Behavioral fingerprinting of the naked mole-rat uncovers signatures of eusociality and social touch

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Summary

The East African naked mole-rat (*Heterocephalus glaber*) lives in large and extremely cooperative subterranean colonies. However, both the biology that drives their social interactions and the behaviors that define their social hierarchy are poorly understood. Here, we study the spontaneous solitary and social behaviors of naked mole-rats using automated animal tracking coupled with unbiased behavior discovery and experimenter-determined behavior quantification. With this approach, we find that reproductive and non-reproductive castes engage in distinct spontaneous behaviors. Further, the relative usage frequencies of a specific animal's spontaneous behavior can be used to estimate its rank in the colony's dominance hierarchy. Strikingly, we discovered that face touch is a prominent form of social interaction—naked mole-rats actively engage in face-to-face contact hundreds of times in a single 10-minute social pairing. We speculate that face-to-face contact might be related to social recognition, as we observe it performed during interactions in which naked mole-rats need to identify each other. Lastly, to demonstrate the specific importance of face-to-face contact, we show that social housing conditions lead to widespread activation of the mechanosensory ion channel Piezo2 in neurons that innervate the face, but not the rest of the body. Together, these findings support the importance of both caste and rank for the organization of spontaneous behavior in naked mole-rats, and they show that face-to-face contact is a prominent social behavior in these animals.

Introduction

The naked mole-rat (*Heterocephalus glaber*) is one of the most social mammals in the animal kingdom. These animals live in large colonies consisting of a breeding queen, 1-3 breeding males, and multitudes of nonreproductive workers (Buffenstein et al., 2021; Jarvis, 1981). Naked mole-rats engage in a range of collective behaviors, from digging and transporting food to parental care of the offspring (Buffenstein et al., 2021; Faulkes et al., 1997; Watarai et al., 2018). Their behavior is also reflective of their dominance hierarchy, with the top-ranked queen dominating subordinates through shoving and biting (Clarke & Faulkes, 2001). Whether naked mole-rats display a true caste-like organization of behavior or if behaviors are more fluid across colony members is still being actively investigated (Siegmann et al., 2021; Toor et al., 2022). Moreover, how their complex repertoire of behavior interacts with their dominance hierarchy remains unresolved.

The sensory world of naked mole-rats is distinct from most above-ground animals, and the sensory basis of social communication remains obscure. One recent study demonstrated that vocalization is a prominent form of naked mole-rat social communication, with animals engaging in dozens of unique vocalizations that are specific to a given colony, and with the colony dialect maintained by the presence of the queen (Barker et al., 2021). Although olfaction is an important form of social communication for many rodents, the olfactory epithelia and bulb of naked mole-rats is greatly reduced compared to other mammals (Onyono et al., 2017), and their olfactory epithelium is dispensable in a standard odor-based discrimination task (Toor et al., 2015). Although olfaction seems diminished in these animals, as well as vision (they are blind because of fossorial living) (Hetling et al., 2005), their sense of somatosensation is quite prominent. In fact, although furless, naked mole-rats have facial vibrissae and vibrissa-like body hairs that are similar in morphology, and these vibrissae are exquisitely sensitive to deflections (Crish et al., 2003). Additionally, the somatosensory cortex of naked mole-rats is 3-fold expanded compared to other mammals (Catania & Remple, 2002; Xiao et al., 2006), suggesting a heightened importance of somatosensation. How these intact—or even heightened—sensory modalities (namely, somatosensation and audition) coordinate to guide communication, enforce dominance, facilitate maintenance of social hierarchy, and support social recognition, is unknown.

Given that naked mole-rats have evolved an extreme lifestyle of cooperative underground living, subtle and unpredictable aspects of behavior might be missed using conventional animal behavior methods which often require experimenter insight and/or supervision. Fortunately, modern tools in computational neuroethology allow unbiased quantification of behavior. For example, it is now feasible to use machine learning approaches such as SLEAP or DeepLabCut to automatically track multiple animals in social contexts (Lauer et al., 2022; McKenzie-Smith et al., 2023; Pereira et al., 2022). Multi-animal tracking is crucial for understanding naked mole-rat behavior because they occupy a particularly extreme end of the social spectrum. Downstream of animal tracking, a new tool called keypoint-MoSeq defines animal behavior as a sequence of repeatedly

used, fundamental modules (also known as “syllables”) which are strung together in specific combinations (Weinreb et al., 2023; Wiltschko et al., 2015). By decomposing the structure of behavior in this way, naked mole-rats can be compared on an individual basis through metrics such as the frequency of syllable use and syllable transition probabilities.

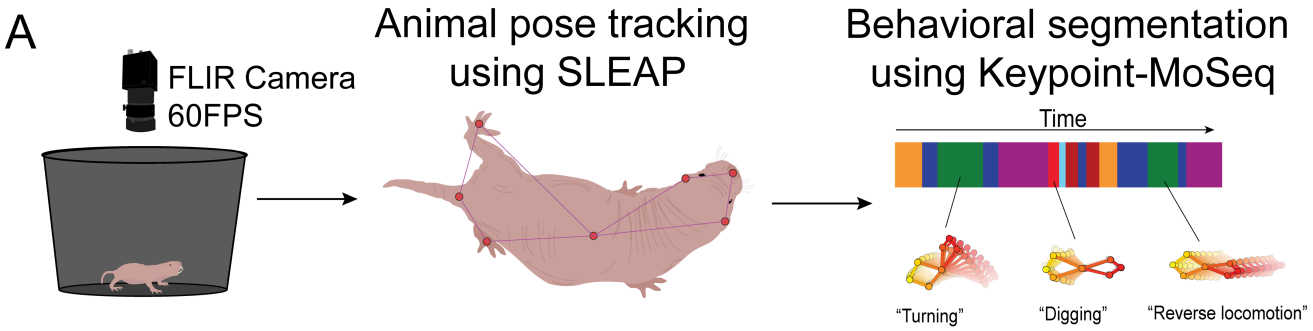
Here, we used these approaches to elucidate the structure of spontaneous behavior in naked mole-rat colonies. We found that naked mole-rats engage in caste and rank-specific behaviors. Intriguingly, we identify face-to-face contact as a prominent form of social interaction in naked mole-rats and speculate that it is important for social recognition.

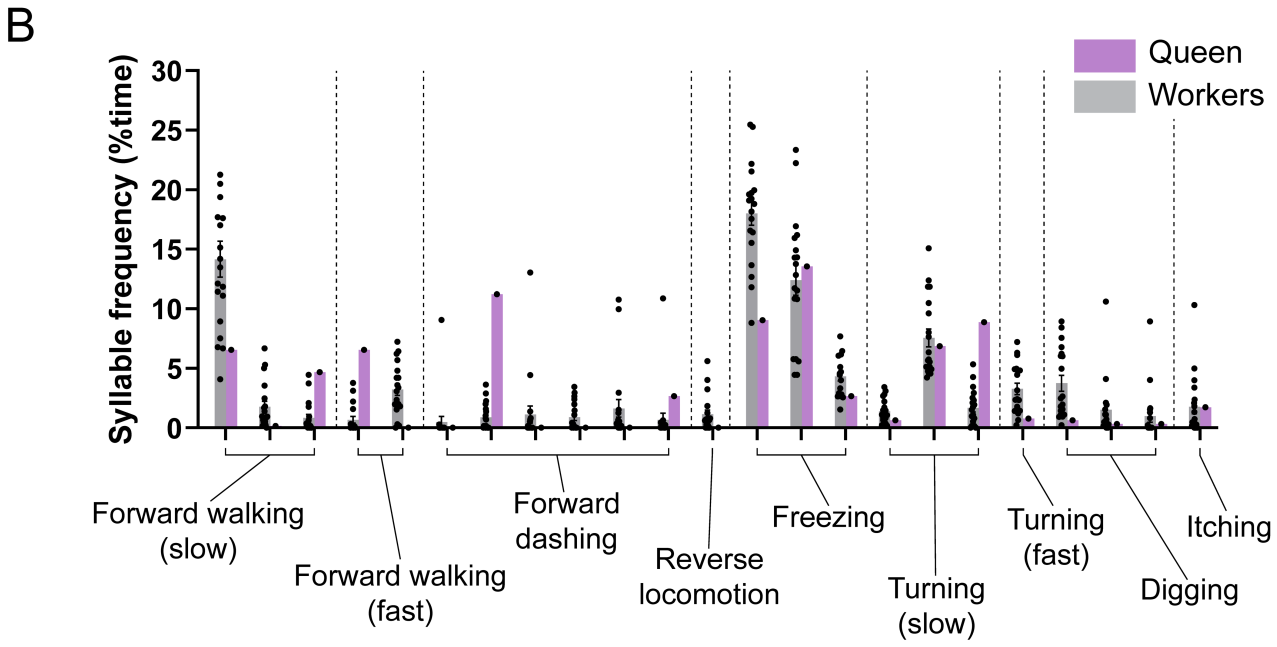
Results

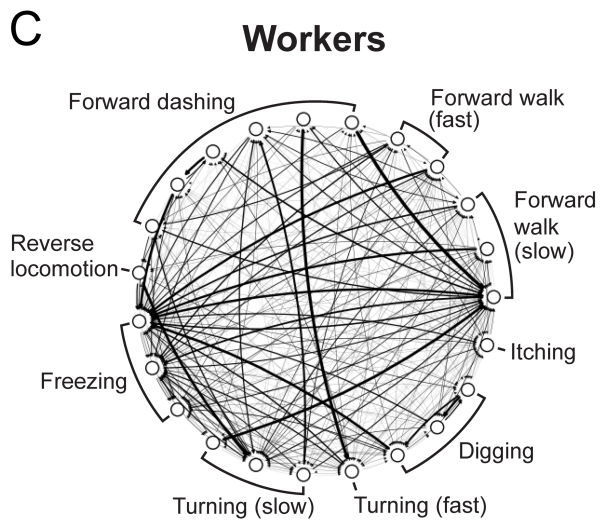
Keypoint-MoSeq identifies behavioral fingerprint of naked mole-rats

We began with a pipeline consisting of the pose estimation software SLEAP and the unsupervised algorithm keypoint-MoSeq. Figure 1A illustrates this pipeline: (1) animals were videotaped in an open field to capture spontaneous behaviors which were then (2) fully pose-tracked using SLEAP with a skeleton of seven nodes (Video S1), and finally (3) fed into keypoint-MoSeq to identify and detect behavioral syllables throughout the duration of the video. The open field arena was filled with terrarium sand to facilitate naturalistic digging behavior. For training data, we used videos from both male and female workers, as well as the queen. Keypoint-MoSeq identified 20+ behavioral syllables shared by all naked mole-rats. Syllables were grouped into categories through manual assignment, generating nine categories of syllables that allowed us to gain a broader view of naked mole-rat behavior without sacrificing the ability to make observations on a per-syllable basis.

