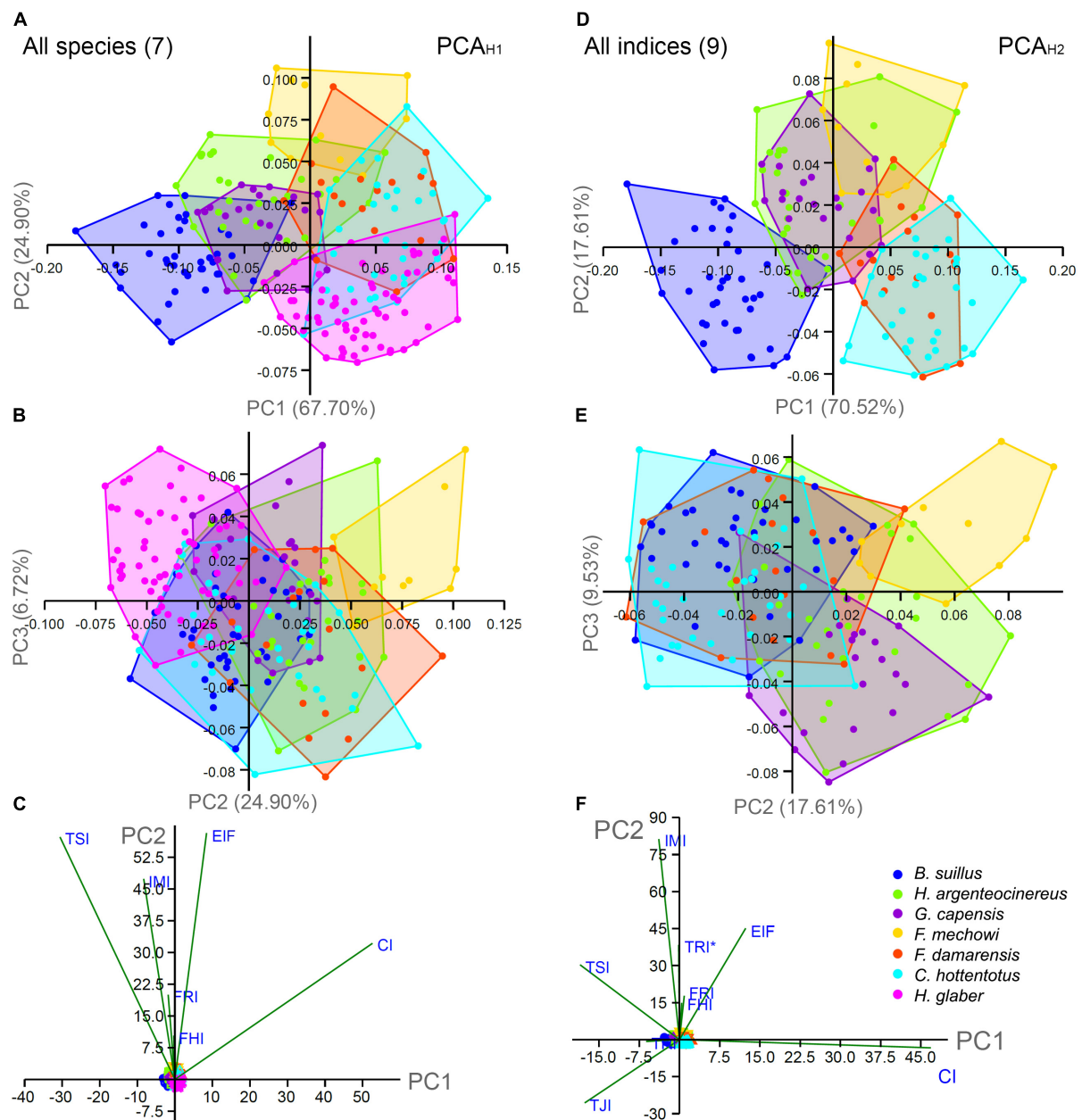The PCA including all species (PCA_H1) generated six components where the three first explained 99.33% of the

Ordination analysis for the morpho-functional indices of the hindlimb including all species (PCA$_{H1}$) and all indices (PCA$_{H2}$). **(A)** Principal components (PC1 and PC2) of PCA$_{H1}$. **(B)** PC2 and PC3. **(C)** Biplots for PC1 and PC2. **(D)** PC1 and PC2 of PCA$_{H2}$. **(E)** PC2 and PC3. **(F)** Biplots for PC1 and PC2.

variance (**Table 5** and **Figures 6A–C**). The PC1 represented 67.71% of the total variance, and it was able to separate *B. suillus* from all other social species, although not able to completely separate it from *He. argenteocinereus* and *G. capensis*. The CI showed the highest contribution ($r^2 = 0.85$), although TSI also showed high contribution ($r^2 = 0.57$) in this axis. Individuals of solitary species distributed mostly in the negative side of PC1, and were associated with higher TSI index, while the social species mostly occupied the positive side of the axis

only and were associated with a higher CI index. The PC2 (25.90%) was able to differentiate between the largest social species (*F. mechowii*) in the positive side and the smallest social species (*H. glaber*) in the negative side, with the exception of a few individuals (**Figure 6A**). The PC2 is mainly associated with increased EIF, FRI and IMI, although EIF showed the highest correlation ($r^2 = 0.87$). Thus, *F. mechowii* showed higher EIF, TSI, and IMI as compared to *H. glaber*. The largest bathyergid, *B. suillus* distributed evenly on both sides

of the axis, being intermediate between *F. mechowii* and *H. glaber*.

