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Original scientific article

The impact of light-dark cycles and caging-systems on behavior and corticosterone excretion in the naked mole rat (Heterocephalus glaber)

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Summary

The naked mole rat *(Heterocephalus glaber)* (NMR) is increasingly becoming an important animal model in biomedical research. Housing NMRs optimally in captivity is therefore important. The present study was designed to establish the effects of varying photoperiods and cage designs on feed consumption, body weight, behavior and fecal corticosterone. The study period was 35 days, and a total of 54 NMRs were used. The main behaviors observed were huddling, patrolling/exploring, feeding, licking, climbing and mouth carrying. The average daily and weekly feed consumption was 5.8 and 40.7 grams per animal respectively. Significant differences in fecal corticosterone were noted on day 2 between group DL (12 hours of darkness and 12 hours of light) (60.66 ± 9.2 nmol/g) and group DD (24 hours darkness) (19.57 ± 1.5 nmol/g) at $p < 0.001$, on day 3 between group LL (24 hours light) (24.27.2 ± 1.3 nmol/g) and DL (49.71 ± 1.6 nmol/g) at $p < 0.05$, and day 35 between group DL (52.60 ± 4.5 nmol/g) and DD (27.10 ± 5 nmol/g) at $p < 0.05$. These results indicate that photoperiodism affected stress levels of NMRs and hence the difference in fecal corticosterone levels. It is therefore recommended that NMRs should be housed preferably under darkness to allow for full expression of their NMR-specific behaviors and to reduce stress. Caging system did not affect behavior, feed consumption, growth or stress levels.

Introduction

Naked mole rats *(Heterocephalus glaber)* (NMRs) belong to the order Rodentia and family Bathyergidae (Jarvis and Sherman 2002). Adults weigh 30-50 grams (Jarvis and Sherman 2002). They inhabit the semi-arid parts of Kenya, Ethiopia, Somalia (Jarvis and Sherman 2002; Jarvis and Bennett 2017) and Djibouti (Mulatu 2018). NMRs live in underground burrows, with tunnels known to be the longest among similar-sized animals (Buffenstein et al. 2021). The tunnel's length can be up to three kilometers, depending on the food available and colony size (Brett 1991).

NMRs within a colony communicate through specific odors and complex vocalizations (Jarvis and Sherman 2002). Their average body temperature is 32° C, although this temperature changes with

Figure 1: Naked Mole Rats housed at South Eastern Kenya University

the ambient temperature, a poikilothermic feature (Buffenstein et al. 2021). They have tiny eyes with limited vision capabilities, although they can detect light and darkness (Nikitina et al. 2004; Hetling et al. 2005). Their cerebral cortex has only a small area for visual processing (Browe et al. 2020; Vice et al. 2021). They possess around 100 sensory body hairs, mostly located on the tail and muzzle, to help navigate and keep informed about their surroundings (Jarvis and Sherman 2002; Mulatu 2018; Vice et al. 2021). Their hearing capability is also reduced compared to other mammals (Pyott et al., 2020), with the external ear pinnae missing and a small canal leading to the middle ear (Mason et al. 2016; Mulatu 2018). Their gestation period is 66 to 74 days (Jarvis and Sherman 2002), with a litter size averaging 13 (Smith and Buffenstein 2021). NMRs can live more than 30 years in captivity (Lewis and Buffenstein 2016; Ruby et al. 2018), which is five times longer than similar-sized rodents (Edrey et al. 2011).

NMRs are eusocial and live in colonies, with a single colony composing up to 300 individuals of overlapping generations. A colony typically consists of one breeding female, up to three male breeders, and a vast number of non-breeders. The non-breeders contribute by caring for the young and doing other tasks to ensure the survival of the colony. It has been suggested that the breeding female suppresses the reproductive abilities of other females within the colony. The exact mechanism is not fully understood, although reports suggest that suppression occurs through the transfer of pheromones from the queen to other females or by the queen's behavior towards other females (Jarvis and Sherman 2002; Buffenstein et al. 2021; Smith and Buffenstein 2021).

Despite their lack of the photoreceptive hormone melatonin, due to their atrophied pineal gland and non-functional melatonin receptors (Oosthuizen and Bennett 2022), NMRs can discriminate between light and dark (Nikitina et al. 2004; Hetling et al. 2005; Mooney et al. 2021). Their eyes are hypo-functional and cannot visualize objects because of the poorly developed retina, but can respond to light, although only at higher levels compared to rats and mice (Crish et al. 2006; Vice et al. 2021). They possess photoreceptors that can transmit light signals to the suprachiasmic nucleus in their hypothalamus, but because of their small eyes, the photoreceptors are few (Oosthuizen and Bennett 2022).

