

1 **Title Page**

2 **Title: The role of ambient temperature and light as cues in the control of**
3 **circadian rhythms of Damaraland mole-rat**

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15 **Keywords:** entrainment, masking, locomotor activity, subterranean, mole-rat

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28 **Abstract**

29 Light is considered the primary entrainer for mammalian biological rhythms, including
30 locomotor activity (LA). However, mammals experience different environmental and light
31 conditions, which include those predominantly devoid of light stimuli, such as those
32 experienced in subterranean environments. In this study, we investigated what
33 environmental cue (light or ambient temperature (T_a)) is the strongest entrainer of
34 circadian rhythms, by using LA as a proxy, in mammals that experience a lifestyle devoid
35 of light stimuli. To address this question, this study exposed a subterranean African mole-
36 rat species, the Damaraland mole-rat (*Fukomys damarensis*), to six light and T_a cycles in
37 different combinations. Contrary to previous literature, when provided with a reliable light
38 cue, Damaraland mole rats exhibited nocturnal, diurnal, or arrhythmic LA patterns under
39 constant T_a . While under constant darkness and a 24-hour T_a cycle mimicking the burrow
40 environment, all mole-rats were most active during the coolest 12-hour period. This
41 finding suggests that in a subterranean environment, which receives no reliable photic cue,
42 the limited heat dissipation and energy constraints during the digging activity experienced
43 by Damaraland mole-rats make T_a a reliable and consistent "time-keeping" variable. More
44 so, when providing a reliable light cue (12 light: 12 dark) to Damaraland mole-rats under
45 a 24-hour T_a cycle, this study presents the first evidence that cycles of T_a affect the LA
46 rhythm of a subterranean mammal more strongly than cycles of light and darkness. Once
47 again, Damaraland mole-rats were more active during the coolest 12-hour period
48 regardless of whether this fell during the light or dark phase. However, conclusive
49 differentiation of entrainment to T_a from that of masking was not achieved in this study,
50 and as such, we have recommended future research avenues to do so.

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57 **Introduction**

58 Endogenous biological rhythms of approximately 24 hours, also known as circadian
59 rhythms, are produced by the suprachiasmatic nucleus (SCN), a group of ten thousand or
60 so cells at the base of the hypothalamus. However, circadian rhythms must be
61 synchronised to external cues, such as changes in light or ambient temperature (T_a).
62 Environmental cues, also known as entrainers or *zeitgebers*, are signals that are linked to the
63 24-hour day-night cycle imposed by the rotation of the Earth (Bhadra, Thakkar et al.,
64 2017; Paganelli, Petrarca et al., 2018). These cues help to synchronise the endogenous
65 circadian clocks and make them oscillate with a period (τ) of 24 hours (Bhadra, Thakkar
66 et al., 2017; Paganelli, Petrarca et al., 2018). Without such *zeitgebers*, these endogenous
67 circadian clocks would begin to free-run and thus desynchronise with the environment,
68 reducing an organism's fitness in its environment (Gander, Mulrine et al., 2016). The
69 entrainment of this endogenous circadian rhythmicity has arisen to provide intrinsic
70 fitness advantages, such as maintaining rhythmicity of internal biological processes such
71 as metabolism and core body temperatures (Refinetti & Menaker, 1992; Serin & Acar Tek,
72 2019), and extrinsic fitness advantages, such as predator avoidance or food availability
73 (Fraker, 2008; Refinetti, 2015). One crucial biological rhythm that is ubiquitous amongst
74 animals is that of locomotor activity (LA).

75
76 Light (photic cues) is arguably the most influential *zeitgeber* for the entrainment of circadian
77 rhythms (Refinetti, 2015). Mammalian photoentrainment of the SCN and their internal
78 biological processes and behaviours, such as LA, has been exhaustively studied (See
79 Peirson, Thompson et al. (2005) for review). However, mammals inhabit all environments
80 on Earth, including those devoid of photic stimuli, such as caves and underground burrow
81 systems (reviewed by Abhilash, Shindey et al. (2017)). Nevertheless, these mammalian
82 species still possess a functional circadian clock often entrained to a τ of 24 hours
83 (Abhilash, Shindey et al., 2017). However, the *zeitgebers* needed to entrain mammalian
84 species inhabiting environments absent of reliable photic cues are still relatively unknown
85 (Beale, Whitmore et al., 2016; Abhilash, Shindey et al., 2017). Recent studies have