Figure 1B illustrates the syllable usage profile of eighteen animals within a colony, including the queen. Most syllables were locomotory in nature, with naked mole-rats devoting a large portion of their time to forward locomotion. Animals also commonly change direction via turning. Interestingly, the most common syllables used by the workers were freezing, indicating a bout of pausing or startling which interrupts forward locomotion (Video S4). This freezing can be interpreted as an anxiety-like behavior in which mole-rats startle to a sudden environmental stimulus or exercise caution when exploring the environment. Naked mole-rats can also engage in forward dashing, a quick forward movement (Video S2). One identified syllable group which is relevant to subterranean life is digging (Video S3). Mole-rats engage in a stereotyped sequence consisting of digging with the forepaws, pushing the sand towards the rear, and then kicking it away with the hind paws. Digging reflects the naked mole-rat’s natural inclination to construct tunnels and maintain the colony chamber system (Buffenstein et al., 2021; Montoya-Sanhueza et al., 2022). Reverse locomotion is another syllable that is consistent with colony behavior; naked mole-rats are known for their ability to navigate tunnels equally well while moving in reverse (Eilam et al., 1995).







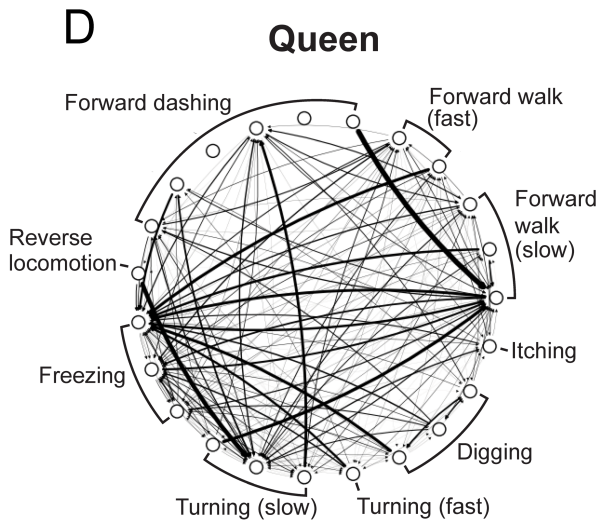


Figure 1: **Using keypoint-MoSeq to characterize behavioral syllables of worker naked mole-rats and their queen**

(A) The SLEAP-to-MoSeq pipeline. Animals were video recorded from above in an open field bucket with black sand, and then fully pose-tracked using SLEAP. Tracked videos were analyzed using keypoint-MoSeq to identify syllabic and grammatical structure of behavior (n=19 animals). **(B)** Syllable usage profile among the nonreproductive workers and the queen of their respective colony. Syllables of similar function were placed into one of 9 broad groups; the Y-axis shows the frequency (% time) each naked mole-rat used each MoSeq syllable. The queen uses forward dashing syllables more frequently than nonreproductive workers. Two of the forward dashing syllables were upregulated in the queen vs. the workers. Freezing syllables, behaviors linked to anxiety-like phenotypes, were lower in the queen compared to nonreproductive workers. The queen exhibited a lower incidence of digging syllables, a behavior linked to colony maintenance [n=18 workers, 1 trial each, n=1 queen, 1 trial]. **(C)** MoSeq syllable transition plot of the worker naked mole-rats. Lines indicate sequential transitions between the two connected syllables, and greater line thickness corresponds to higher frequency. **(D)** The transition plot of the queen naked mole-rat. Comparison of the patterns of syllable transitions shows that the queen has a distinct syllabic grammar compared to the nonreproductive workers.

The queen naked mole-rat exhibits a hyperactive, low anxiety-like phenotype

To determine whether individual spontaneous behavior contains caste information, we asked whether the behavioral profile of the queen naked mole-rat differed from that of the nonreproductive-caste members of her colony (Figure 1B). We discovered that the queen more frequently engaged in fast locomotion than the nonreproductive workers. This result includes a higher frequency of forward dashing syllables and a higher incidence of fast walking syllables. This data is consistent with previous reports that the queen hyperactively patrols her colony as a way of maintaining her status (Clarke & Faulkes, 1997; Reeve, 1992). Interestingly, it appears that this behavior is hard-wired, as the queen is hyperactive in even a non-social setting. As shown in Figure 1B, the queen engages in anxiety-like behaviors less frequently than the workers. This result suggests that the queen is less anxious, which is consistent with data in mice exhibiting that more dominant animals are less prone to anxious behavior (Horii et al., 2017). The queen also engaged in digging less frequently than the workers, suggesting that certain colony maintenance tasks are unique to the workers. To supplement our keypoint-MoSeq analysis, we also used the AnyMaze software to characterize the open field behavior of the queen (Figure S2A). The queen exhibited higher average locomotion speed than workers, entered the center zone more frequently than workers, and her pro-exploratory behavior was more sustained (Figure S2B). Together, these data show that the queen has a high-locomotion, low anxiety-like phenotype linked to her unique reproductive status within the colony.

We next aimed to detect ethological signatures at higher levels of behavioral organization. This behavioral “grammar” was determined by obtaining the probability of each syllable transitioning into every other syllable. Matrices for entire groupings of animals (e.g. “all nonreproductive workers”) were also generated (Figure S3). As shown in Figures 1C and D, we displayed this data as bigram probability plots and compared the syllable transition dynamics of all nonreproductive workers to those of the queen, similar to a recent study on vocal emission in gerbil families (Peterson et al., 2023). The workers exhibited more transition types than the queen, potentially reflecting the diversity of

specific roles that the workers play within the colony. Many of the most common transitions among workers also had a higher frequency in the queen. Furthermore, the workers possessed many transitions that the queen never exhibited, suggesting the existence of worker-specific syllable sequences that the queen does not utilize. We also tested 5 different queens from the McCloskey lab in this setup, and the queen behavior of these animals was also distinct from workers and slightly distinct from the Abdus-Saboor lab queen (Figure S4). Differences in behavior may reflect the different genetic lineages of the queens between labs, a possibility that warrants further study. Together, these results indicate that the queen has a behavioral fingerprint distinct from the workers and shows that caste-like signatures exist not only at the level of fundamental syllabic units, but at higher levels of behavioral structure.

Differential in dominance rank correlates with win% in crawl-over tube test

Having verified differences at the most extreme caste division (queen vs. workers), we next wondered whether behavioral stratification also existed among the workers themselves. Mole-rat colonies can be divided into "ranks" based on dominance testing in which a more dominant mole-rat crawls over a more subordinate mole rat in a tube. To determine the dominance hierarchy within our colony, we used the tube test assay which is the standard in the field (Clarke & Faulkes, 1997, 1998; Hite et al., 2022; Toor et al., 2015). One observation that immediately caught our attention (which we will return to later in the paper) is that face-to-face contact always preceded the crawl-over decision (Figure 2E, left). A total of 18 mole-rats were used for every possible pairwise combination of animals, and with ten trials for each tube test, this amounted to ~2,000 individual trials. From this data, overall crawl-over win% was used to construct a dominance hierarchy (Figure 2A) with higher win% animals assigned higher ranks. Worker win% ranged from less than 5% to over 85%, and the queen exhibited the second-highest win% and was assigned a rank of 2. It should be noted that the queen did not adopt the subordinate posture and "lose" tube tests; she would force herself underneath the other naked mole-rat. These relative ranks were then used for all subsequent experiments. As shown in Figure 2A, we then plotted the tube test results from every pairwise encounter in a matrix to determine how disparity in rank affects the outcome of each tube test. The results indicated that in 84.96% of encounters, the more dominant naked mole-rat won. Each encounter was assigned a rank differential, which corresponds to the distance between two naked mole-rats in the dominance hierarchy. Another metric which we created, win differential, quantified how lopsided the victory/loss of a given tube test was (win differential = $|\#wins_{animal1} - \#wins_{animal2}|$). To determine how rank differential correlates with win differential, we plotted these two metrics (Figure 2B). The data showed that in a given tube test encounter, the greater the difference in rank between the two animals, the more asymmetrical the win is for the victorious animal (linear regression, $R^2 = 0.7784$). This indicates that dominance rank in a naked mole-rat colony includes elements of determinism and stochasticity; higher-ranked animals typically win, lower-ranked animals typically lose, and the win percentage predictably converges to 50% for evenly-ranked pairs.

Spontaneous behavior in isolated naked mole-rats maps onto dominance rank

With the repertoire of behaviors for each animal identified, we next asked if individual naked mole-rats might cluster based on spontaneous behavioral profile. In order to answer this question, we used an elastic net regression to determine how well rank could be predicted from syllable usage (Figure 2C). Strikingly, we found that the rank of an animal predicted by leave-one-out cross-validation had a correlation of 0.75 with actual rank, indicating that MoSeq syllable frequency strongly predicts the dominance rank of a naked mole-rat in isolation. In order to determine which syllables contributed most to the fits in the regression, we next examined the corresponding regression weights of all behaviors (Figure 2D). This showed that the higher-ranking animals tended to use certain syllables more or less frequently than lower-ranking animals. Of particular note was digging, which higher-ranking animals used less frequently. This result is consistent with our previous observation that colony maintenance behaviors, such as digging, are relegated to lower-ranking individuals. Digging may be a caste-like signature that correlates with rank, and is rarely engaged in by dominant animals such as the queen. Taken together, these results indicate that spontaneous behavior, even in isolation stratifies with individual dominance rank. These results suggest that dominance vs. subordinance induces an entire brain and behavior state change in naked mole-rats that is maintained even outside of the social context.

We next tested whether the frequency or nature of spontaneous behaviors was altered by the social context. To do this, we studied workers using multi-animal SLEAP tracking followed by behavioral segmentation with keypoint-MoSeq (Figure S5A). In general, as we increased from 3 to 6 naked mole-rats we saw a decline in most behavioral syllables (Figure S5B-G). However, we saw an increase in forward slow walking as animal density increased, suggesting animals might be spending more time to stop and investigate one another socially and less time doing non-social individualistic behaviors (Figure S5F).

Throughout these experiments, we noticed that snout-to-snout touch was quite common, especially during dominance testing. Using high-speed cameras, we captured videos of naked mole-rats during the tube test (Figure 2E, Video S5). We found that snout touch interactions always occur first when two animals encounter each other, which is then followed by a crawl-over. Strikingly, the time between snout touch and initiation of the crawl-over was dependent on the rank differential between the two naked mole-rats. When a high-dominance and a low-dominance animal encounter each other, the latency between snout touch and crawl-over is extremely fast (mean latency = 105.8 ms ± 0.0273 SEM). Comparatively, when the animals are similarly ranked, they spend a greater time engaging in face touch (mean latency = 297.3 ms ± 0.0492 SEM) (Figure 2F). These results show that the speed of sensory processing during tube encounters occurs on very short timescales, and suggest that face touch might carry salient information about naked mole-rat identity. In other words, more closely ranked animals may need more time to gather information to determine the rank of their interlocutor in the tube.