NMRs are gaining importance in biomedical research and bioprospecting (Judd and Sherman 1996; Jarvis and Sherman 2002; Mulatu 2018). As they are long-lived, they are used as models for longevity. They are also used in cancer research, since they resist induced tumorigenesis, due to their gene and protein stability and their ability to resist oxidative damage (Seluanov et al. 2018; Shepard and Kissil 2020; Miura et al. 2021), as well as in hypoxia studies, since they can tolerate hypoxia and hypercapnia for longer periods than mice and humans, and without developing the associated pulmonary edema (Park et al. 2021). They can recover from eighteen minutes of anoxia (Browe et al. 2020). NMRs are also used in osteoarthritis studies. Their cartilage contains upreg-

ulated hyaluronan, which facilitates greater resistance to osteoarthritis than in mice (Taguchi et al. 2020). Another field where the NMR is found to be useful is in pain research. Notably, they do not respond to some types of noxious stimuli, such as acid-induced cutaneous pain, by licking the affected part (Buffenstein et al. 2021; Lewin et al. 2021). Similarly, they do not display pain behavior when exposed to capsaicin, even though they possess receptors with TRPV1 ion channels that can be stimulated by capsaicin (Park et al. 2008; Lewin et al. 2021). Given this increased use and apparent importance of the NMR in biomedical research, with consequently increased number of NMRs housed in captivity, it is of utmost importance to optimize welfare by housing them in conditions as close as possible to their natural habitat.

Housing captive NMRs in environments that mimic their natural habitat ensures their survival, expression of normal behavior and reproduction, in addition to ensuring that there is no negative influence on experimental results due to stressful conditions (NRC, 2011). Stress in animals can be assessed by measuring levels of corticosterone in body fluids or feces since stress causes the release of corticosteroids into the bloodstream after stimulation of the hypothalamic-pituitary-adrenal (HPA) axis (Kant et al. 1987). These measurements are however, not without limitations, including how to obtain the sample without stressing the animal, as well as the need for laboratories for analysis and the challenge of interpreting results (DeVries et al. 2003; Siswanto et al. 2008; Abelson et al. 2009; Joëls et al. 2018).

Physiological measures alone may not reveal sufficient information, and adding behavioral measurements provides more information about the animal's experience. Hence, combining several measures ensures we can better assess if an animal's welfare has been compromised.

This experiment aimed to study the behavior and fecal corticosterone excretion of NMRs under varying photoperiods and in various caging system designs. It was hypothesized that a stressful environment to NMRs would stimulate the HPA axis and subsequently increase corticosterone production and induction of nocifensive behaviors.

Materials and methods

Ethical statement

The following permits were acquired before starting experiments: research approval (ref: KWS/BRM/5001), NMR capture permit (ref: KWS/904), ethical permit (ref: FVM BAUEC/2020/275) from ethics committee, Faculty of Veterinary Medicine, University of Nairobi. The study also adhered to prevention of cruelty to animals act, chapter 360, laws of Kenya, and where applicable to European Directive 2010/63/EU on the protection of animals used for scientific purposes.

Animals and housing

Wild adult NMRs were sourced from their natural habitat in Makueni County, Kenya, then transported to South Eastern Kenya University. Animals were acclimatized to the laboratory environment for one month, during which each colony was housed separately in single cages (70 x 50 x 20 cm in length, width and height respectively) with two connected compartments. NMRs live as colonies in their wild habitat, and thus we housed them similarly in captivity. Housing colonies in separate cages also helps to prevent inter-colony aggression. Fine wood shavings were used as bedding; no other enrichment was provided. They were fed daily on a fresh diet of sweet potatoes, carrots and Irish potatoes chopped into large pieces to stimulate gnawing and chewing. No water was provided since they obtain water from their food (Jarvis and Sherman, 2002). The temperature in their microenvironment was 28-31°C, with humidity between 50-70% to maintain their soft skin. Buckets of water were strategically placed in the animal room to maintain high humidity. Animals were housed under alternating twelve hours of light and darkness. Clinical observations were performed on a daily basis by a veterinarian. After acclimatization, the NMRs were transferred into cages of different designs and under varying photoperiods to serve as the treatment variables for the study. All other microenvironment conditions such as temperature, humidity and the use of bedding material were not varied. NMRs were handled by the same experimenter throughout. No queens were noted based on phenotypic characteristics (enlarged body when pregnant), but no confirmatory hormonal assays were conducted.

Study design

Fifty-four NMRs were housed in three groups, with 18 animals in each. NMRs in each group were randomly assigned into three cages with dimensions as shown in Figure 2. Each cage had six animals shown as dots on the figure. Each cage acted as one experimental unit, and there were thus three experimental units in each group for comparing photoperiods and caging systems. Sample size (n=3) was selected based on the resource equation approach (Mead 1988).

The experimental period was 35 days. Animals were marked numerically on their backs using a permanent marker. During experiments, males and females were equally distributed to avoid sex bias, and only those weighing above 20 grams were used.

Behavioral observations

Counts for each behavior were recorded from each experimental unit, to allow calculation of the total counts for the group. Behavioral data were collected daily during the first four days and subsequently at three-day intervals. Observations were done visually following the scan sampling method and recorded into a check sheet (Martin and Bateson, 1986). The behaviors for NMRs housed in 24 hours of light (LL) and 12 hours darkness/ 12 hours light (DL) were observed as either present or absent at intervals of two minutes between 9.00 to 9.30 hours. Animals housed in 24 hours of darkness (DD) were also observed between 9.00 to 9.30 hours using red lighting, which is considered invisible to NMRs. The observed behaviors are defined in Table 1. These definitions are important to allow for others' clear understanding, consistency, and reproducibility (Lacey et al. 1991).