The second PCA including all indices (PCA$_{H2}$) generated five components where the three first explained 97.68% of the total variance (**Table 5** and **Figures 6D,E**). The PC1 (70.53%) was positively correlated with CI ($r^2 = 0.86$) and EIF ($r^2 = 0.61$), and negatively correlated with TRI ($r^2 = 0.62$) (**Supplementary Table 2**). Social species distributed mostly in the positive side of this axis, while the negative side was mostly occupied by solitary species. *Bathyergus suillus* showed higher TSI and TJI as compared to other species. The other solitary species (*He. argenteocinereus* and *G. capensis*) occupied an intermediate position between *B. suillus* and social species (**Figures 6D–F**). The PC2 contributed only with 17.62% of the variance and the greatest positive correlations were obtained for IMI ($r^2 = 0.75$) and TRI* ($r^2 = 0.64$) (**Supplementary Table 2**). In this axis, *F. mechowii* occupied the positive sector, while most individuals of *C. hottentotus* (the second smaller species in this study) occupied the negative sector. In this sense, *F. mechowii* showed higher IMI and TRI* as compared to *C. hottentotus* and *B. suillus*.
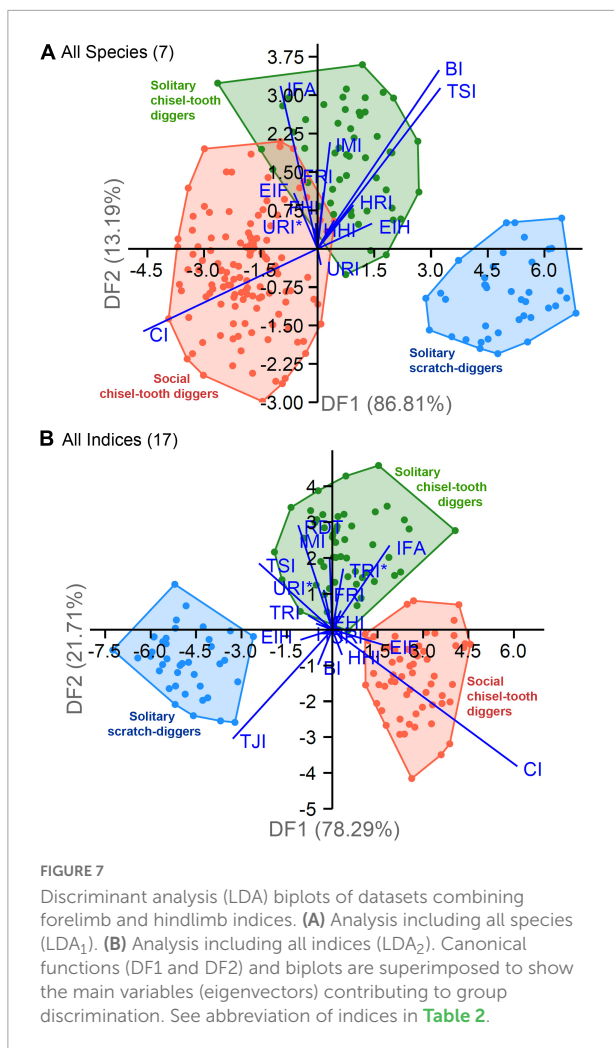
Discriminant analysis (forelimb and hindlimb)

The LDA$_1$ (all species) showed a significant separation between groups [Wilks' $\lambda = 0.063$; $F_{(26, 402)} = 45.72$; $p < 0.001$], and the variables that most contributed to such discrimination (i.e., variables with the largest standard deviation) were BI, CI, TSI, and IFA. Two canonical functions were obtained, from which the first function (DF1) accounted for 86.81% of the variance, and was positively correlated with BI and TSI, and negatively correlated with CI (**Table 6** and **Figure 7A**). The second function (DF2) accounted for 13.19% of the variance, and was also positively correlated with BI and TSI, and negatively correlated with IFA. All individuals in the scratch-digging group and many of the solitary chisel-tooth digger group were on the positive side of DF1, reflecting a trend for longer functional ulna (BI), larger tibial tuberositiy (TSI), and shorter tibia in relation to the femur (CI). The rest of the individuals in the solitary chisel-tooth digger group, whereas most of the social chisel-tooth diggers were on the negative side, mainly indicating a larger tibia (CI) (**Figure 7A**). The DF2 separated most solitary chisel-tooth diggers on the positive side from most of the scratch-diggers in the negative side, indicating a larger olecranon (IFA) in solitary chisel-tooth diggers. Jackknifed cross-validation of group assignments produced a 93.06% of correctly classified individuals (**Supplementary Table 3**). In the scratch-digging group, all individuals were correctly classified. The solitary chisel-tooth diggers and social chisel-tooth diggers groups were not completely separated by the analysis; 15 individuals (6.94%) were misclassified. Most

TABLE 6 Loadings, eigenvalues, and proportion of variance explained by each function of the discriminant analysis, including all species (LDA$_1$) and all indices (LDA$_2$).