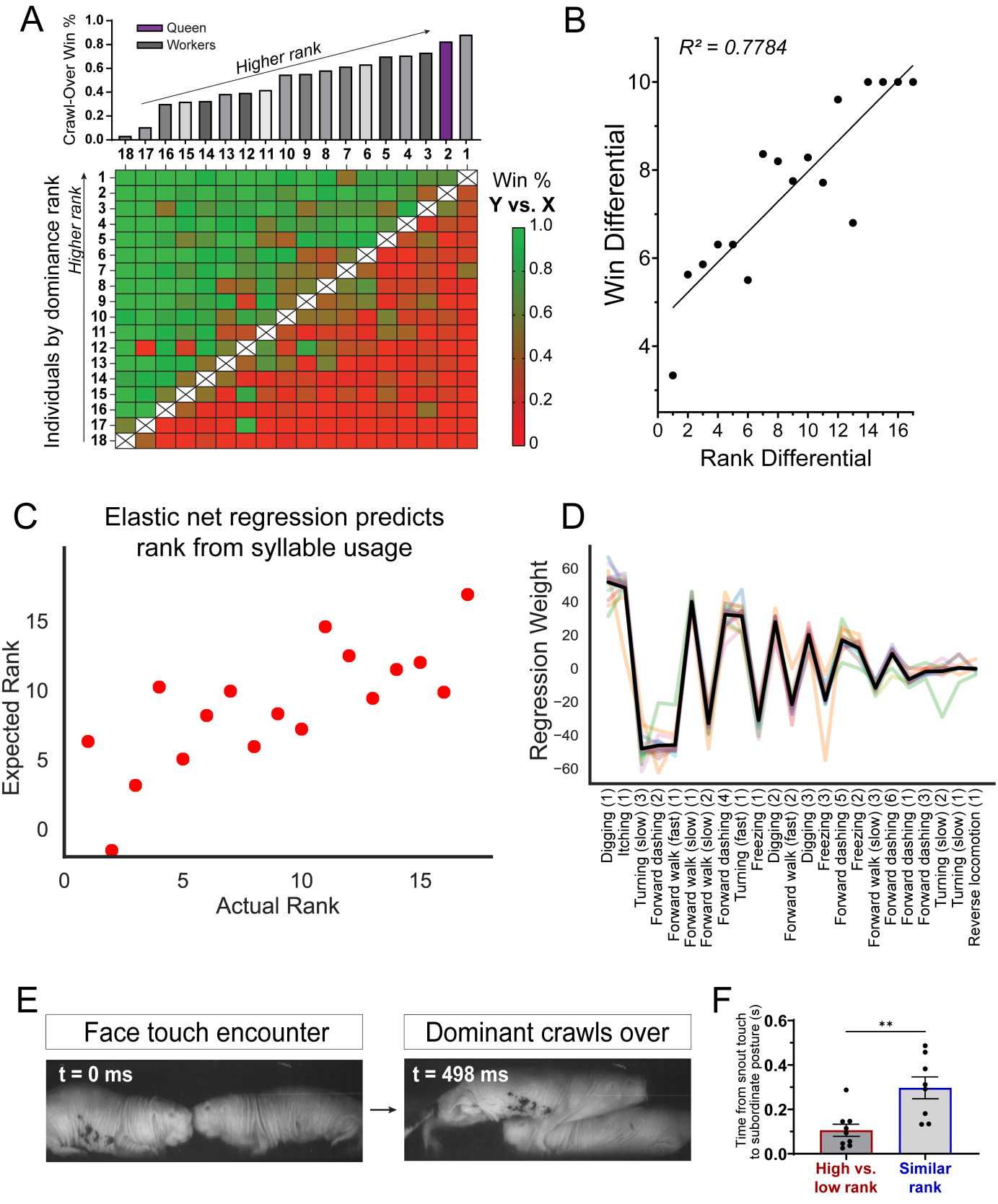


Figure 2: Spontaneous behavior in isolation maps on to dominance rank

(A) Dominance hierarchy and pairwise tube testing plot. Each pairwise tube test consisted of 10 consecutive trials, with color corresponding to win% of column Y vs. column X. Higher-ranked animals consistently "won" vs. lower-ranked individuals (n=18 animals). **(B)** Linear regression shows that the higher the rank differential between a pair of animals in the tube test, the greater the win differential is [R^2 = 0.7784]. **(C)** Elastic net regression successfully predicts the rank of a naked mole-rat from its usage of MoSeq syllables. Cross validated rank prediction showed a correlation of 0.75 with actual rank. This suggests that spontaneous behavior in the open field is correlated with dominance rank. **(D)** Regression weights of the fits in (C), indicating the relative importance of each behavioral syllable. Individual fits = lines in color, mean fit = black line. **(E)** High-speed videography of tube test for dominance. Naked mole-rats engage in a snout-to-snout interaction upon encounter in the tube, followed by the subordinate adopting an arched-back posture and the dominant animal crawling over. **(F)** Quantifying the time between snout touch and subordinate posture. In pairings between animals with high differential in rank, latency is significantly faster than in animal pairings of similar rank [n = 9 tube tests for similar-rank pairs, 10 tests for high v. low rank pairs, two-tailed t-test, p=0.0350, Grubb's test removed 2 outliers at alpha = 0.05].

Active face touch is a prominent form of social interaction in naked mole-rats

We next examined snout-to-snout interactions beyond the context of the tube test (Figure 2E), as pairs of animals socialized in an open field (Video S6). To quantify face touch interactions as animals freely interacted in a bucket, when two snout nodes (tracked by SLEAP) entered within a shared circular region with a diameter of 20 pixels, which corresponded to a distance of 11.21mm, this was registered as a face touch interaction (Figure 3A,B). To create a control for comparison to real face touch interactions, we generated "phantom" plots of the number of face touch interactions expected by chance alone. Remarkably, we discovered that naked mole-rats frequently socialize using active face touch, with an average of 106.8 interactions per 10 minutes (Figure 3C,D). The frequency of this behavior points towards its primacy in their social interactions.

Next, to more deeply probe the social relevance of face-to-face contact, we decided to investigate face touch in the context of foreign encounters, since naked mole-rats are extremely xenophobic (O'Riain et al., 1996; O'Riain & Jarvis, 1997). For this experiment, we established additional naked mole-rat colonies donated from colleagues around the country. Interestingly, when we performed the experiments with foreign naked mole-rats in the same bucket used with familiar animals (Figure 3E), we noticed a doubling of active face touch (Figure 3F,G). This result is consistent with our observations in a two-chamber preference assay where we show that naked mole-rats show increased interest in investigating a foreign naked mole-rat as opposed to a familiar conspecific (Figure S6). Thus, like mice, naked mole-rats appear to prefer novelty and may have sufficient social memory to remember members of their home colony, as well as members from other colonies. In the open arena testing, where the animals can physically interact, foreign naked mole-rats' social interactions were often aggressive and involved biting and shoving. We speculate that the active face touch might be critical for social familiarity recognition, and when a certain cue, or desired signal is not reciprocated from one animal

to another during the face touch, antagonism is unlocked. Taken together, we have identified active face touch in naked mole-rats as a prominent form of social interaction, and the full significance of this behavior will require extensive follow-up investigation.

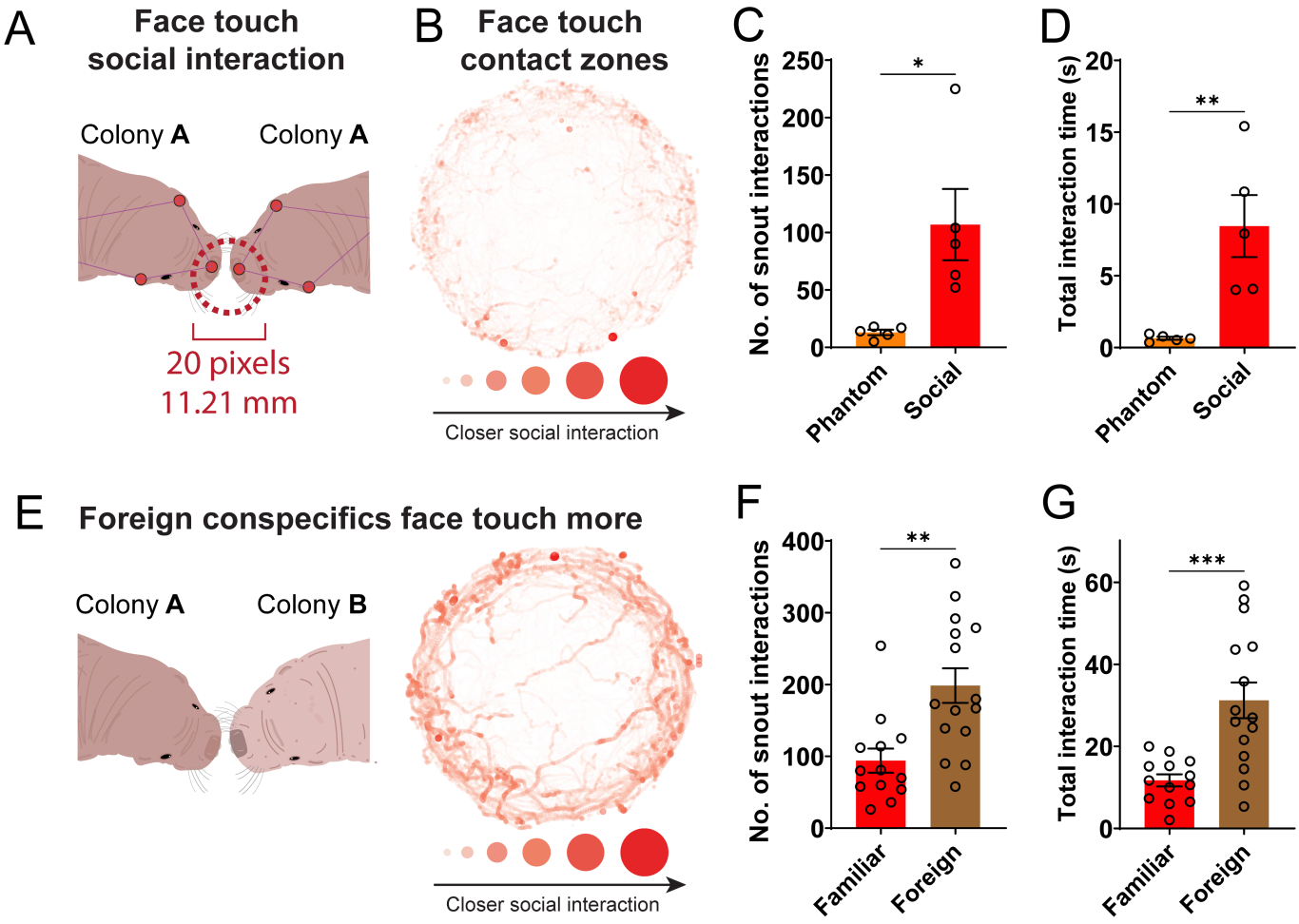


Figure 3: Face touch is a prominent form of social interaction in naked mole-rats

(A) Quantification of face touch interaction. A face touch interaction was defined as a continuous event where two naked mole-rat snout nodes (as tracked by SLEAP) were within 20 pixels of each other. **(B)** Representative visualization of face touch interaction hotspots of two familiar naked mole-rats. Areas of higher interaction indicated by larger and darker red circles. **(C-D)** Two familiar naked mole-rats engage in numerous face touch interactions in a 10-minute window. Both the number of snout interactions and the total duration of social interaction was much higher than by random chance alone (phantom control condition). Total Interaction time, [two-tailed t-test, p = 0.0166, n=5 pairs]. Number of interactions [two-tailed t-test, p = 0.0069, n=5 pairs]. **(E-G)** Naked mole-rats engage in a unique, pro-investigatory behavioral phenotype when engaging with foreign conspecifics from other colonies. Two foreign naked mole-rats engage in significantly more snout interactions (F) [two-tailed t-test, p = 0.0019], and spend a longer total duration socializing (G), than do two familiar workers [two-tailed t-test, p = 0.0005, n = 13 familiar pairs, 15 foreign pairs].