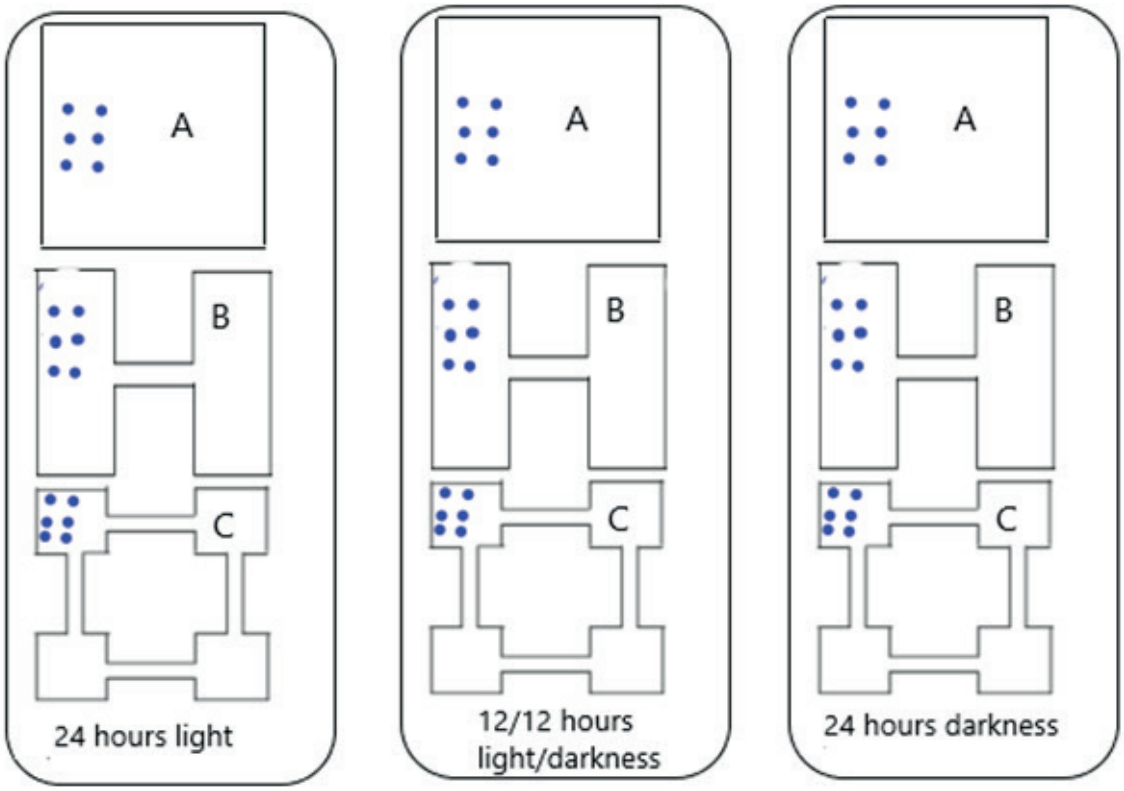

Figure 2: Graphical representation of the study design which allowed comparison of the effects of cage design and photoperiodism. Cage type A consisted of a single cage chamber measuring 70 x 50 x 20 cm in length, width, and height, respectively. Cage type B involved cage type A partitioned into two equal chambers and joined by a tunnel. Cage type C involved cage type A partitioned into four equal chambers joined by tunnels measuring 30 x 5.5 x 5.5 cm length, width, and height, respectively. Each cage was one experimental unit and contained six NMRs. A dot on the figure represents each NMR.

Table 1: Definition of behaviors observed in Naked Mole Rats

Behavior	Definition
Yawning	Open mouth gaping for a few seconds while in relaxed state
Shoving	Push objects along the floor of the cage using the muzzle
Pulling objects	Biting and then dragging objects along the floor towards the animal
Patrolling	Walking along the edges of the cage repetitively
Licking	Pass the tongue over own animal's external parts
Huddling	In groups, NMRs lie on the back, side, or abdomen with their eyes closed
Urination	Removal of urine through the anogenital area
Defecation	Expelling solid material through the anogenital area
Feeding	Biting smaller portions of feed with teeth and then swallowing
Passing over	One animal walking over the other
Mouth carrying	Biting, lifting, and then moving objects from one point to another
Teeth grinding	Sliding upper and lower teeth repetitively that produces sound
Vocalization	Making sounds by mouth
Climbing	Front feet step on vertical cage walls and make repetitive movements while hind feet step on the floor of the cage
Scratching	Rub external animal parts using front or hind paws

Body weight and feed consumption

Individual NMRs were weighed weekly. This enabled calculation of weekly average weights per cage, which was the experimental unit. Feed consumption per experimental unit was measured on the same days as fecal sampling for corticosterone analysis. Daily feed consumed was calculated as:

(amount fed - remaining feed after 24 hours)
-22,2% amount fed (moisture loss)

Moisture loss from feed was due to the high room temperatures necessary for NMR comfort.

Fecal corticosterone

Fecal droppings from each experimental unit were collected and pooled for hormone analysis. Pooling allowed enough fecal matter for analysis since NMRs practice coprophagy and void small pellets of feces. Feces in each experimental unit were collected and pooled daily for the first five days and thereafter at three-day intervals. Hence, fifteen samples were collected per experimental unit. Fecal hormone extraction was done according to Altmann et al. (2002).