86 highlighted the importance of T_a as a powerful *zeitgeber* of circadian rhythms in mammals
87 that are not provided with regular photic cues (Hart, van Jaarsveld et al., 2021; Finn, Janse
88 van Vuuren et al., 2022). However, the question arises; which *zeitgeber* (light or T_a) is the
89 strongest entrainer of circadian rhythms in mammals that inhabit environments
90 exclusively devoid of photic cues?

91
92 African mole-rats (family Bathyergidae), have been the ideal model mammal family for
93 exploring the circadian rhythms and their *zeitgebers* of mammals exposed to non-photoc
94 environments (See Oosthuizen and Bennett (2022) for review). African mole-rats are a
95 group of exclusively subterranean rodents endemic to the African continent (Bennett &
96 Faulkes, 2000). The subterranean niche provides mole-rats with two main advantages,
97 these being shelter from above-ground predators and muted temperature ranges (Bennett
98 & Faulkes, 2000). However, the lack of sensory cues and the disadvantages associated with
99 high relative humidity, poor ventilation, low oxygen and high carbon dioxide partial
100 pressures make it simultaneously a challenging environment (Bennett & Faulkes, 2000;
101 Ivy, Sprenger et al., 2020; Hart, Bennett et al., 2023).

102
103 Several studies have investigated the circadian rhythms of African mole-rats (See
104 Oosthuizen and Bennett (2022) for review). Most studies have investigated the effect of
105 light on LA patterns and shown evidence of circadian rhythmicity and entrainment by
106 light in African mole-rats. These studies have revealed that African mole-rats possess
107 endogenous rhythms of LA, which are capable of entraining to light and allude to the
108 presence of a functional circadian system (See Oosthuizen and Bennett (2022) for review).
109 Interestingly, under laboratory-based studies in particular, the most common LA pattern
110 observed in African mole-rats is nocturnality (Oosthuizen & Bennett, 2022). However,
111 these studies have also revealed that the degree of LA rhythmicity and its ability to entrain
112 to light varies between species and within species (Oosthuizen, Cooper et al., 2003), with
113 sex (Haupt, Bennett et al., 2017) and reproductive status (Oosthuizen & Bennett, 2015).
114 Previous studies have also concluded that circadian rhythms in African mole-rats are

115 affected by T_a (Oster, Avivi et al., 2002; Lövy, Šklíba et al., 2013; Šklíba, Lövy et al., 2014;
116 van Jaarsveld, Bennett et al., 2019; Hart, van Jaarsveld et al., 2021; Finn, Janse van Vuuren
117 et al., 2022). This leads to the possibility that without reliable photic cues, African mole-
118 rats may rely on the daily rhythm of soil temperature cycles throughout their burrow
119 systems, as although the subterranean niche is considered to express a muted temperature
120 range, a temperature range still exists (Roper, Bennett et al., 2001). Soil temperature cycles,
121 even though muted, have been predicted to have a disproportional effect on African mole-
122 rat behaviour and physiology mainly due to their increased sensitivity to changes in T_a ,
123 due to their reduce heat dissipating abilities (Wallace, van Jaarsveld et al., 2021). Recently,
124 the strength of T_a as a *zeitgeber* of LA circadian rhythms depends on the sensitivity of a
125 species to daily T_a changes (El Allali, Achaâban et al., 2013; Farsi, Achaâban et al., 2020;
126 Farsi, Harti et al., 2020; Hart, van Jaarsveld et al., 2021; Bennett, Hart et al., 2024).