Active face touch in social interactions involves Piezo2 mechanosensation in face-innervating sensory neurons

Finally, to determine the cellular basis of face touch within the home colony, we used the FM 1-43 dye whose fluorescence intensity is a direct readout of the activation of the mechanosensitive ion channel Piezo2 (Villarino et al., 2023) (Figure 4A). We tested either an isolated animal or an animal that remained in its home colony with ~30 other naked mole-rats. The control and experimental animal were each given an intraperitoneal injection of FM 1-43 and were euthanized 24 hours later. We then collected tissue from the dorsal root ganglion (body-innervating sensory neurons) and trigeminal ganglion (face-innervating neurons). In skin sections from snout skin of the face, within the social

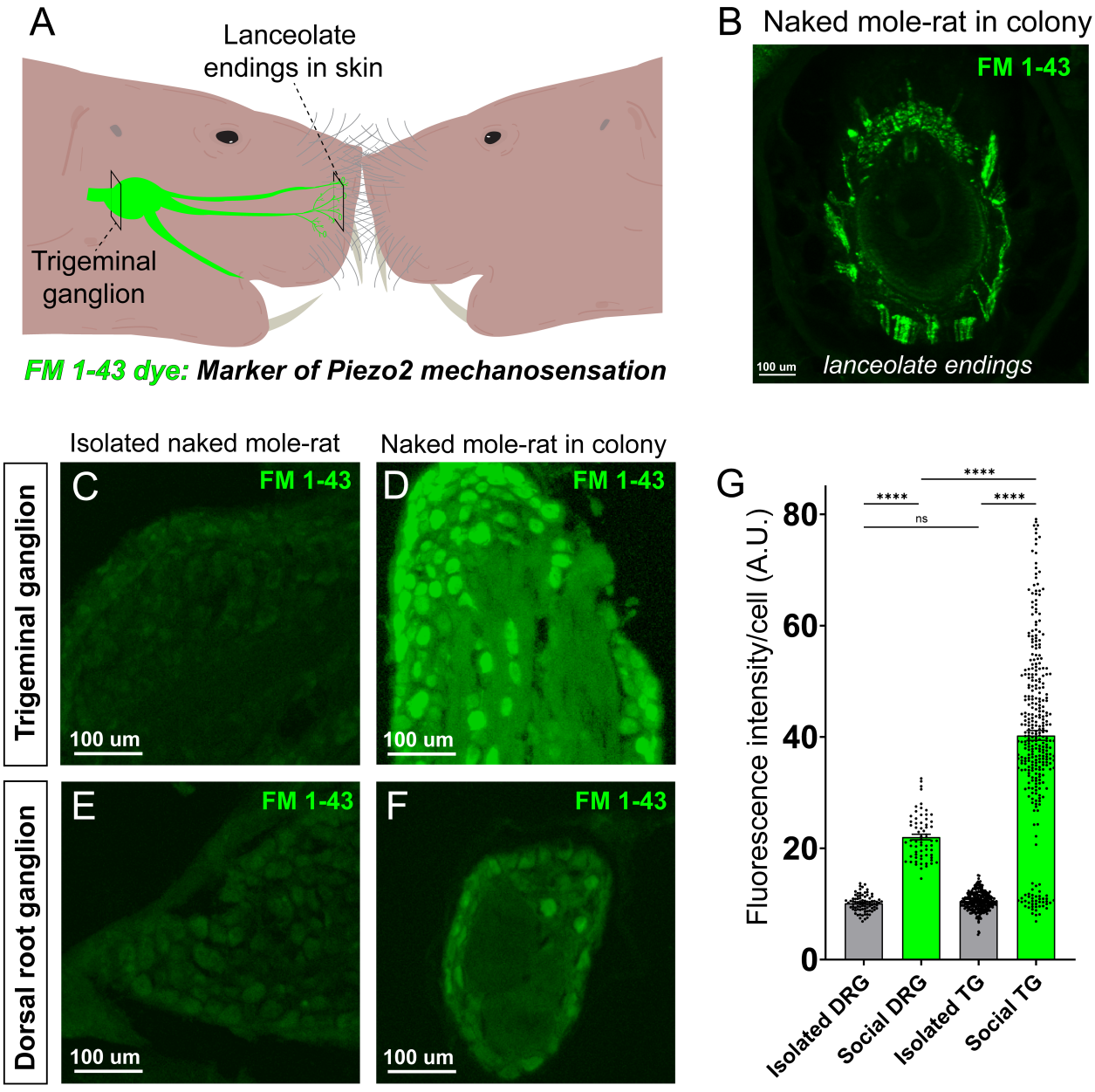


Figure 4: Face touch in the social setting is performed with involvement of Piezo2

(A) Schematic depicting peripheral innervation underlying face touch interaction in animals injected with mechanosensitive dye FM 1-43. Cryosections were obtained from the trigeminal ganglia innervating the face, and its peripheral sensory endings in the snout skin. **(B)** Activated lanceolate endings in the snout skin of naked mole-rat housed within social setting of home colony and injected with FM 1-43. Fluorescence indicates Piezo2-mediated mechanosensitive activity in snout whiskers. **(C)** Cryosection of trigeminal ganglion of naked mole-rat isolated from colony (and therefore isolated from snout-to-snout interactions) injected with FM 1-43. **(D)** Trigeminal ganglion section of FM 1-43-injected mole-rat housed in home colony and exposed to snout-to-snout interactions. **(E)** Dorsal root ganglion section of isolated naked mole-rat. **(F)** Dorsal root ganglion section of social naked mole-rat. **(G)** Comparison of peripheral neuron activation between naked mole-rats that were socially isolated vs. social (C-F). Quantifying fluorescence intensity per cell showed substantially higher Piezo2-dependent activity in the TGs and DRGs of social naked mole-rats than in socially isolated animals. In the social condition, the TG had substantially higher activity than the DRG [One-Way ANOVA, $p<0.001$, n=3 DRGs and 3 TGs per condition].

setting, we observed strong FM 1-43 fluorescence in lanceolate sensory terminal endings that associate with facial vibrissae (Figure 4B). These sensory endings in the face most likely emerge from Aβ mechanoreceptors, as these are the main mechanosensory endings in this snout region as previously described (Park et al., 2003). Strikingly, when we analyzed the trigeminal ganglia we saw robust and widespread staining of the FM 1-43 dye in the social setting, but not when animals were alone, although in a similar home environment (Figure 4C, D). However, it should be noted that we could see a few positive cells in the isolated setting when the gain and intensity were turned high on the confocal microscope. In the dorsal root ganglion, although we saw more FM 1-43 positive cells in the social setting vs. the isolated setting, the fluorescence intensity and number of fluorescent cells was greatly reduced compared to the trigeminal ganglion (Figure 4C-F). Together, these results support the finding that active face touch, which is a highly mechanosensory behavior, is prominent when naked mole-rats socially engage. Moreover, naked mole-rats appear to use the same molecular machinery for mechanosensation, Piezo2, as other mammals despite being quite evolutionarily distant and divergent.

Discussion

Although naked mole-rats have captivated biologists for decades, mainly for their longevity, resilience to cancer, and apparent indifference to certain types of pain (Buffenstein, 2008; Buffenstein et al., 2021; Freire Jorge et al., 2022), the biology of their social interactions has been less studied. Here, we argue that their spontaneous behavior is reflective of their biological caste and rank, and we establish face-to-face contact as a key social behavior—perhaps indispensable for the stability of the naked mole-rats' reproductive and social hierarchies. Thus, these features of their biology and behavior make them an ideal model system for understanding the basis of complex social systems.

Caste-like organization of behavior in naked mole-rat colonies

One of the earliest studies of naked mole-rat behavior from Osman Hill and colleagues in 1957 revealed that digging was specific to certain animals in the colony and that these animals would dig in a cooperative manner (Hill et al., 1957). In 1996, a rare "disperser" caste was identified, where certain males grow in size, perform little work in the colony, and eventually disperse and leave to establish a new colony (O'Riain et al., 1996). These, and related observations, have been substantiated over the years by different groups, showing a division of labor within naked mole-rat colonies, with behaviors that seem linked to dominance rank. Given these observations and the presence of a single breeding queen and sexually repressed workers, it is widely acknowledged that naked mole-rats are eusocial and appear to arrange behaviors and duties by caste membership such as queen, breeding male, worker, soldier, and disperser (Jarvis, 1981; Mooney et al., 2015; Pepper et al., 1991). However, it remains debated in the field whether naked mole-rats truly organize behavior by caste akin to eusocial ants and bees, where caste-specific behaviors are so hard-wired that they are encoded in the genome (Warner et al., 2019). Indeed, a recent study shows that behavioral tasks appear more fluid amongst non-reproductive workers with no evidence of task specialization (Siegmann et al., 2021).