We validated the corticosterone ELISA kit (EIA-4164, DRG, Germany) prior to use in this study. Samples were processed according to the manufacturer's instructions. Assay results were read using an ELISA reader (ELX800UV, BioTek, Inc-USA) at 450 nm. The assay kit had cross-reactivity to progesterone (7.4%), deoxycorticosterone (3.4%), 11-dehydrocorticosterone (1.6%), cortisol (0.3%), and pregnenolone (0.3%). Thus, the values reported may partially represent these substances.

All samples from the same experiment were measured in the same assay to avoid inter-assay variation, and samples from different test periods were randomized when placed onto ELISA plates. A single experimenter was responsible for acquiring all fecal samples, which were run in duplicate. All samples were diluted 1:16 with methanol to fit within the assay range of the kit used. A linear regression performed on the standard concentration to percent corticosterone demonstrated excellent linearity (R2 ≥0.98%).

Statistical analysis

For each behavior, statistical differences in values between the groups were calculated using the online Kruskal-Wallis test with Dunn's multiple comparison tests calculator (Kruskal Wallis test calculator - with post-hoc Dunn's test multiple comparisons, statskingdom.com) that check for differences between the median values (Figure 3A and 3B). Statistical differences between the groups in feed consumption, cumulative weight gain and fecal corticosterone values (Figures 4 to 6) were calculated using two-way ANOVA and with Bonferroni post hoc tests. The tests were done using Graph Pad Prism 5.0 statistical software. Data were checked for normality. A p-value of less than 0.05 was considered significant.

Results

Behavioral observations

The main behaviors observed are shown in Figures 3A and 3B. Huddling behavior was significantly higher ($p < 0.05$) in the groups housed under 24-hour light compared to the groups housed under 24-hour darkness, i.e. the effect of photoperiodism (Figure 3A). However, no behavior showed significant differences when comparing groups housed in single, double, or four-chambered cages, i.e. the effect of cage design.

Body weight and feed consumption

Housing under different light or darkness regimes only significantly ($p < 0.05$*) affected individual feed consumption between LL and DL groups on the second day, with reduced food consumption in the LL group (Figure 4). Cage design did not have any effect on feed consumption. Average daily and weekly feed consumption was 5.8 and 40.7 grams per NMR, respectively.

Significant differences in cumulative weight gain were noted when comparing individual experimental units on day 28 between LL1 and DL4 ($p < 0.05$), DL2 and DD2 ($p < 0.05$), LL2 and DL4 ($p < 0.01$), DL4 and DD2 ($p < 0.001$). On day 35, significant differences ($p < 0.05$) in weight gain were noted between LL1 and DL4; LL2 and DL4; DL2 and DD2. Additionally, significant differences were noted between DL4 and DD2 ($p < 0.01$), as shown in Figure 5. These findings indicate that weight gain was mostly higher in experimental units housed in either constant light or constant darkness compared to those housed in alternating light/darkness.

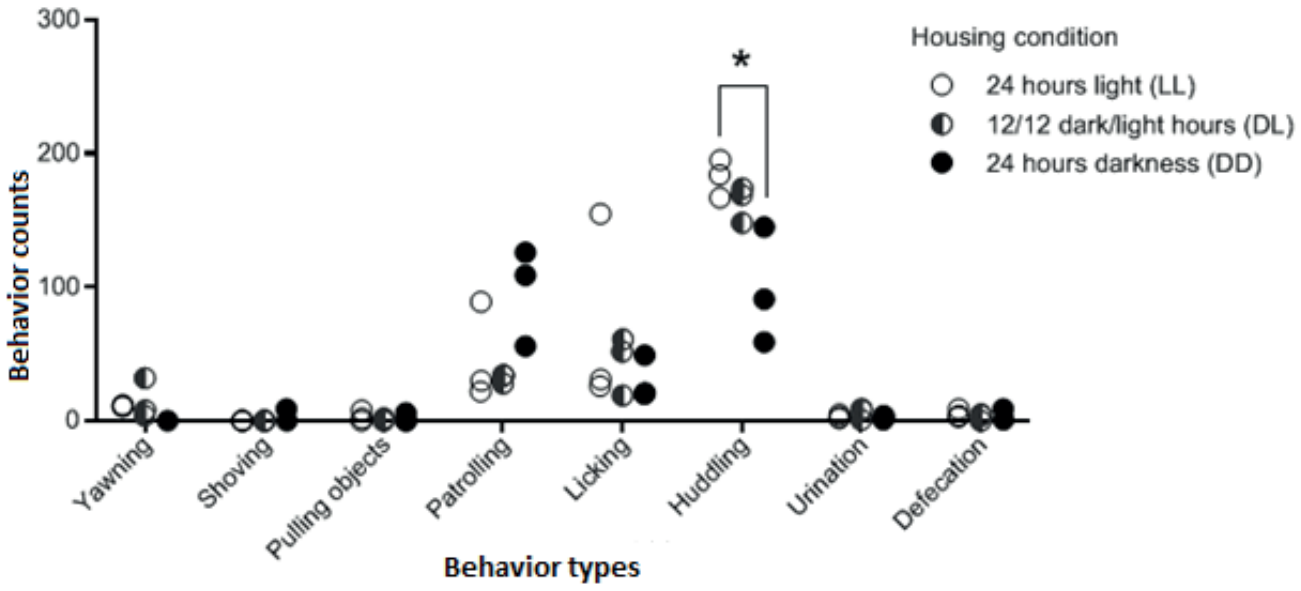

Figure 3 A: Behaviors observed after housing NMRs under different light/dark periods. Each dot on the graph represents the total counts for the behavior per experimental unit during the 35 days experimental period. Huddling behavior was significantly different *($p < 0.05$) between LL and DD photoperiods.