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128 The current understanding is that photic cues (light) are the strongest *zeitgeber* of circadian
129 rhythms of African mole-rats. However, without photic cues, T_a becomes the primary
130 *zeitgeber* of circadian rhythms of African mole-rats, particularly LA patterns (Oosthuizen
131 & Bennett, 2022). To date, no study has challenged any African mole-rat species with both
132 light and T_a in antagonistic roles to one another to investigate which is the strongest
133 *zeitgeber* of circadian rhythms of African mole-rat species. As such, this study attempted to
134 address this simple but crucial question. The Damaraland mole-rat (*Fukomys damarensis*)
135 was selected as the ideal model to address this dearth of knowledge as recent findings have
136 shown that this species possesses a functional circadian clock, which can be entrained to
137 photic cues and is affected by T_a (Oosthuizen & Bennett, 2015). Yet, no study has
138 confirmed whether Damaraland mole-rats entrain their LA circadian rhythms to T_a nor
139 has any study tested whether light or T_a is the strongest *zeitgeber*.

140

141 Therefore, this study had three primary aims: First, to confirm if the Damaraland mole-
142 rat is nocturnal under constant T_a in a laboratory environment, as described by Oosthuizen
143 and Bennett (2015). Secondly, to observe whether Damaraland mole-rats can entrain their

144 LA circadian rhythms to T_a in the absence of light. Lastly, to test which is the strongest
145 *zeitgeber*, light or T_a .

146

147 **Materials and methods**

148 *Study species and animal housing*

149 This study used ten Damaraland mole-rats (four males and six females) from 10 separate
150 colonies. All animals used in this study were from the mole-rat captive breeding stock at
151 the University of Pretoria. The animals used in this study were of similar age (4-5 years
152 old), with a body mass range 104-198g (mean: 149.4 ± 8.54 g) with males (mean:
153 158.0 ± 15.1 g) and females (mean: 143.7 ± 10.5 g) possessing a similar body mass ($t = 0.81$,
154 $p = 0.44$). The mole-rats were separated from their natal colony in order for them to
155 familiarise to a solitary lifestyle for at least one year prior to the start of the experiment
156 (Hart, van Jaarsveld et al., 2021). As such, all animals would be free from colony imposed
157 reproductive suppression and would be classed as disperser morphs (Hazell, Bennett et
158 al., 2000), yet females would likely not ovulate regularly due to a lack of access to an
159 unrelated male (Snyman, Jackson et al., 2006; Voigt, Medger et al., 2021; Wallace, Hart et
160 al., 2023). All Damaraland mole-rats were individually housed in large, black plastic
161 containers ($83 \times 44 \times 37$ cm), with nesting material in the form of wood shavings and
162 paper towelling. An *ad libitum* diet of assorted vegetables, consisting mainly of sweet
163 potatoes, was provided (see Bennett and Jarvis (1995)), which provided the animals with
164 their nutritional and water requirements, with the coefficient of digestibility being 90%
165 (Bennett & Jarvis, 1995). Feeding times differed daily to avoid entraining the animals to a
166 fixed feeding time (Golombek & Rosenstein, 2010). Food was replaced daily to avoid
167 increased activity due to hunger-related foraging (Vasicek, Oosthuizen et al., 2005). Plastic
168 containers were cleaned between experimental cycles, with a dim red light ($<1 \text{ lux}$) being
169 used during both cleaning and feeding times under complete darkness (DD) conditions.
170 Dim red light was used as African mole-rats have been observed not to be able to discern
171 between dim red light and DD conditions (Kott, Sumbera et al., 2010) and as such dim
172 red light would unlikely affect our study animals' circadian rhythms. Similar housing

173 conditions have been used in the past while investigating African mole-rat chronobiology
174 (Oosthuizen, Cooper et al., 2003; Oosthuizen & Bennett, 2015; Haupt, Bennett et al.,
175 2017; van Jaarsveld, Bennett et al., 2019; Hart, van Jaarsveld et al., 2021). All Damaraland
176 mole-rats were housed in climate-controlled rooms at the Small Animal Physiological
177 Research Facility at the University of Pretoria. The climate rooms allowed for the
178 duplication of sinusoidal patterns in T_a . At the facility, animals were initially kept in rooms
179 at a constant T_a of 25°C and humidity ($\pm 40\%$), under a square wave light and dark (LD)
180 lighting regime of 10 hours light (400 *lux*) and 14 hours dark (0 *lux*) (10L:14D – Light
181 commencing at 06:00h and being switched to dark at 16:00h). The experimental
182 procedures were approved by the Animal Ethics Committee of the University of Pretoria
183 (EC038-18).