Here we used the modern tools of computational neuroethology to generate an atlas of the structure of spontaneous behavior in a naked mole-rat colony. We divided groups into queen vs. non-reproductive workers, so the question of behavioral division amongst workers was not directly addressed in our study. We observed behavioral syllables and transition matrices from one behavioral syllable to another that segregate by colony rank. For example, the queen's most robust behavioral syllable is a so-called forward dash that she does frequently in the home colony and even in isolation, suggesting that it is a hard-wired behavior and not merely contextual. This behavior may be linked to colony dominance and reproductive suppression of subordinates. The queen also rarely digs. The frequency of behavioral syllable usage could be used to determine the relative dominance or submissive status of a given naked mole-rat amongst 18 colony members, demonstrating that behavior and rank are inextricably linked. Therefore, our studies here using an unsupervised machine learning pipeline support the claims of behavior organized in a caste-like fashion in naked mole-rat colonies. How do these seemingly hard-wired behaviors become flexible, such as a female worker's emergence into queenhood, or a male worker's emergence from worker to breeder? This remains an open and exciting question.

The role of active face touch in naked mole-rat social behavior

We observed naked mole-rats engaging in an active form of face touch hundreds of times over a short window. Extrapolating from these results, naked mole-rats likely perform face touch with colony members thousands of times a day. Why do naked mole-rats engage in face touch so frequently? Although a blind animal might accidently bump into another animal in a crowded space, the frequency of these interactions rises far above chance. In addition, it is possible that the close face contacts are made in an effort to smell a volatile or pheromone cue on the other animal, or even to gain closeness to hear a unique vocalization. Nonetheless, we speculate that face touch is a form of social

communication that the animals perform because it is rewarding and/or important for animal identification. Indeed, we observe an increased amount of face touch when two foreign naked mole-rats encounter one another—which might be due to increased efforts in trying to identify the stranger. The somatosensory system of the naked mole-rat is exquisitely tuned to respond to face touch by detecting movement on the skin through activation of vibrissae-innervating sensory neurons (Crish et al., 2006; Park et al., 2003). Our results here with the FM 1-43 dye in the trigeminal ganglion of naked mole-rats in a social setting is consistent with abundant face touch and direct mechanical activation of the somatosensory system. This result shows that the naked mole-rat, separated by approximately 30 million years of evolution from the standard lab mouse, uses the same molecular machinery, Piezo2, for mechanosensation. Moreover, the face, including the externally located incisor teeth, have a heightened representation in the somatosensory cortex compared to tactile inputs from other parts of the body (Catania & Remple, 2002). Thus, the naked mole-rat face is an ideal locus for gathering tactile sensory signals and, potentially, other sensory modalities as well as for transmitting this information to their expanded somatosensory cortex and other cortical and sub-cortical areas.

What about the role of other sensory systems in the social behavior of naked mole-rats? Active face touch seems unlikely as a sensory cue, in isolation, to be rich enough to serve as an animal identification code. A recent landmark study in 2021 identified 25 unique vocalizations in naked mole-rat colonies (Barker et al., 2021), largely consistent with reports from an earlier study (Pepper et al., 1991). These vocalizations constitute a dialect that is culturally transmitted and maintained by the presence of a queen, and animals from the same colonies display vocal patterns of call and response (Barker et al., 2021). Indeed, spending any brief time amongst naked mole-rats, an investigator will easily discern an audible soft chirp. To what degree naked mole-rats use these vocalizations as a system for individual identification or to guide social behaviors or social hierarchy remains unclear, especially since hearing capacity is diminished and auditory structures in the naked mole-rat outer and inner ear are greatly reduced (Heffner & Heffner, 1993; Mason et al., 2016; Okanoya et al., 2018). The role of olfaction in naked mole-rats remains controversial, with some studies showing olfactory-mediated colony preference and food localization (Judd & Sherman, 1996; O'Riain & Jarvis, 1997) and others studies showing no reduction in olfactory mediated behaviors when the olfactory epithelium is ablated (Toor et al., 2015). We hypothesize that animal recognition and social communication is multimodal, with varying contributions from olfaction, vocalization-audition, and somatosensation. How these sensory modalities integrate to drive social interactions in naked mole-rats is a fascinating question worthy of intense investigation.

Methods

Naked mole-rats husbandry and behavioral testing

All experimental testing was performed in compliance with the Guide for the Care and Use of Laboratory Animals (NIH). All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University. Naked mole-rats were housed in a climate-controlled room maintained at 80-85° F and approximately 15-20% humidity, on a 12-hour day/light cycle. All experiments were conducted in this environment to ensure naturalistic conditions for the animals. Naked mole-rats were fed a daily diet of sweet potatoes, celery, and apple, and this diet was supplemented twice a week with ProNutro cereal enriched in vitamins (Bokomo, 100g Pronutro/16g protein mix). Naked mole-rat colonies were housed in interconnected habitats consisting of mouse and rat cages joined by plexiglass tubing, with the number of chambers determined by the size of the colony. All naked mole-rats were microchipped using RFID transponders to track identity. Animals that were used for open field recording and dominance testing ranged from 3.09 to 12.34 years of age, and both sexes were equally tested. Breeding pairs were formed by housing a female and male naked mole-rat together in a rat cage. Naked mole-rats were generously provided by Dr. Rochelle Buffenstein, Dr. Dan McCloskey, and Dr. Thomas Park.

Open field recording setup

For all open field recordings, naked mole-rats were recorded in a circular arena consisting of a linear low-density polyethylene (LLDPE) tank with a diameter of 17” (United States Plastic Corp., catalog #14317). The arena was filled with black terrarium sand (ZooMed ReptiSand, Midnight Black color) approximately ¼” deep to simulate a naturalistic digging medium for the animals. For even illumination, a 21-inch diameter ring light was positioned above the arena. Video on the arena was obtained using a BlackFly S USB3 FLIR camera (catalog #BFS-U3-13Y3M-C USB 3.1; 1.3 megapixels) with an 8mm UC Series Fixed Focal Length Lens (Edmund Optics; catalog #33-307). The camera was mounted on a tripod above the center of the arena, and videos were acquired at 60 FPS and 1280x1024 resolution. Videos were recorded using the Teledyne FLIR Spinnaker SDK SpinView software. For videos of isolated animals, naked mole-rats were placed in the center of the arena and recorded in segments of 10 minutes. 19 animals, including the queen, were videotaped for the SLEAP-to-MoSeq pipeline. For social contexts, naked mole-rats were introduced to the arena at opposite sides to allow for natural snout-to-snout encounters. The machines used for recording were running Windows 11 Pro, with a NVIDIA® GeForce RTX™ 3060 GDDR6 GPU, and an Intel® Core™ i7-11800H CPU processor.

Pose estimation using SLEAP

Recorded videos were labeled using SLEAP, with a universal skeleton used for all naked mole-rats. This skeleton consisted of seven nodes: snout, left ear, right ear, centroid (torso), left hindpaw, right hindpaw, and base of the tail. All images were converted to

grayscale upon import. We labeled 2,749 frames with which we then trained a model using the multi-animal top-down pipeline type. Our receptive field in the centroid model configuration was 76 pixels. Frames used to train this model were derived from videos of both isolated animals, videos with multiple animals, videos of male and female workers, and videos of the queen. The model was also trained on frames of animals with variable coloration to account for pigmentation variability in naked mole-rats. In order to run inference on every frame, we culled the max instances to the number of naked mole-rats in the respective video, enabled the “simple” cross-frame identity tracker method, and used the built-in functionality to connect single-track breaks. Any additional errors in tracking were corrected using the manual track proofreading function in SLEAP. All files were exported in HDF5 format for further analysis. The machine used to train the SLEAP model was running Windows 11 Pro, an NVIDIA GeForce RTX 3060 GPU, and an Intel Core i7-12700K CPU processor.

Unsupervised behavioral segmentation using keypoint-MoSeq

We launched the keypoint-MoSeq program interface using Jupyter notebooks and used the SLEAP-tracked files (HDF5) and their corresponding videos (AVI) as inputs. We set the snout node as the anterior body part and the base of the tail node as the posterior body part. Seventeen total videos and their corresponding HDF5s were loaded and noise was calibrated using a confidence threshold of 0.5, prior to model initialization. During the step in which a PCA model was fit to keypoint coordinates, 90% of the variance was explained by 5 components. Prior to initiating training of the AR-HMM model, the hyperparameter kappa was set to $1e^6$. The AR-HMM model was then trained for 50 iterations. Following this, we then used the results of this AR-HMM fitting for initialization of a full model in which all parameters were updated via Gibbs sampling. This full model was trained using a kappa of $1e^6$ and was run for 300 iterations. Once trained, this full model was applied to all subsequent videos and HDF5s in the data set, and all results were exported in CSV format. Trajectory plots and crowd movies were generated to visualize all syllables identified by MoSeq, and a dendrogram illustrating syllable similarity was exported. MoSeq identified 23 syllables which were discernible as true behavior. These 23 syllables were then grouped into nine broad categories based on phylogenetic similarity on the dendrogram, and we gave them names that best corresponded to the nature of the behavior. These groups were: forward walking (slow), forward walking (fast), forward dashing, freezing, turning (slow), turning (fast), reverse locomotion, itching, and digging. Frequency of syllables were compared between groups using a two-way ANOVA with multiple comparisons.

Open field assay for anxiety-like and exploratory behaviors

To quantify anxiety-like and exploratory behaviors, we employed the same open field videos used in the SLEAP-to-keypoint-MoSeq pipeline. We used the ANY-maze software (https://www.any-maze.com) to track isolated animals as they moved about the circular arena. The center zone of the arena was defined as a circle of 9” diameter in the center of the arena with the remaining area defined as the periphery. Entries into the center were

counted when a portion of the animal entered the zone, and other metrics such as average and max velocities were automatically calculated by the software. The time of each 10-minute video was binned into 2-minute segments, and the number of center entries per bin was calculated. All values were compared using a student's two-tailed t-test at 95% confidence interval.

Syllable transition matrices

Syllable transition probabilities were derived from the raw frame-by-frame CSV spreadsheet generated by keypoint-MoSeq ("moseq_df.csv"). Using in-house code written in Python (https://github.com/abdus-saboor-lab-code/NMR-Notebooks) the first and final frames bounding each behavioral syllable were identified. For each type of syllable, the total number of subsequent syllable transitions was obtained. The probability of a given Syllable A transitioning to a given Syllable B was calculated using the formula P(A→B) = (number of transitions A→B) / (total number of transitions). Syllable transition probabilities were calculated for every pairwise combination and assembled into 22 x 22 syllable transition matrices. Transition matrix data was visualized as bigram probability plots using open-access code from Peterson et al. (2023).