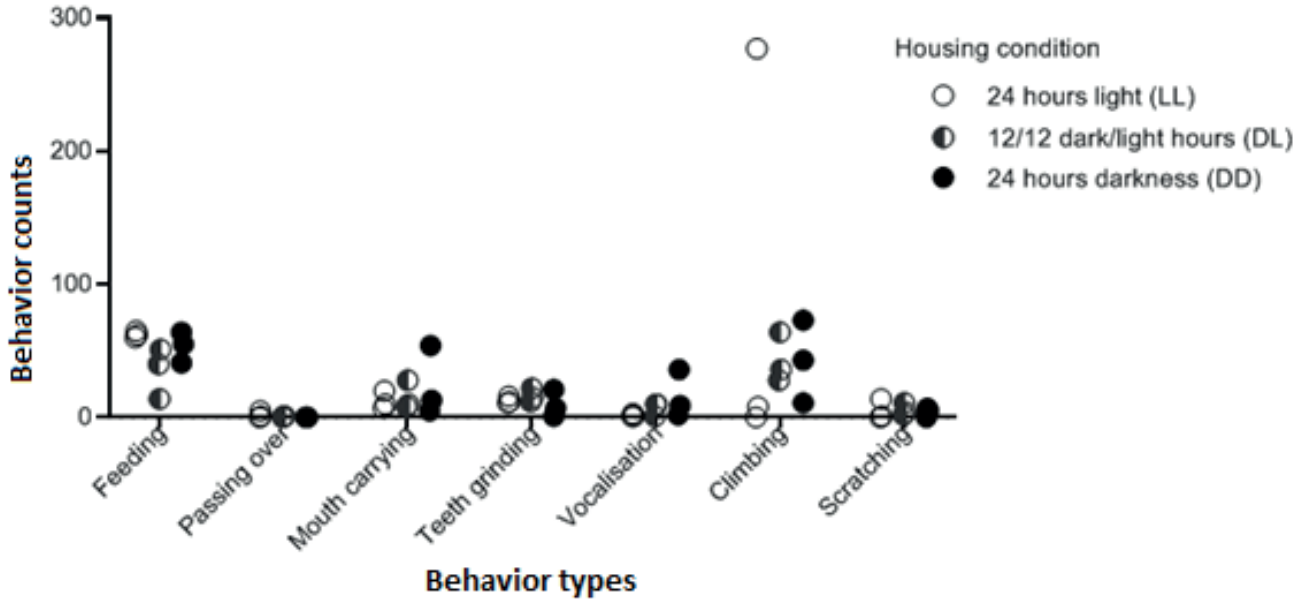

Figure 3 B: Other behaviors observed after housing NMRs under different light/dark periods. No significant differences were noted.