184

185 *Experimental protocols*

186 All Damaraland mole-rats were subjected to six different cycles in sequence (Figure 1),
187 where each respective cycle ranged from 17 to 32 days (see Table 1). *Cycle 1* consisted of
188 a 10L:14D lighting regime (identical to regular housing conditions) at a constant T_a of
189 25°C. In order to determine whether the circadian rhythm of LA of the Damaraland mole-
190 rat could entrain T_a cycles without light, all animals were subjected to DD with simulated
191 T_a cycles (Figure 1). *Cycle 2* mimicked the natural range of underground burrow T_a (18-
192 28°C) that these animals would experience (Roper, Bennett et al., 2001) (Figure 1 – nadir
193 at 03:00h). In *Cycle 3*, mole-rats were subjected to constant conditions (DD, $T_a = 25^\circ\text{C}$)
194 (Figure 1). *Cycle 3* differentiated whether any observed rhythms were due to masking or
195 entrainment (Figure 1). The T_a cycle was then moved forward by six hours (nadir at
196 09:00h) in *Cycle 4* to examine whether rhythms would re-entrain to the temperature
197 stimulus (Figure 1). To determine which *zeitgeber* (light or T_a) was the most dominant
198 entrainer of Damaraland mole-rats LA circadian rhythm, the T_a cycle of *Cycle 5* (nadir at
199 09:00h) remained the same as *Cycle 4*. However, during the hottest 12 hours (24-28°C),
200 lights were turned on (on: 14:00h and off: 02:00h); while during the coldest 12 hours (18-
201 23°C), lights were turned off (Figure 1). For *Cycle 6*, the T_a cycle was inverted (12-hour

202 advance – nadir at 21:00 h), resulting in the hottest 12 hours in the dark while the coldest
203 12 hours in the light (Figure 1).

204
205 **Table 1. Number of days spent in each experimental cycle.**

Cycle	Number of days
<i>Cycle 1</i>	17
<i>Cycle 2</i>	27
<i>Cycle 3</i>	29
<i>Cycle 4</i>	32
<i>Cycle 5</i>	23
<i>Cycle 6</i>	29

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207

208 *Recording of locomotor activity*

209 The LA of each Damaraland mole-rat was detected using an infrared motion detector
210 (Quest PIR internal passive infrared detector; Elite Security Products [ESP], Electronic
211 Lines, UK). The motion detector placed over each container was such that all LA on the
212 floor space of the container was detectable. The recorded LA readings were collected
213 every minute and subsequently relayed to a nearby computer employing the programme
214 VitalView (VitalView™, Mini Mitter Co., Inc., Sunriver, OR, USA; www.minimitter.com).
215 Activity measurements were analysed and depicted as double-plotted actograms using
216 ActiView Biological Rhythm Analyses 1.2 software (Minimitter Co., Inc., Sunriver, OR,
217 USA).

218

219 *Statistical analyses*

220 Actograms and *tau* (τ) of LA were calculated using ClockLab™ (Actimetrics, Evanston, Il.
221 USA) and ActiView™ Biological Rhythm Analysis 1.2 (Mini Mitter Co., Inc., Sunriver,
222 OR, USA; www.minimitter.com). All statistical analyses were performed in R version 4.1.3
223 (R Core Team, 2023), and statistical significance was assumed at $p \leq 0.05$.

224

225 The proportion of activity per hour was calculated using Equation 1 (Hart, van Jaarsveld
226 et al., 2021) and presented as a percentage. Prior analysis an arcsine transformation on the
227 proportion of activity per hour was completed. To assess whether the proportion of
228 activity within the cold phase (12-hours) (18-23°C) varied between *Cycle 2, 4, 5* and *6*, a
229 generalised linear mixed-effects model (GLMM) was conducted with cycle, sex and body
230 mass as predictor variables.