Pairwise tube testing for dominance

For each pairwise tube test, two naked mole-rats were removed from the colony and placed on opposite ends of a tube. A trial consisted of the two animals encountering each other in the tube, and concluded with one animal (the winner) crawling over the other (the loser). The next trial began by resetting the two naked mole-rats to their starting positions, and 10 trials were conducted for each pairwise combination of animals. 18 total naked mole-rats were tested, with pairwise tests for every possible combination of animals (nCr = 153 combinations). For each pairwise test, the rank differential was calculated as the absolute value of the difference in rank (rank differential = $|rank_{animal1} - rank_{animal2}|$). Another metric, win differential, was calculated as the absolute value of the difference in the number of wins (out of 10 trials) between the winner and loser naked mole-rat (win differential = $|\#wins_{animal1} - \#wins_{animal2}|$). For some tube tests, a high-speed camera was used to capture the encounter at 750 - 2000 FPS with a Photron FastCAM Mini AX 50 170 K-M-32GB camera with a Zeiss 2/100M ZF.2 mount attachable lens. This camera was monochrome 170K, had 32 GB memory, and was controlled using a Dell laptop with Photron FastCAM software (PFV4). The time from first snout touch to adoption of subordinate posture was calculated using Photron PFV4.

Predicting rank from behavioral syllable usage

We used elastic net regression to identify the behavioral syllables that best predict rank. For each animal, we fit the model weights to data from all other animals and then evaluated its accuracy on the held-out animal. For all animals, we use a shared regularization parameter (commonly called alpha) of 0.001, and the ratio of L1 and L2 penalties of 0.9.

Snout-to-snout interactions in open field

Snout interaction data was generated using custom code written in R (https://github.com/abdus-saboor-lab-code/NMR-Notebooks), applied to HDF5 files generated by SLEAP. This code extracted the x and y coordinates of each skeletal node. Using these coordinates, the distance between any two given nodes in pixels was calculated using the Pythagorean theorem $=\sqrt{((x2-x1)^2+(y2-y1)^2)}$. A snout interaction was defined as any continuous instance in which two snout nodes were ≤20 pixels within each other, without any frames during the interaction where the distance was >20 pixels. Using these criteria, the code calculated the total number of snout interactions between all of the snout nodes in a given HDF5 file. Snout interactions were visualized by plotting the xy trajectories of the snout nodes over time and creating “hotspots” of activity where closer interactions were represented by larger-diameter, higher-opacity circles. These hotspots were centered on the midpoint between a given snout interaction between two nodes.

Social preference assay

Social preference assay was conducted in a custom chamber created from a polyethylene tank with dimensions of 12” width, 18” length, and 12” height (United States Plastic Corp., catalog# 14828). Two acrylic cage-like chambers were located at far corners of the bucket and held the naked mole-rats to serve as preference stimuli. The 10-minute trials were videotaped using the same BlackFly S USB3 FLIR camera as in the open field testing, and the investigating naked mole-rat was fully tracked using ANY-maze software (https://www.any-maze.com). The investigating naked mole-rat was placed in the bucket and was free to move and investigate either cage. During an initial habituation phase, the test subject was allowed to freely explore the paradigm for 15 minutes. Before each of the subsequent recording sessions, the social stimulus animal (i.e. in- and out-colony conspecifics) was placed in the respective chamber, which physically confined its movement and allowed limited interaction with the investigating animal through the barred fence. The test subject was then re-introduced to the center point of the field (opposite to the chamber location) at the start of each video recording. The chamber was disinfected in between trials.

FM 1-43 injection and histology

In order to administer the Piezo2-selective dye, naked mole-rats were scruffed and injected intraperitoneally (IP) with 1.12 mg/kg FM 1-43 (Thermo Fisher). For the socially isolated condition, the animal was removed from the home colony for 6 months and housed in a rat-sized cage within the same climate-controlled room as that of the colony. After 6 months, the animal was injected with FM 1-43. For the social condition, the injected naked mole-rat was housed in the home colony. Prior to tissue collection, both animals were perfused using 4% PFA, and tissue was harvested from trigeminal ganglia (TG), dorsal root ganglia (DRG), and snout skin. Tissues were fixed overnight in 4% PFA and then overnight in 30% sucrose in PBS. All tissues were then frozen in OCT until

sectioning. For cryosectioning, TGs and DRGs were sectioned at 30 uM thickness, and snout skin was sectioned at 100 uM thickness, with cuts made parallel to the surface of skin. All tissues were mounted on SuperFrost microscope slides and imaged on a Leica confocal microscope. For TGs and DRGs, ImageJ was used to quantify fluorescence intensity and count cell bodies. The number of cells imaged from each condition were as follows; DRG (socially isolated), 83 cells; DRG (socially paired), 66 cells; TG (socially isolated), 273 cells; TG (socially paired), 334 cells.

Data availability

All data and code are available upon request.

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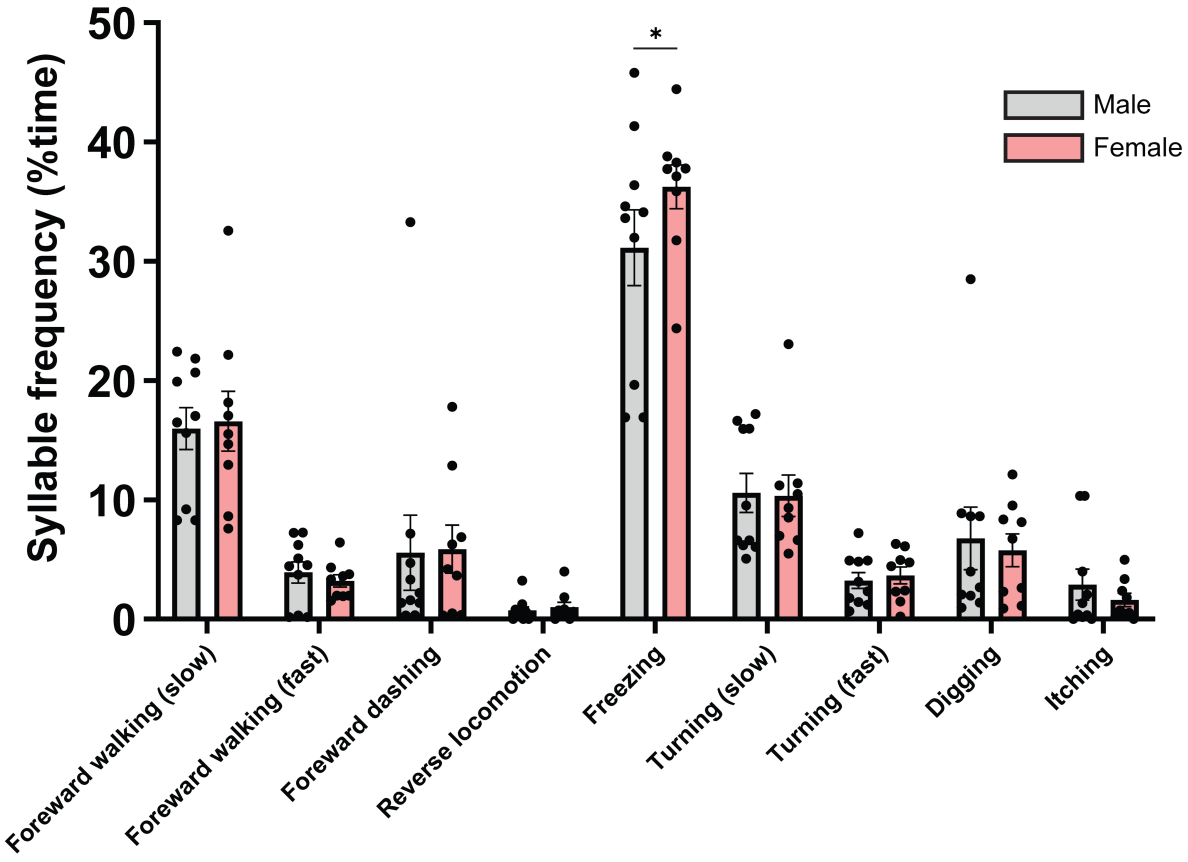
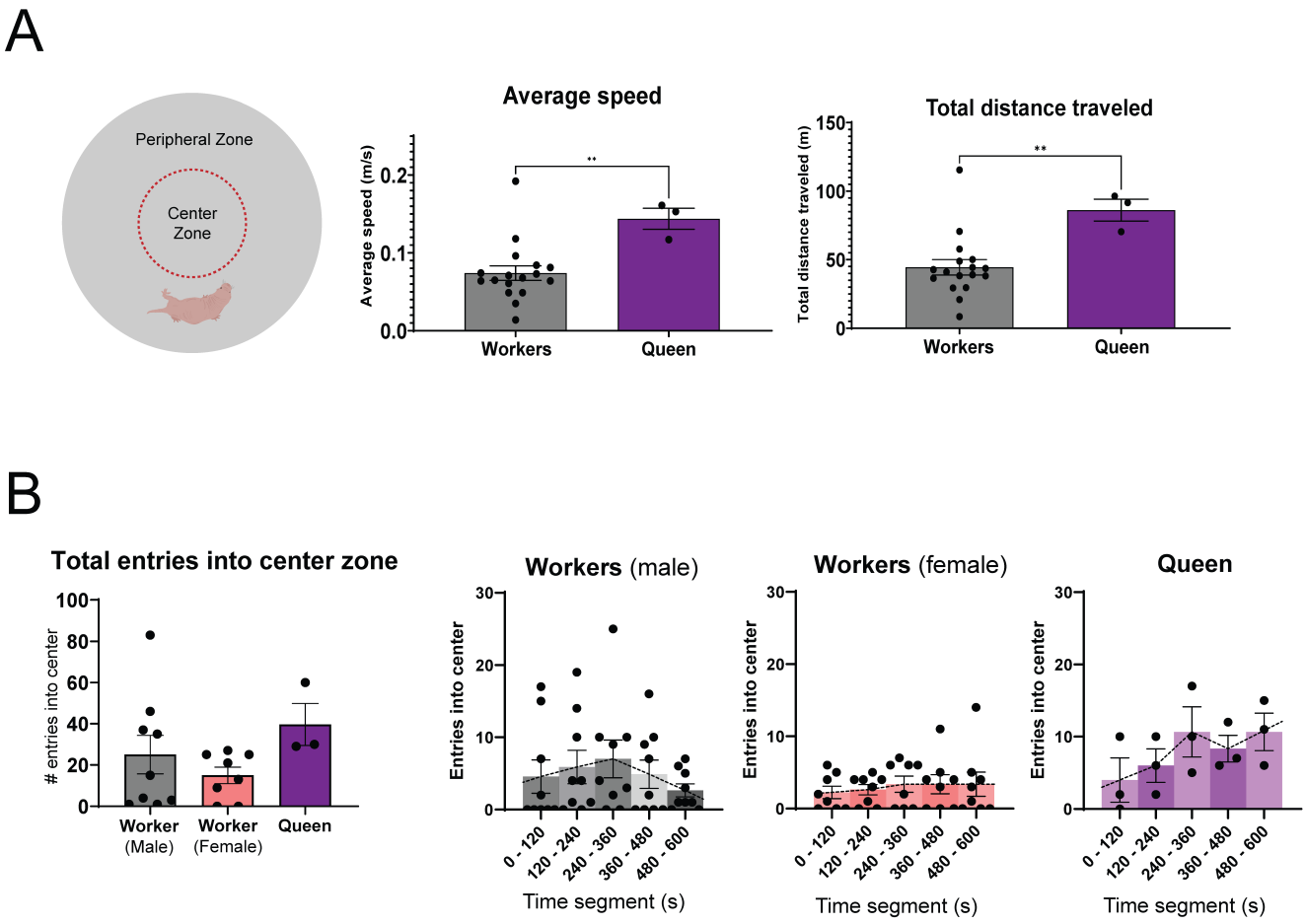
Supplemental Figure 1



Supplemental Figure 1: Comparing sex differences in nonreproductive workers using MoSeq syllable profile

Syllable usage profile for all nonreproductive workers, split into male and female categories. Syllable categories were summed together to gain a broader view of behavior, and to make comparison easier. Male and female workers had very similar overall MoSeq syllable usage. One notable difference was that females exhibited slightly more freezing behavior [two-way ANOVA, $p = 0.0453$]. Males: n=10, females: n=9.