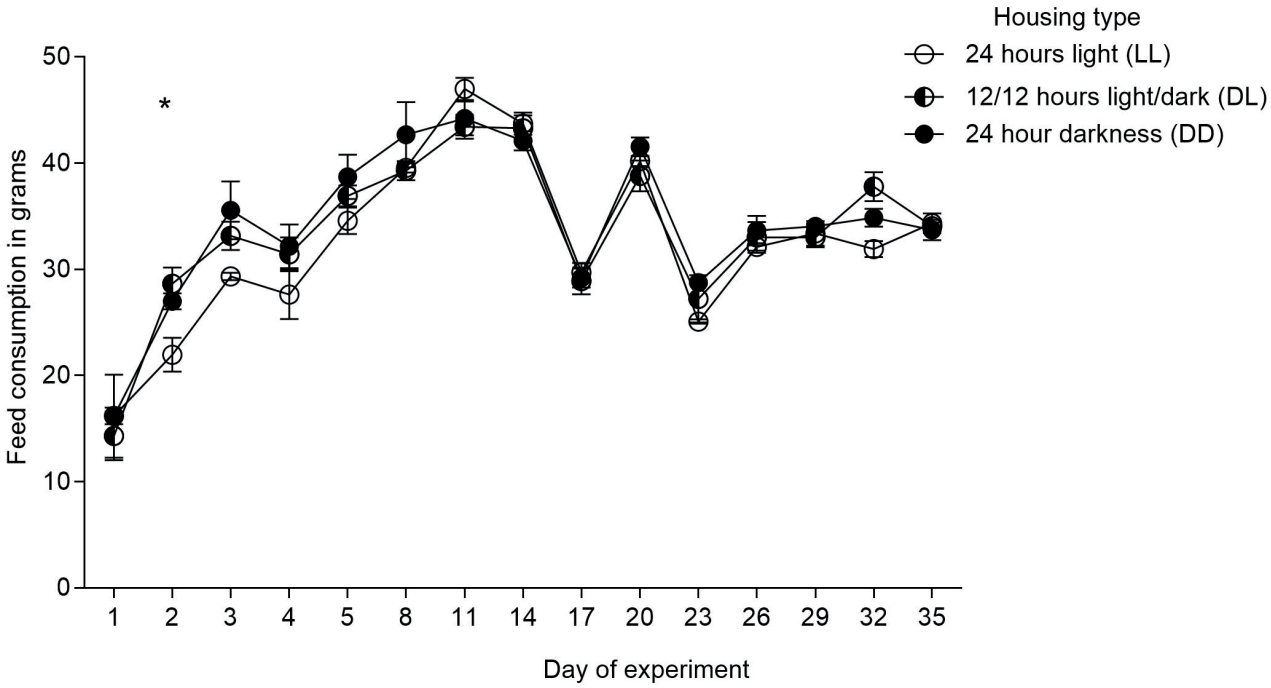

Figure 4: Mean ± SEM of feed consumed by NMRs. Data is presented as averages for the groups for the first five days, followed by three-day averages during the 35-day experimental period. Significant differences ($p < 0.05$) were only observed between LL and DL photoperiod groups on day 2. Please note that the x-axis is not linear and that the time intervals change after Day 8.

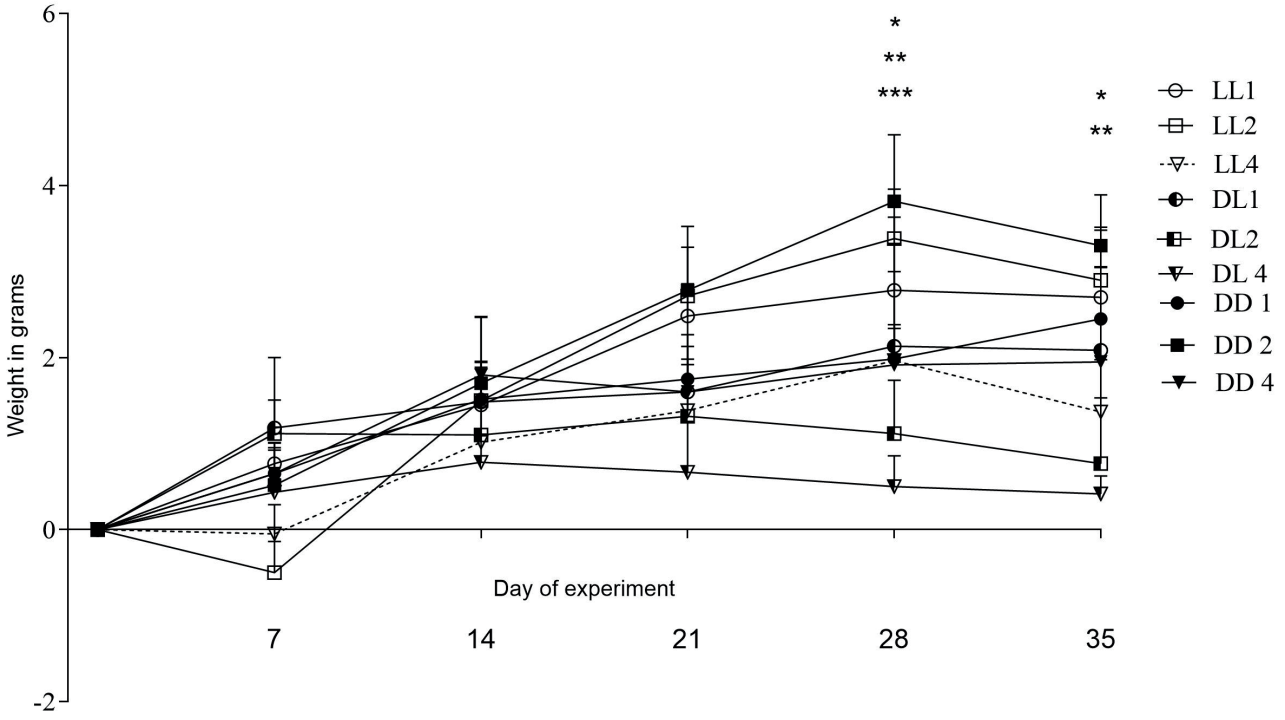

Figure 5: Differences in weekly body weights per experimental unit. Significant differences ($p < 0.05$*, $p < 0.01$**, and $p < 0.001$***) were observed between experimental units on day 28 and 35. LL1, DL1 and DD1 indicate cages with one compartment. LL2, DL2 and DD2 indicate cages with two compartments. LL4, DL4 and DD4 indicate cages with four compartments.