Indices	Loadings	
	DF1	**DF2**
LDA$_1$ (All species)		
HRI	32.555	1.564
HHI	−0.069	−14.316
EIH	39.250	−21.578
IFA	−25.404	52.713
URI	−27.326	14.961
URI*	17.591	40.070
BI	3.799	20.380
FHI	23.022	36.098
FRI	−11.049	3.388
EIF	−40.133	−4.540
TSI	11.116	−5.465
CI	−19.187	−8.105
IMI	−7.965	9.263
Eigenvalue	6.737	1.024
%	86.810	13.190
LDA$_2$ (All indices)		
RDT	−6.280	12.595
HRI	−31.797	13.908
HHI	9.433	−14.700
EIH	−40.159	−18.942
IFA	52.712	36.691
URI	17.597	8.433
URI*	−33.044	34.425
BI	10.076	15.153
FHI	−31.999	86.971
FRI	19.414	0.427
EIF	47.882	−11.748
TSI	−7.540	−0.727
TRI	−1.809	−0.580
TRI*	−7.128	−31.353
TJI	−8.965	−34.811
CI	10.419	−22.708
IMI	4.092	6.615
Eigenvalue	9.266	2.569
%	78.290	21.710

misclassifications (11) were found within the social chisel-tooth digger group, where several specimens of *F. mechowii* were misclassified as solitary chisel-tooth diggers. None of the individuals in the analysis were misclassified as scratch-diggers and none of the individuals of *H. glaber* were misclassified.

The LDA$_2$ (all indices) also showed a significant separation between groups [Wilks' $\lambda = 0.027$; $F_{(34, 266)} = 39.53$; $p < 0.001$], and the variables that most contributed to such discrimination were CI, TSI, TJI, IFA, and IMI. Two canonical functions

FIGURE 7
Discriminant analysis (LDA) biplots of datasets combining forelimb and hindlimb indices. **(A)** Analysis including all species (LDA$_1$). **(B)** Analysis including all indices (LDA$_2$). Canonical functions (DF1 and DF2) and biplots are superimposed to show the main variables (eigenvectors) contributing to group discrimination. See abbreviation of indices in **Table 2**.

were obtained, from which the first function (DF1) accounted for 78.29% of the variance, and was positively correlated with CI, and negatively correlated with TSI and TJI (**Table 6** and **Figure 7B**). All individuals of the scratch-digging group were associated with the negative side of DF1, indicating a trend for a larger tibial spine (TSI), shorter tibio-fibular fusion area (TJI), and shorter tibia (CI) as compared to the other groups. Social chisel-tooth diggers occupied the positive side of DF1, while solitary chisel-tooth diggers occupied both sides of the DF1. The second function (DF2) accounted for 21.71% of the variance, and was negatively correlated with IFA and IMI, and negatively correlated with CI (**Table 6**). The exclusion of *H. glaber* allowed a clear separation between social and chisel-tooth diggers. Solitary chisel-tooth diggers were on the positive side of DF2, indicating a trend for larger olecranon process (IFA), larger tibia, and longer hindlimb proportions (IMI), while the other two groups a trend for shorter tibia (CI) and smaller tibio-fibular fusion area (TJI). Jackknifed cross-validation produced a 97.37% of correctly classified individuals (**Supplementary Table 3**). As in LDA$_1$, all specimens in the

first group were correctly classified, although only 4 individuals (0.97%) (mostly pertaining to the solitary chisel-tooth digger group) were misclassified. This represents a considerably low number of misclassifications, probably related to the inclusion of additional indices (i.e., RDT, TRI, TRI*, and TJI), which showed significant explanatory power in previous multivariate analyses (**Tables 3–5**). Thus, the inclusion of humeral and tibio-fibular indices in LDA$_2$ represents a 4.31% increment in discriminant power respect to LDA$_1$. It is likely that the exclusion of *H. glaber* from LDA$_2$ also contributed to a better discrimination between groups, since this species showed a highly variable forelimb and hindlimb morphology, which overlap other species (**Figure 5**).

Sex differences

Only two species exhibited significant sex differences in BM, *B. suillus* and *F. damarensis* (**Supplementary Table 4**), although no statistical differences were detected for morpho-functional indices for most bathyergids (**Supplementary Table 5**), as well as for *H. glaber* (U = 0.560; *p* = 0.715).

Discussion

The multivariate analysis of morpho-functional indices of the humerus, ulna, femur and tibia-fibula of all bathyergid genera including seven species showed significant differences within the family, although no significant differences between sexes. *Heterocephalus glaber* showed the highest level of morphological disparity in the family, lacking several discrete features. Because of the high level of disparity of *H. glaber*, the following discussion focuses firstly on the main differences among bathyergids, while the functional anatomy of naked mole-rats is treated separately in the next section.

One of the most remarkable findings of this study is that several forelimb indices appeared equally or more specialized in chisel-tooth diggers (including social species) rather than in the scratch-digger species, which do not agree with our initial expectations. Chisel-tooth diggers presented highly developed fossorial features like those of specialized scratch-diggers: including a prominent deltoid tuberosity, enlarged olecranon process, and fused tibia-fibula (Lehmann, 1963; Hildebrand, 1985; Samuels and Van Valkenburgh, 2008; Steiner-Souza et al., 2010). This suggests that despite their primary digging mode, their limb anatomy is also under strong selection for burrowing (see also Montoya-Sanhueza et al., 2022a). Some well-developed indices observed in chisel-tooth diggers were the olecranon process (IFA), the anteroposterior robustness of the ulna (URI*) and the relative position of the deltoid tuberosity (RDT) (**Figure 3** and **Supplementary Table 1**). Similar results were obtained from the ordination analysis of the forelimb, where IFA and RDT contributed greatly to separate species and