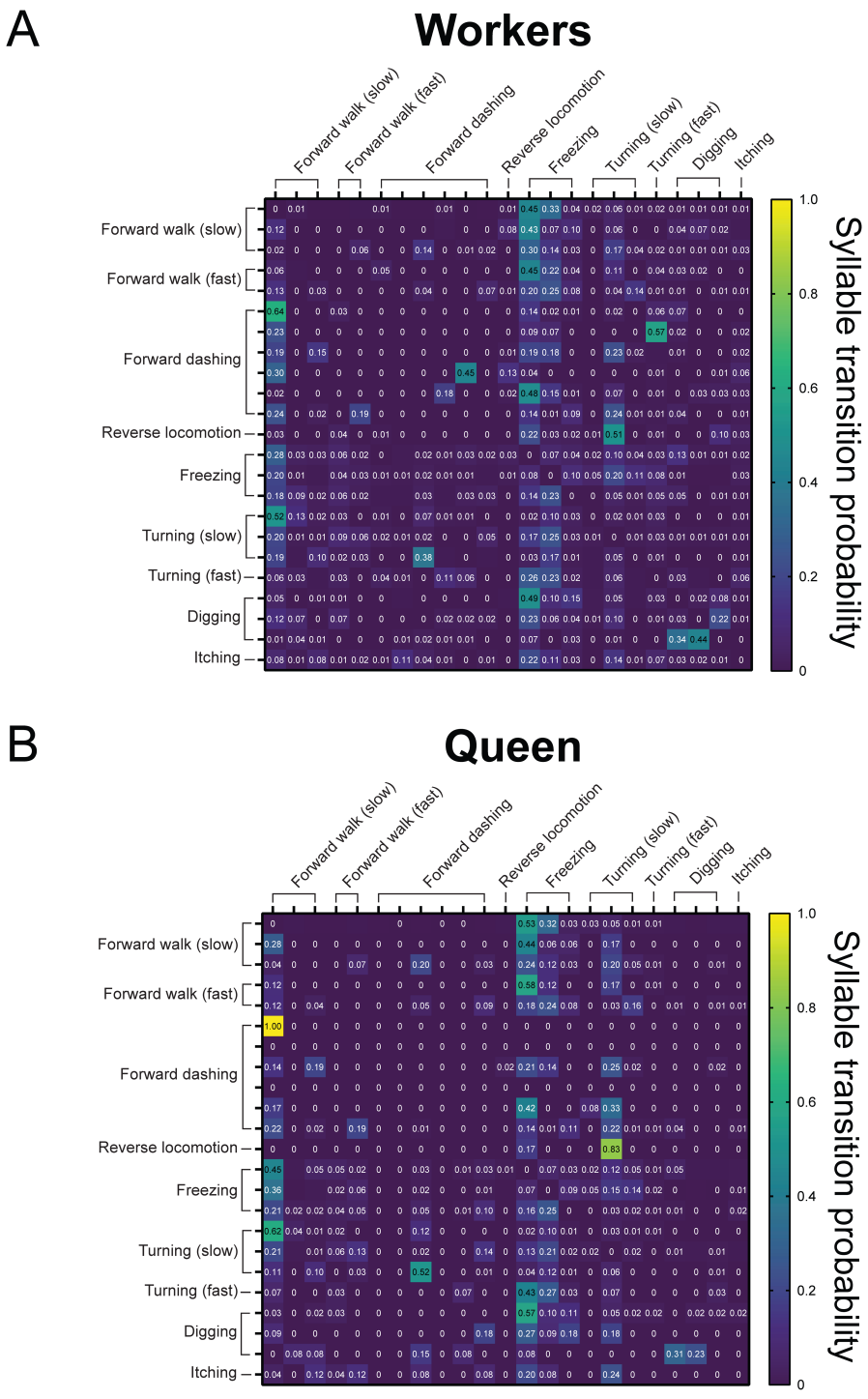
Supplemental Figure 2



Supplemental Figure 2: The queen mole-rat shows a pro-exploratory, low anxiety-like phenotype in the open field

(A) Open field locomotory test analyzed by AnyMaze software. Naked mole-rats were able to crawl around the peripheral zone and could explore the center zone. The queen naked mole-rat exhibited higher average speed [two-tailed t-test, p = 0.0074] and traveled more distance than the nonreproductive workers [two-tailed t-test, p = 0.0075]. **(B)** The queen exhibits a pro-exploratory phenotype. Worker naked mole-rats entered the center zone less frequently than the queen, with female workers the least exploratory, the queen the most exploratory, and the male workers intermediate. The queen had a sustained phenotype, exploring more often over time. More exploration suggests a lower anxiety-like phenotype in the queen. Workers: n=17, 1 trial per animal. Queen: n=1, 3 trials of single queen].

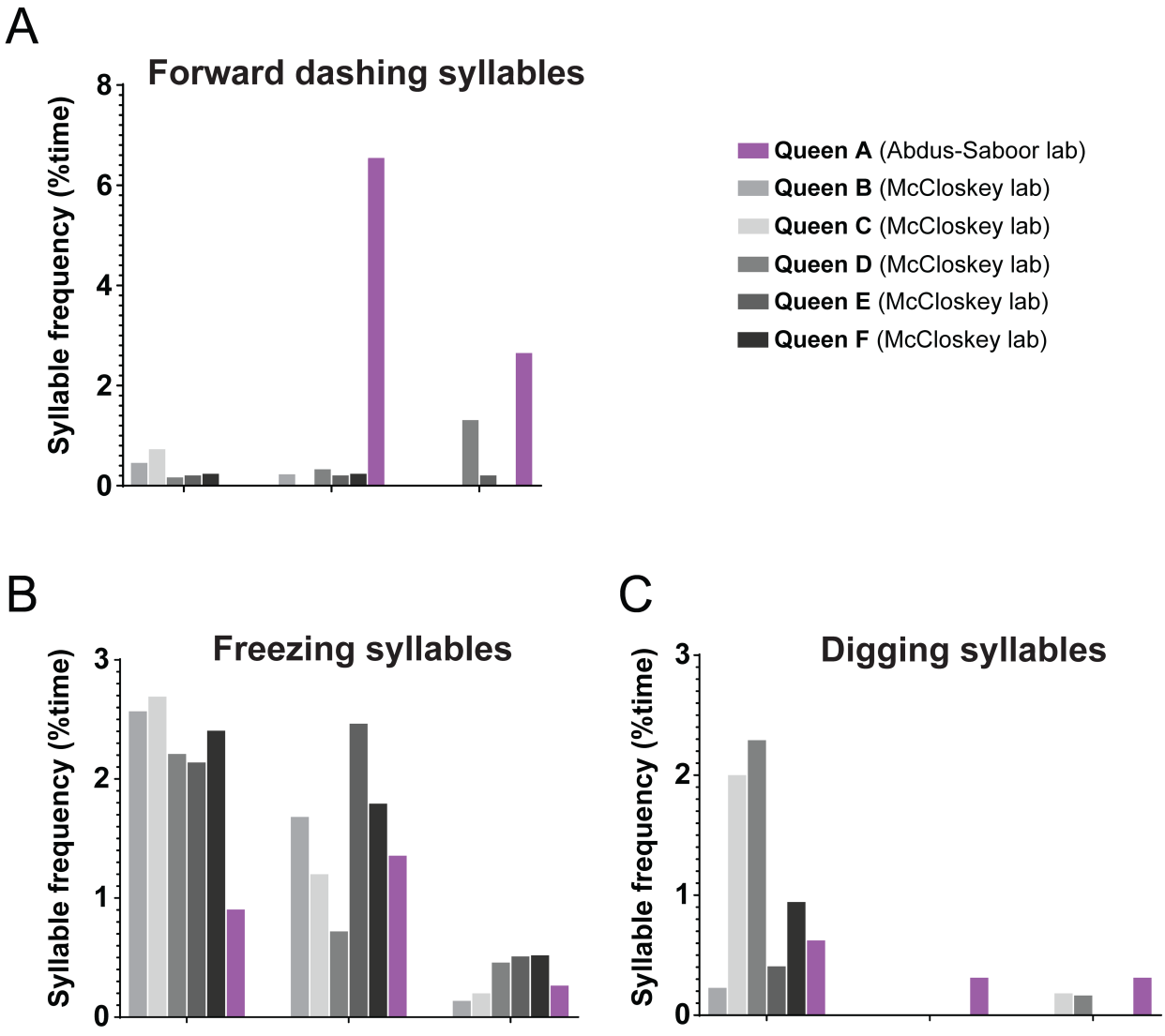
Supplemental Figure 3



Supplemental Figure 3: Comparing raw syllable transition matrices between queen mole-rat and nonreproductive workers

(A) Syllable transition matrix of nonreproductive workers. Transition probabilities represent the percent of the time that a given incoming syllable (Y axis) is followed by a given outgoing syllable (X axis). **(B)** Syllable transition matrix of the queen naked mole-rat. Same as (A), but for the queen. Workers: n=17, queen: n=1.