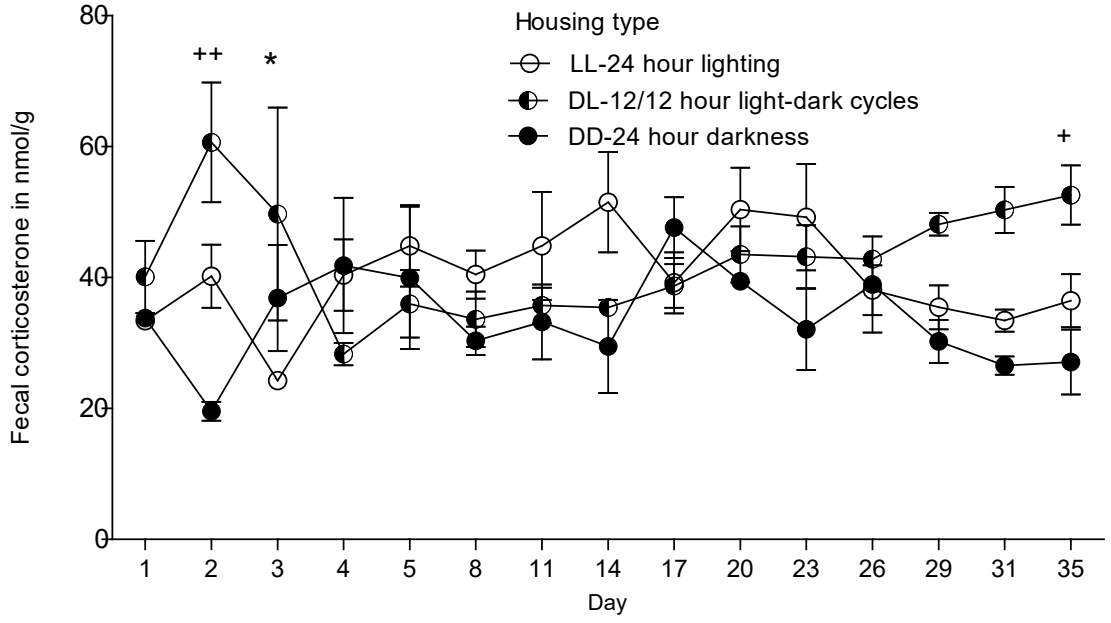

Figure 6: Daily mean ± SEM values of fecal corticosterone for the three groups housed with different photoperiods. Significant differences were noted on day 2 between DL and DD ++ ($p < 0.001$), on day 3 between LL and DL * ($p < 0.05$) and on day 35 between DL and DD + ($p < 0.05$). Please note that the x-axis is not linear and that the time intervals change after Day 8.

Fecal corticosterone

When fecal corticosterone values were compared on a daily basis (Figure 6), significant differences were noted on day two between group DL (60.66 ± 9.2 nmol/g) and DD (19.57 ± 1.5 nmol/g) at $p < 0.001$, on day three values between group LL (24.27.2 ± 1.3 nmol/g) and DL (49.71 ± 1.6 nmol/g) at $p < 0.05$ and on day 35 between group DL (52.60 ± 4.5 nmol/g) and DD (27.10 ± 5 nmol/g) at $p < 0.05$. These results indicate that altering the photoperiod significantly affected fecal corticosterone levels. However, no significant difference was found when fecal corticosterone was checked for the effects of cage design.

Overall, the DD group had significantly lower fecal corticosterone ($p<0.05$) than DL and LL. The means were 33.80 ± 1.64, 42.59 ± 1.77, and 40.15 ± 1.54 nmol/g for DD, DL and LL respectively (Figure 7).

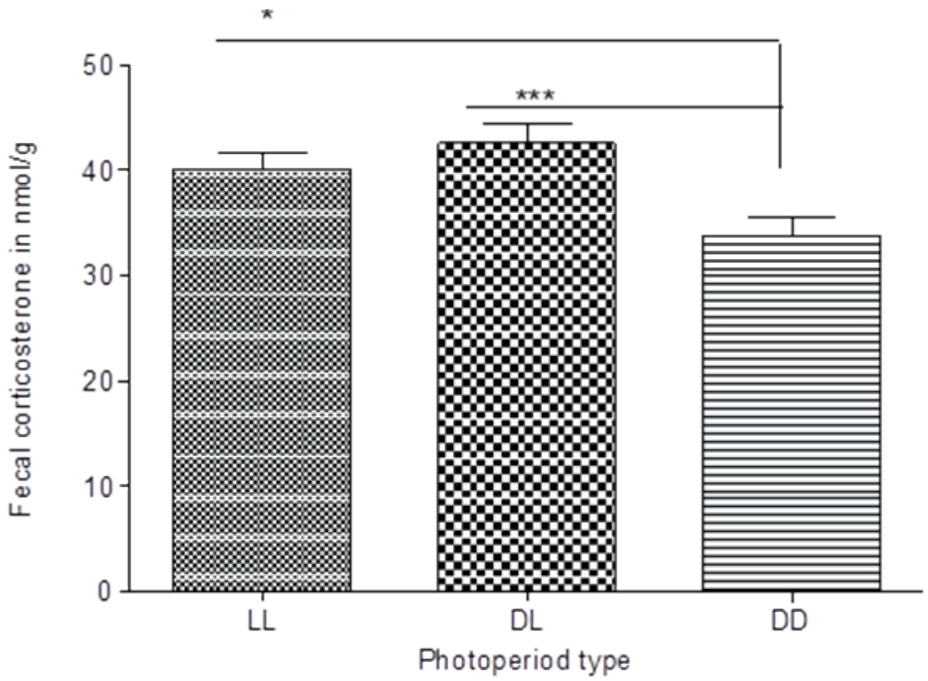

Figure 7: Mean fecal corticosterone values for the entire 35-day study period. DD values were significantly lower ($p<0.05$) than the values for DL and LL.