ecomorphological groups (**Figures 5F**, **6F**, **7B**). A high IFA index reflects a relatively larger olecranon process of the ulna, and consequently indicates larger areas for the attachment of the *mm. triceps brachii* and *m. epitrochlearis.* These muscles are directly related to elbow extension for generation of stronger mechanical advantage, hence is a good estimator of the parasagittal scratch-digging ability in mammals (Vizcaíno et al., 1999, 2016). The highest IFA values were observed in the solitary chisel-tooth digger *He. argenteocinereus*, which also showed the most distally located deltoid tuberosity (RDT) and thickest anteroposterior diameter of ulna (URI*) (**Table 3** and **Figure 3**). The largest social bathyergid, *F. mechowii* also showed a large olecranon process. These results are unexpected since *Heliophobius* and *Fukomys* are primarily chisel-tooth diggers that use their forelimbs secondarily for burrow construction (e.g., Jarvis and Sale, 1971; Van Wassenbergh et al., 2017). Although, *Bathyergus* is the only scratch-digger of the family which uses primarily its long fore-claws for breaking up the soil (Davies and Jarvis, 1986), it showed the lowest IFA values, thus having a comparatively shorter olecranon process (**Figure 3**). A high URI* reflects a relatively thicker anteroposterior cross-sectional shape of the ulna and an increased mechanical advantage for resisting bending strains, thus reducing fracture risks during burrowing (Montoya-Sanhueza et al., 2019, and references therein). This may also increase the area available for the attachment of muscles involved in pronation and supination of the forearm, and flexion of the manus and digits, important for scratch-digging. The highest URI* values were found in *He. argenteocinereus* (and *H. glaber*), allowing them sustain higher strains during parasagittal scratch-digging (**Figure 3**). The projected and distally located deltoid tuberosity (RDT) is other important trait characterizing highly specialized fossorial mammals, since is principally adapted to accommodate enlarged *mm. pectorales* and *mm. deltoidei*, thus increasing the in-lever ability and power-stroke (flexion/retraction) of the arm (Hildebrand, 1985; Álvarez et al., 2012). The more distally located deltoid tuberosity was observed in *He. argenteocinereus,* and indicates a stronger flexion of the humerus, and therefore a stronger ability of the forelimb for pulling the substrate out against the body. In general, all solitary species (and the social *C. hottentotus*) showed a more distal location of this feature as compared to the highly social species of genus *Fukomys*, with *B. suillus* showing an intermediate level (**Figure 3**).

A similar pattern was observed for hindlimb indices, where chisel-tooth diggers exhibited equally similar or higher indices as compared to *B. suillus*. In general, the indices that most contributed to species differences were associated with the tibia-fibula, like the tibio-fibular fusion (TJI), mediolateral diameter of the diaphysis (TRI), size of the tibial spine (TSI), and size of the tibia-fibula in relation to the femur (CI). In the femur, only the size of the epicondyles (EIF) contributed most to interspecific differences (**Figure 4**). Among these indices, the TJI, TSI, and EIF were equally developed or more specialized

in chisel-tooth diggers than in *B. suillus*. The TJI reflects the degree of distal fusion between tibia and fibula, and represents a novel index to assess the robustness of the hindleg. The fused (ossified) condition is found in burrowing or swimming animals that use their hindlimbs in action against a resistant medium such as earth or water, and this contrasts with the condition of many terrestrial and arboreal mammals where the fibula remains separated, and is relatively mobile to increase agility and the range of limb/pes motion (Carleton, 1941; Moss, 1977; Stein, 2000). Larger fusion areas would increase the resistance for bending and torsional loads imposed by strong pulling of muscles, which may be advantageous in fossorial animals. Apart from *H. glaber,* which lacks an ossified tibio-fibular fusion, most bathyergids showed a larger fusion area as compared to *B. suillus* (**Figure 4**), suggesting that larger body sizes may not determine the ossification of such bones. The TSI reflects the strength of the leg and the relative width available for the insertion of the *m. semitendinosus* and *m. semimembranosus* in the tibial spine, so that wider surface areas increase the in-lever action to retract muscles and hence increase the strength of the knee (Samuels and Van Valkenburgh, 2008). A larger tibial spine was found in large bathyergids (e.g., *B. suillus*, *G. capensis* and *F. mechowii*), while the lowest values occurred in the smaller species (e.g., *C. hottentotus* and *H. glaber*) (**Figure 4**). A similar trend was found by Sahd et al. (2020), with *B. suillus* having higher TSI (0.491) as compared to *C. h. natalensis* (0.422). This relationship suggests a positive trend with body size, a pattern that was also reported among caviomorph rodents with different digging capabilities (Elissamburu and Vizcaíno, 2004). The EIF indicates the relative area available for the origin of the *m. gastrocnemius* used in the flexion of the knee and ankle. The larger epicondyles among bathyergids were found in social taxa, especially in *F. mechowii*, with solitary species showing relatively narrower epicondylar widths (**Figure 4**). Similar qualitative observations have been reported by Sahd et al. (2019), were the epicondyles of the social *C. h. natalensis* appeared more robust and prominent as compared to the solitary species *B. suillus* and *G. capensis,* although posterior quantification showed similar high values between *C. h. natalensis* and *G. capensis* (Sahd et al., 2020).