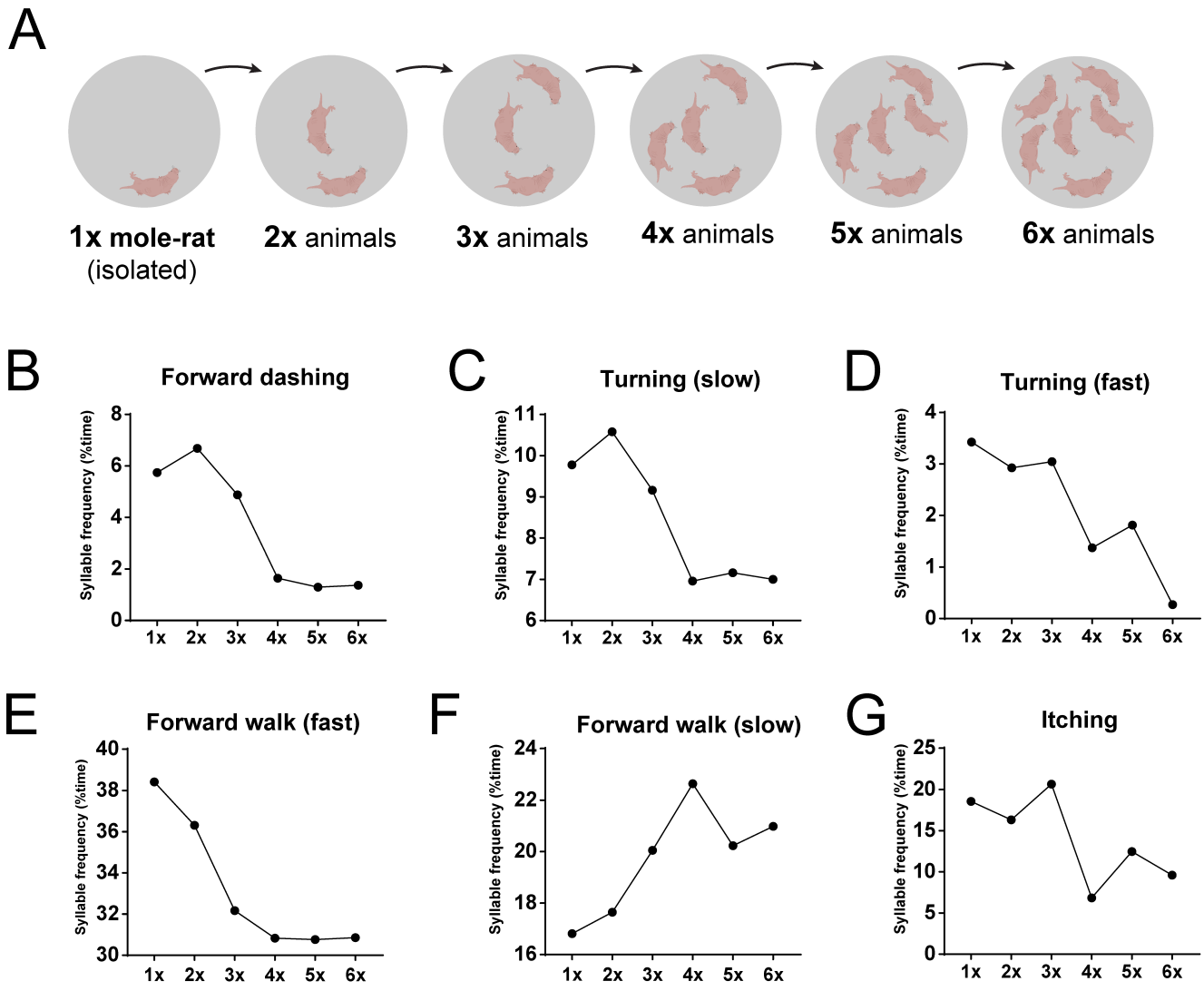
Supplemental Figure 4



Supplemental Figure 4: Queens from different mole-rat colonies have distinct MoSeq behavioral phenotypes

(A) Comparing the forward dashing syllables between queen in the Abdus-Saboor lab, and five queens in the McCloskey lab. The use of forward dashing was higher in the Abdus-Saboor queen than any of the McCloskey queens. **(B)** Comparing the freezing syllables among different queens. Freezing syllables were generally higher in queens from the McCloskey lab. **(C)** Comparing digging syllables among queens. McCloskey queens generally dug more than the Abdus-Saboor queen, but only for one of the three digging syllables. n=1 trial per animal.

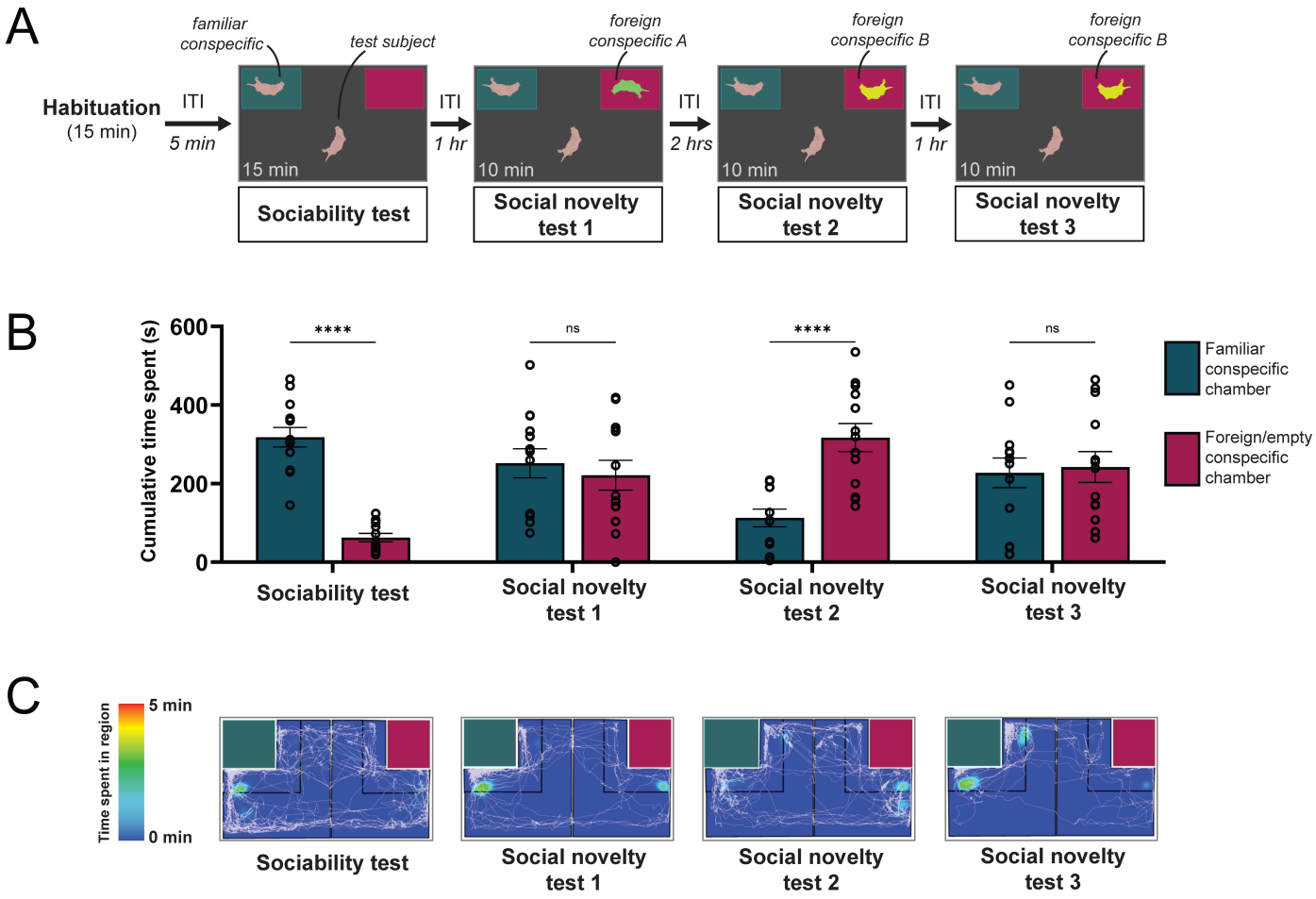
Supplemental Figure 5



Supplemental Figure 5: Increasing social presence affects naked mole-rat behavioral profile

(A) Social titration paradigm. A single naked mole-rat was placed in the open field for 10 minutes. Every 10 minutes, an additional conspecific was added to the arena, up to 6x animals. All conditions were videorecorded and run through SLEAP and keypoint-MoSeq. **(B-G)** Syllable use per animal with increasing social presence. Most MoSeq syllables decreased in frequency as the number of naked mole-rats increased, including forward dashing, turning (slow), turning (fast), forward walking (fast) and itching. Forward walking (slow) was the only syllable to increase in frequency as the social presence of naked mole-rats increased. n=2 videos per condition.

Supplemental Figure 6



Supplemental Figure 6: Naked mole-rats exhibit strong place preference for conspecifics

(A) Experimental design of social preference assay. After a 15-minute habituation period to two empty chambers and a 5-minute inter-trial interval (ITI), the test subject naked mole-rat underwent a sociability test with a familiar conspecific vs. an empty chamber for 15 minutes. Three subsequent social novelty tests then allowed the test animal to choose between a familiar vs. a foreign conspecific from another colony. **(B)** Naked mole-rats exhibit strong social preference and show signs of social memory towards foreign animals. Investigating animals overwhelmingly prefer to investigate a familiar conspecific vs. an empty chamber and spend similar time investigating a familiar vs. foreign conspecific. During social novelty test 2, preference for the foreign conspecific is higher, but this preference is lost during the subsequent social novelty test 3. This loss of preference suggests habituation to the foreign conspecific, potentially due to social memory. **(C)** Representative heat map of investigating animal during each test condition. Color indicates areas of density of the animal's centroid over time. White lines indicate the trajectory of the snout over time [n=13 test subject naked mole-rats and respective total trials, unpaired t-test for all four conditions; sociability test, $p<0.001$; social novelty test 1, $p=0.5682$; social novelty test 2, $p<0.001$; social novelty test 3, $p=0.7891$].

Supplemental Video 1: Open field SLEAP tracking of two naked mole-rats. Sample of two naked mole-rats fully identity-tracked by SLEAP, using the methodology described in Figure 1A. One animal (blue skeleton) engages in digging behavior, and the other (orange skeleton) explores the arena.

Supplemental Video 2: MoSeq syllable – forward dashing. Tiled compilation of one of the forward dashing syllables identified by keypoint-MoSeq. Each tile represents a single instance of the animal engaging in a forward dash.

Supplemental Video 3: MoSeq syllable – digging. Tiled compilation of one of the digging syllables identified by keypoint-MoSeq.

Supplemental Video 4: MoSeq syllable – freezing. Tiled compilation of one of the forward dashing syllables identified by keypoint-MoSeq.

Supplemental Video 5: High-speed video of tube test for dominance. Sample of two naked mole-rats of similar rank during the tube test, as described in Figure 2E. The interaction begins with a snout-to-snout touch, followed by adoption of subordinate posture by the lower-rank animal, and crawl-over of the higher-rank animal. Video was recorded at framerate of 750FPS.

Supplemental Video 6: Snout interaction of two familiar naked mole-rats. Sample of snout interactions between two naked mole-rats from the same colony, as described in Figure 3A. Two snout interactions occur in this video, and prominently involve face touch.

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