Discussion and conclusion

Previous NMR studies have shown various behaviors are associated with certain activities (Lacey et al., 2017; Pepper et al., 2017). The associations include licking with grooming, yawning and huddling with resting, defecation and urination with elimination, patrolling, passing over and climbing with locomotion, mouth carrying and pulling objects with transport, and shoving with agonistic behavior. Teeth grinding was associated with communication, although this behavior can be agonistic if accompanied by biting. Grinding behavior also serves to sharpen and maintain teeth. Passing over also implies dominance, where dominant animals pass over the subordinate, especially in areas of the cage where space is limited, e.g. in the tunnels (Sherman, Jarvis and Alexander, 1991; Clarke and Faulkes, 1999; Lacey et al., 2017). Therefore, most of the behaviors observed were non-agonistic. It should be noted that these behaviors are species specific and may not be similar to those of other species. The present study also indicates that housing in light or darkness did not affect most NMR behaviors during the experimental period.

The LL group showed significantly ($p<0.05$) higher huddling than the DD group. Huddling in NMRs is associated with saving energy and water, and also to facilitate thermoregulation (Yahav and Buffenstein, 1991). NMRs being nocturnal, there is a possibility they found constant light uncomfortable for carrying out colony maintenance tasks and rested by huddling. Observations were made only during a particular time of the day for the entire study period

and may not represent what happens at other times, this calls for future studies.

Different photoperiods are reported to influence food intake and growth rate in other rodent species, thereby affecting the physiology of the animals (Yang et al., 2007; Tavolaro et al., 2015). However, in this study, photoperiodism did not affect food intake. The large variations in feed intake around 20 days points to the possibility that an event happened that altered the environment in the entire animal room, hence disrupting feeding behavior in all cages in the room, although no such event was observed by the experimenter.

Housing in either constant light or constant darkness positively affected weight compared to housing in alternating light/darkness. This may indicate that NMRs easily adapt to one type of light compared to alternating light periods, which is reported to affect the adrenal clock (Otsuka et al., 2012; Fisk et al., 2018).

The present study did not quantify hormone levels in feces at various times of the day within a 24-hour period. Instead, NMRs were exposed to constant light, darkness or 12 hour alternating light and darkness for the entire study period of 35 days, with feces collected once a day. The observation that fecal corticosterone significantly differed between the experimental groups shows that photoperiod influenced fecal corticosterone levels in NMRs. The exact mechanisms to explain the effect were not investigated. However, it is reported that NMRs entrain to light (Riccio and Goldman, 2000b, 2000a), which could explain the observed effect. It is reported that NMRs also use feeding time as zeitgeber for circadian rhythmicity (Ghosh et al., 2021).

The use of ELISA kits to measure fecal corticosterone in animals is an established method that has been successfully reported in mice and rats, although the measured values are not absolute and may vary between different types of kit because of their differing sensitivity. Nevertheless, ELISA kits are very useful since they allow measurements of relative values of these hormones in animals. The level of fecal corticosterone is considered proportional to the level of stress in animals (Abelson et al., 2016).

The finding that NMRs housed under complete darkness had less fecal corticosterone on some days indicates that housing NMRs in light or varying dark: light environments may cause stress in the animals. We recommend that NMRs should therefore be housed under dark conditions. Light exposure has been reported to influence animal hormonal responses (Fisk et al., 2018). In rats, exposure to light can induce stress and increase corticosteroids like corticosterone and cortisol (Mohawk, Pargament and Lee, 2007). Corticosterone levels in mice have been reported to vary under different light-darkness cycles, which subsequently affect the animal's physiology (Yang et al., 2007). This occurs because by varying light-darkness periods, the adrenal clock is affected (Otsuka et al., 2012; Fisk et al., 2018). Corticosterone undergoes metabolism in the digestive tract and, therefore, the corticosterone values presented here represent corticosterone metabolites.

In conclusion, the findings in this study demonstrate that NMRs should be housed under one type of photoperiod, preferably constant darkness where it was observed that NMRs show less stress and positive weight gain. Caging design did not affect behavior, feed consumption, weight gain or fecal corticosterone levels. The lack of effect of caging design in the present study should not be seen as a reason for not providing the animals with a complex cage design when considering animal housing. More studies are needed to elucidate the possible effect of caging system on the animals' physiology and welfare.

Limitations

Although the design of this study allowed cross investigation for the effects of different photoperiods and cage systems, comparison between photoperiods without any possible influence of caging system, and vice versa, was limited due to few experimental units. Hence, the factors investigated in this study need to be investigated in more detail in the future.

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We wish to thank Gilbert Mwanthi for caring for the NMRs in the laboratory and Hesbon Odongo for guidance on hormone ELISA assays.

Conflict of interest

The authors declared no potential conflict of interest.

Author contributions

RM conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis

tools or data; wrote the paper. TK conceived and designed the experiments; contributed reagents, materials, analysis tools or data, reviewed and approved the paper. KA conceived and designed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools, reviewed and approved the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Most of the data are presented in this paper.

Ethical considerations

During the entire study, we adhered to the principles of 3Rs to the greatest extent possible. While it was not possible to replace the use of animals, the sample size was selected to ensure the number of animals used was justifiable. Regarding refinement, several attempts were made to minimize any pain and suffering to the animals. Additionally, during capture, transport, acclimatization and experimental periods, the NMRs were handled carefully by skilled personnel to avoid incidences that could evoke fear, anxiety and stress. See also the Ethical Statement in the Materials and Methods section.

Consent for publication

Not applicable.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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