Because of its primary digging mode, the long bones of *B. suillus* are expected to generate stronger forces and undergo higher mechanical strains during elbow extension as compared to chisel-tooth diggers, therefore their limbs were expected to exhibit higher bone robustness and fossorial ability. Although *B. suillus* exhibited high specialization for several indices, these were not significantly higher than chisel-tooth diggers. This does not support our hypothesis of higher functional specializations in bathyergid scratch-diggers, and rather suggests an "equally" important role of limbs for digging in chisel-tooth diggers. This does not implies that chisel-tooth diggers actually use primarily their claws and forelimbs for loosening soils. Actually, the claws of the pes and manus of these chisel-tooth diggers are significantly reduced as compared to *Bathyergus* (**Figure 1**),

with *C. hottentotus*, *He. Argenteocinereus,* and *H. glaber* rather showing a lower claw length index as compared to other fossorial rodents (see Samuels and Van Valkenburgh, 2008). This suggests that more specialized limbs (stylopods and zeugopods) in chisel-tooth diggers may be advantageous for other phases of the digging process. For example, the generation of forward forces for biting soils, shoulder/neck support, body support, and transporting/removing soils out of the burrows may be equally or energetically more demanding tasks as compared to breaking up soils solely (see below), and therefore undergo high selective pressures. Similarly, since several indices were equally, or more developed in social species, our results neither support the hypothesis of lower specialization in social species as compared to solitary ones due to sharing the costs of burrowing. Although the LDA (excluding *H. glaber*) was able to significantly discriminate ecomorphological groups and characterize solitary chisel-tooth diggers from social chisel-tooth diggers by having increased IFA, RDT, and TSI (**Figure 7B**), this only represents a 21.71% of the variance in the sample.

An explanation for such high levels of specialization in mole-rats with different digging modes may lie with the substrates these species occupy. The relatively smaller olecranon of *B. suillus* may be associated with the soft soils in which this species lives, mostly composed of loose sandy soils (Davies and Jarvis, 1986; Bennett et al., 2009), in comparison with the much harder soils (clays and loams) occupied by chisel tooth-diggers (e.g., Bennett et al., 1988; Bennett and Faulkes, 2000; Šumbera et al., 2008, 2012; Lövy et al., 2012). Living in such soft substrate conditions could reduce the energetic requirements of digging (Vleck, 1979; Luna and Antinuchi, 2006; Zelová et al., 2010), and therefore preclude higher specialization. In this respect, important advances on the understanding of the digging habits and the postcranial morphology of subterranean mammals have been done in the South American tuco-tucos of the rodent family Ctenomyidae (Vassallo, 1998; Steiner-Souza et al., 2010; Vassallo et al., 2021). Several studies have found a more specialized (e.g., robust) humeral and cranial morphology in *Ctenomys* species inhabiting harder soils, whereas a more slender humeral morphology in species inhabiting softer soils (e.g., Steiner-Souza et al., 2010). In particular, Vassallo (1998) found that *Ctenomys talarum*, a species living in hard soils exhibits relatively larger *mm. triceps brachii* as compared to *Ct. australis*, which lives in sandy and friable soils. Because the triceps group inserts into the olecranon process, these muscles are directly associated with the generation of greater out-forces during scratch-digging (Vassallo, 1998), and consequently a larger olecranon may represent an important feature in chisel-tooth digging mole-rats to compensate for burrowing in harder substrates. Similar observations have also been reported for intraspecific assessments among populations of *Ct. minutus* living in different habitat types (and soil hardness); the population living in softer soils has a less specialized skull morphology, humeral morphology, as well as estimators of

bite force as compared to the population living in harder soils (Kubiak et al., 2018). Among bathyergids, Barčiová et al. (2009) also suggested that the morphological variations in skull morphology (e.g., relatively shorter rostrum) found in different populations of *He. argenteocinereus* may be related to differences in soil parameters between localities (e.g., soil hardness), and Kraus et al. (2022) revealed how habitat characteristics are responsible for high bite force across different mole-rat species.

Overall, the above information indicates that burrow construction in harder soils imposes stronger constraints on limbs (e.g., ulna and tibia-fibula), so that such species may require increased limb specialization as species living in softer soils, even when they are primarily chisel-tooth diggers. Stronger ulnar flexion in chisel-tooth diggers may be beneficial in environments where the soils may reach a high degree of compaction and/or become heavier due to changes in soil properties, such as increased moisture during the rainy seasons. For example, the net cost of transporting soils depends on several parameters, including moisture content, soil density, adhesion and viscosity among others (Vleck, 1979; Luna and Antinuchi, 2006). When soils are wet, particularly during the rainy seasons, these increase considerably their mass and viscosity (Collis-George, 1959; Marcy et al., 2013), making its excavation more demanding. Recent assessments on the effects of soil compactness on burrowing performance in talpid moles indicate that increased soil compactness impedes tunneling performance, resulting in reduced burrowing speed, shorter tunnels, shorter activity time and less time spent burrowing continuously (Lin et al., 2017). Moreover, the successive components of the entire burrowing sequence are most likely not energetically equivalent, and some activities such as pushing off of the soils along the tunnel and throwing it out may represent the most energy-consuming phase during the entire burrowing process (Gambaryan and Gasc, 1993). In fact, the estimations of the energy costs of burrowing in pocket gophers (Vleck, 1979) and tuco-tucos (Luna et al., 2002) have shown that although the cost of breaking the soil (shearing) is *per se* energetically higher than transporting it (pushing), the transport phase represents a considerably longer process (in terms of time), hence increasing its total costs throughout the entire burrow construction. In African mole-rats, above-ground digging activity has been associated with seasonality (Jarvis et al., 1998; Bennett and Faulkes, 2000), suggesting that overall digging activity increases during the rainy season, since soils are easier to loosen as compared to the more compacted soils of the dry season. Therefore, it is possible that both the transport and removal of soils out of the tunnels represents energetically demanding activities in Bathyergidae, so the forelimb and hindlimb undergo strong selective pressures and increased specialization, regardless of their primary digging mode. Indeed, such level of limb specialization in chisel-tooth diggers is in concordance with their augmented dental and cranial specializations adapted to breaking up harder substrates

(McIntosh and Cox, 2016a). Although the evolution of a highly specialized chisel-tooth digging apparatus in bathyergids may be highly associated with the exploitation of harder soils (McIntosh and Cox, 2016a), it is known that chisel-tooth diggers can occupy a wide range of habitats including softer substrates, e.g., *F. damarensis* (Bennett and Jarvis, 2004; Lövy et al., 2012; Thomas et al., 2016). This suggests that there are no strong apparent ecological constraints on chisel-tooth mole-rats to occupy a diverse range of habitats and soil conditions, which is most likely promoted not only by the extreme specialization of their cranial and dental anatomy, but also by their highly specialized appendicular morphology.

Locomotion

Three indices (CI, BI, IMI) reflect important aspects associated with locomotion. In particular, the CI gives an indication of how well the hindlimbs are adapted for speed (Howell, 1965), and it showed an important amount of variation among species. The lower CI values (i.e., shorter tibia-fibula in comparison to the femur) were found in solitary species, while the higher values (i.e., larger tibia-fibula) were found in social species, especially in the smaller ones (*F. damarensis* and *C. hottentotus*) (**Table 3** and **Figure 4B**). Longer zeugopods are typically found in surface-dwelling mammals, particularly in cursorial ones (Howell, 1965), although the social bathyergids still showed lower values as compared to surface-dwelling species (Samuels and Van Valkenburgh, 2008). This suggests that social species may have faster propulsive forces as compared to solitary species, allowing the capacity for faster movements and locomotion. Apart from indicating leaping ability in particular cases like saltatorial mammals, the IMI has not shown clear functional correlates in mammals, especially those associated with either higher speed or lack of it (Howell, 1965). This author reported an IMI of 0.75 as the generalized condition for large terrestrial mammals, so that the hindleg is longer than the foreleg, with bipedal jumpers showing the lowest ratios (0.32–0.50). Bathyergids have higher IMI values (0.87) as compared to terrestrial mammals, thus evidencing relatively symmetrical limb proportions, although the hindlimb is still slightly longer than the forelimb (**Figure 4**). Lehmann (1963) also found that the length of the hindlimbs of the fossorial *Geomys*, *Ctenomys*, and *Tachyoryctes* are nearly equal to the length of the forelimbs, differing from non-fossorial forms like *Rattus*, in which there is a tendency for the hindlimbs to be longer. This high interlimb symmetry is also related to the similar proportion between humerus and ulna (BI), although it is probable that by adding the scapular length to the length of the forelimb, as other studies have suggested (e.g., Lilje et al., 2003), the IMI ratio would change, making the proportions in bathyergids even more symmetrical. Montoya-Sanhueza et al. (2019) described

the limb development of *B. suillus* and discussed the function of symmetric limb proportions in subterranean mammals, suggesting that symmetrical limb proportions may be related to maximizing equal bidirectional locomotion within burrows by emphasizing equal propulsive action of the forelimbs and hindlimbs, without changing body posture and orientation (Eilam et al., 1995). Overall, the high IMI of bathyergids and other fossorial mammals, along with their usually short limbs, may not represent a fossorial adaptation for excavation *per se*, but a morphological specialization for bidirectional locomotion in narrow spaces and a dense medium (Howell, 1965; Eilam et al., 1995). Although the locomotory dynamics of bathyergids have not been assessed, these characteristics may compromise the locomotor capabilities of mole-rats in comparison to terrestrial mammals (Howell, 1965; Samuels and Van Valkenburgh, 2008). Montoya-Sanhueza et al. (2019) pointed out the apparent lower locomotor capabilities of *B. suillus* in comparison to the ctenomyid *Ct. talarum*, which suggests that living underground not always results in reduced locomotor capabilities. Further research on locomotory dynamics of subterranean mammals, would help us understand the relationship between morphology and locomotion in this particular ecomophological group of mammals.

The limb phenotype of *Heterocephalus glaber*: Functional implications

The limb anatomy of *H. glaber* lacks two important discrete fossorial limb adaptations, the projected deltoid tuberosity and distal tibio-fibular fusion (Montoya-Sanhueza et al., 2022a). This precluded the measurement of RDT, TRI, TRI*, and TJI. However, *H. glaber* accumulated most of the interspecific differences in morpho-functional indices in the family (**Figures 3, 4**). Out of seven forelimb indices, four were significantly different in *H. glaber,* showing a comparatively less robust humeral diaphysis (low HRI), smaller humeral head (low HHI), narrower epicondyles (low EIH) and "shorter ulna" (functional length, without olecranon process) as compared to the humerus (low BI) (**Table 3** and **Figure 3**). Because the different morphology of the humeral diaphysis in *H. glaber*, a slightly different calculation for the HRI was also obtained, making comparisons not straightforward among bathyergids. In most species, the HRI was measured around 58–60% the length of the humerus from the proximal epiphysis (because the presence of the deltoid tuberosity), although in *H. glaber* this was taken at 50% of the total length. This represents a more proximal measurement as compared to the other bathyergids, however because of the even thinner morphology of the humerus at lower locations (e.g., 58–60%) in *H. glaber*, a comparable measurement with other bathyergids would result in even thinner estimations. Related to this section of the

bone, the cross-sectional shape of the humerus at 50% of the bone length in bathyergids is strongly influenced by the deltoid tuberosity, resulting in a rather triangular shape. This section in *H. glaber* is more elongated and ellipsoidal (see Montoya-Sanhueza et al., 2021a), thus probably compensating for the lack of a prominent tuberosity. Yet, the diaphysis of *H. glaber* is considerably thinner as compared to other bathyergids. Regarding the low BI, several individuals of *H. glaber* showed a curved ulna, which may explain the low values of this index. Although *H. glaber* (and *He. argenteocinereus*) showed the highest URI* among bathyergids, reflecting a relatively thick anteroposterior ulna, it is likely that this index may have also been influenced by the short curved ulna. Overall, the forelimb of *H. glaber* show smaller areas for the insertion of pectoral and deltoid muscles, and reduced robustness suggesting a comparatively less efficient scratch-digging ability. Regarding the hindlimb, *H. glaber* showed the smaller tibial spine (low TSI) among bathyergids (**Figure 4**). This feature, along with the lack of distal tibio-fibular ossification, and thinner femur (also in *F. damarensis*) reflected a reduced robustness of the hindlimb.

Altogether, these features indicate a considerably reduced fossorial ability for the limbs of *H. glaber,* probably affecting multiple functions during burrowing, such as the efficiency of parasagittal scratch-digging, stabilization and shock absorption of the shoulder, resistance for bending and torsional loads imposed by muscles, and retraction/flexion of muscles and strength of the knee (e.g., Carleton, 1941; Samuels and Van Valkenburgh, 2008; Steiner-Souza et al., 2010). Such reduced fossorial ability is in concordance with the reduced claws of this species among bathyergids (**Figure 1**), suggesting a considerable relegated function of limbs to break up soils (see also Samuels and Van Valkenburgh, 2008). Instead, such features indicate a more gracile appendicular morphology in this species and a wider range of motion and flexibility of limbs, which may be beneficial to prioritize bidirectional locomotion and turning around within narrow spaces, particularly when colonies can reach a high number of individuals, e.g., up to 300 individuals (Jarvis et al., 1994). Nevertheless, like other bathyergids, *H. glaber* also exhibits well-developed fossorial traits in their limbs, including a well-developed olecranon process (high IFA) and well-developed third trochanter (Montoya-Sanhueza et al., 2022a), the former being even more developed than in *B. suillus* (**Figure 3**). The long bones of *H. glaber* are also composed of thick cortical walls and experience scarce bone resorption during ontogeny (Pinto et al., 2010; Montoya-Sanhueza et al., 2021a,b), like other bathyergids (Montoya-Sanhueza and Chinsamy, 2017; Montoya-Sanhueza, 2020), allowing them to be more resistant to high impact activities. These latter features may represent important attributes to compensate for the lack of specialization in other regions of the appendicular skeleton.

The reduced robustness in the long bones of *H. glaber* rather resembles that of non-fossorial mammals including their closest outgroup relatives, *Petromys typicus* (Petromuridae), *Thryonomys swinderianus* (Thryonomyidae), and *Hystrix africaeaustralis* (Hystricidae) (Montoya-Sanhueza et al., 2022a). The proximal section of the humerus is also similar to that of terrestrial mammals with a more generalized bone morphology such as the tenrecid *Setifer* and *Tenrec*, while the distal humerus is more similar to that of the erinaceid, *Echinosorex* (Salton and Sargis, 2008). The unfused tibia-fibula of *H. glaber* is more similar to the generalized condition found in hystricognaths (De Graaff, 1979), including *P. typicus* (a rock climber), *T. swinderianus* (semi-aquatic/semi-fossorial), and *H. africaeaustralis* (ambulatorial/semi-fossorial), thus providing further evidence for the basal position of *H. glaber* within the family (Montoya-Sanhueza et al., 2022a, and references therein). Such closely related taxa also have very reduced third trochanters and less specialized olecranon process, although a well-defined deltoid tuberosity (Montoya-Sanhueza et al., 2022a). The lack of a projected deltoid tuberosity in *H. glaber*, and its presence in *P. typicus*, *T. swinderianus,* and *H. africaeaustralis* suggest a considerable reduction during the evolution of *Heterocephalus*, probably occurring in the ancestors or early extinct members of the family. This was proved correct after the analysis of the extinct *Bathyergoides neotertiarius* from the early Miocene of East Africa and Namibia, one of the proposed ancestors of bathyergids (Lavocat, 1973; Bento Da Costa and Senut, 2022). This taxon is more similar to *H. glaber* than to the more recent bathyergids, showing a considerably reduced deltoid tuberosity (although more developed than in *H. glaber*), lack of distal tibio-fibular fusion, a long tail, and a less robust femur with a highly reduced third trochanter (Lavocat, 1973; Bento Da Costa and Senut, 2022). Based on these set of features, it was proposed that *Ba. neotertiarius* had a comparatively reduced fossorial ability (Lavocat, 1973; Bento Da Costa and Senut, 2022), suggesting that early bathyergids may have had a comparatively more restricted use of the subterranean ecotope, and that the evolution of the appendicular fossorial phenotype in Bathyergoidea (Bathyergoididae + Bathyergidae) occurred gradually, and was only fully acquired after the split of *H. glaber* from the rest of the other bathyergids (Montoya-Sanhueza et al., 2022a).

Importantly, the morphological disparity observed in *H. glaber* is unlikely to be the result of prolonged life in captivity. Although life in captivity can have strong effects on the skeleton, particularly associated with disorders of the mineral metabolism and skeletal disuse (O'Regan and Kitchener, 2005), early studies of wild individuals of *H. glaber* have reported a similar phenotype as our captivity sample, e.g., individuality of tibia and fibula, variable shape of the humeral head (flattened and a less pronounced humeral neck), relatively thin distal

humerus, very thin anteroposterior humeral diaphysis, and curved ulna (Parona and Cattaneo, 1893; Hamilton, 1928; Hill et al., 1957). In this respect, previous studies mentioned the fused condition of the distal tibial and fibular epiphyses in bathyergids (De Graaff, 1979; Patterson and Upham, 2014, and references therein), although they did not recognize the particular morphology of *H. glaber* among the family. Muscle activity plays a major role in the stimulation of normal size, shape and architecture of bones, so that muscle force and bone morphology have a reciprocal relationship (Burger and Veldhuijzen, 1993; Whedon and Heaney, 1993; Hillam and Skerry, 1995). Muscle dysfunctions result in alteration of bone composition and geometry, even at prenatal stages (e.g., Sharir et al., 2011). For this reason, it would be expected that living in captivity would affect the normal bone growth of the individuals studied here, especially considering the prolonged time that these colonies have been inbred (more than 20 years), and the fact the such colonies lacked real substrates to dig. However, these colonies show a wide repertoire of digging behaviors. We provide video recordings of active digging (**Supplementary Video 1**) in different members of the naked mole-rat colony housed at the University of Cape Town (Cape Town, South Africa), which demonstrates that members of the colony are able to perform both chisel-tooth digging and scratch-digging, as well as potent synchronized kicks with the hindlimbs, where the animal uses backward locomotion to simulate the expulsion of soils out of the burrow. It is likely that the constant digging in these colonies have had a positive impact on stimulating normal muscle function, and therefore bone growth and morphology. This represents one of the clearest lines of evidence supporting the idea that the phenotype of *H. glaber* has not been considerably affected by being held in captivity, at least by disuse conditions. Other studies using captive individuals from other colonies have also reported normal bone development, at least for femora, vertebrae and cranium (e.g., Dengler-Crish and Catania, 2007; Pinto et al., 2010; Carmeli-Ligati et al., 2019). Overall, the limb phenotype of naked mole-rats from captive colonies is assumed to not vary considerably from that of wild specimens, although we do not discard the possibility that natural digging regimes in the wild, involving different load magnitudes and frequencies of muscular activity, may also have an impact on other aspects of bone structure such as its bone geometry. Further examinations of wild specimens are needed to assess such hypothesis.

Conclusion

The appendicular anatomy of bathyergids shows clear specializations associated with fossoriality. However, our data demonstrates that scratch-digging bathyergids may not require the highest levels of limb bone specialization, and that both solitary and social chisel-tooth diggers can exhibit

equally or more specialized humero-ulnar joints and tibio-fibular features. Differences in habitat characteristics and soil parameters occupied by mole-rats may help explain such differences, suggesting that cranial specializations are not the unique relevant aspect of digging performance, and that the limb anatomy of chisel-tooth diggers represents an important burrowing tool to maximize the entire process of burrow construction. Such organismal combination of cranial and postcranial adaptations may have played an important role on the dispersion and colonization of African mole-rats to a diverse range of habitats in sub-Saharan Africa (and in turn restricted the distribution of some morphologically divergent taxa such as *B. suillus* and *H. glaber*). Despite the enigmatic disparity of *H. glaber* with the rest of the social bathyergids, it is probable that a combination of historical and ecological characteristics in *H. glaber* involving heterochrony, hyperspecialized cranial apparatus, formation of organized digging sequences and large colony sizes, have all contributed to reduce the selective advantage of a highly specialized appendicular anatomy (see Montoya-Sanhueza et al., 2022a). Our results also showed that most interspecific differences were associated with the zeugopodial elements, suggesting that limb specialization is more prone to occur in the lower parts of the appendicular system, and that the stylopods may represent more conservative elements, probably associated with the multifunctional nature of the humerus and femur. The analysis of a large sample of individuals allowed us to uncover the high degree of morphological variation and developmental plasticity within *H. glaber* (see also Montoya-Sanhueza et al., 2022a, 2021b), whereby further studies on these animals should consider the inclusion of representative samples of the population, as well as the inclusion of wild specimens. Additional studies on burrowing behavior and habitat characterization of African mole-rats would greatly contribute to our understanding of the morphological evolution of this group.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because our material is composed of skeletons from old specimens collected many years ago, and/or from previous studies.

Author contributions

GM-S and AC designed the study. NB and RŠ provided mole-rat specimens and supported the experimental procedures. GM-S analyzed the data, prepared the manuscript, acquired images, and created the figures. All authors read, edited, and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.857474/full#supplementary-material

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