231

232 Equation1:

233

$$\text{Hourly activity (\%)} = \frac{\sum_{i=1}^{24} \text{Counts}}{\sum \text{Counts}}$$

234

235

236 Furthermore, total activity counts per day (TACs) were analysed between and within each
237 cycle. Firstly, a GLMM was conducted with cycle (*Cycle 1, 2, 4, 5* and *6*), sex, their two-
238 way interaction (sex*cycle) as predictor variables and TAC as the response variable. Body
239 mass was included as a covariant in this model. Additionally, a separate GLMM was
240 conducted to investigate the effect of light cycle (light vs dark), sex and their two-way
241 interaction on TAC in *Cycle 1*. Body mass was again added as a covariant. Furthermore,
242 separate GLMMs were conducted for *Cycle 2, 4, 5*, and *6* with sex, body mass and T_a as
243 predictors and TAC as the response variable.

244

245 All GLMMs were fitted with a gamma log-link distribution using the *lme4* package in R
246 (Bates, Machler et al., 2014). Furthermore, individual ID was included as a random factor
247 to account for pseudoreplication. Post-hoc comparisons were made using Tukey's
248 Honestly Significant Difference (HSD) tests using the *emmeans* package (Lenth, Singmann
249 et al., 2018).

250

251

252 **Results**

253 Under constant T_a and a 10L:14D lighting regime (*Cycle 1*), Damaraland mole-rats showed
254 nocturnal, diurnal and arrhythmic activity profiles, where only ~46% of activity was
255 confined to the dark phase of the LD cycle (Figure 2). Of the 10 Damaraland mole-rats
256 under observation, four females possessed a nocturnal activity profile (Figure 2a), two
257 males and two females possessed a diurnal activity profile (Figure 2b), whereas the
258 remaining two males possessed no discernable activity pattern [arrhythmic] (Figure 2c).

259

260 In the presence of a natural T_a ramp (*Cycle 2*) under constant DD, LA became concentrated
261 in the cold phase (12-hours) of the cycle (Figures 3 and 4a), displaying ~61% of their
262 activity during this period (Figure 5). Eight of the 10 Damaraland mole-rats displayed a
263 free-running rhythm under *Cycle 3* (constant DD conditions ($T_a=25^\circ\text{C}$)), with an average
264 τ of 24.0 ± 0.12 hours (Figure 4b). During *Cycle 4*, *Cycle 2* T_a cycle which was advanced by
265 six hours, Damaraland mole-rats displayed a phase advance by approximately six hours,
266 and LA again was concentrated in the cold phase (12-hours) of the cycle (Figure 3 and 4c),
267 with the percentage of activity expressed during this time being ~65% (Figure 5).

268

269 Upon the completion of *Cycle 4*, lights were turned on during the hot phase (12-hours) of
270 the T_a cycle (*Cycle 5*); as with *Cycle 4*, LA remained concentrated in the cold phase (12-
271 hours) of the cycle (Figures 6a and 7a), displaying ~66% of their activity during this period
272 (Figure 5). Upon inverting the T_a cycle (12-hour advance), seeing the cold phase (12-hours)
273 of the cycle falling into the light period of the lighting regime, all Damaraland mole-rats
274 again displayed the majority (~70%) of their activity during this period (Figures 5, 6b and
275 7b).

276

277 The cycle was observed not to significantly affect the proportion of hourly activity during
278 the cold phase (12-hours) (Figure 5, $t = 1.90$, $p = 0.06$). Furthermore, no effect of sex ($t =$
279 0.61 , $p = 0.54$) or body mass ($\chi = -0.49$, $p = 0.62$) was reported.

280

281 TAC for *Cycle 1, 2, 4, 5* and *6* was unaffected by body mass ($t = 0.31, p = 0.76$), sex ($t = -$
282 $0.03, p = 0.98$), cycle ($t = -0.01, p = 0.81$) and sex*cycle ($t = -0.72, p = 0.47$). Similarly,
283 TAC for *Cycle 1* was unaffected by sex ($t = -0.91, p = 0.36$), light cycle ($t = 1.77, p = 0.08$)
284 and the two-way interaction of light cycle and sex ($t = 0.08, p = 0.93$). However, heavier
285 individuals were found to have lower TACs compared to lighter individuals ($t = -0.27, p$
286 $= 0.02$) in *Cycle 1*. TAC for *Cycle 2, 4, 5* and *6* were unaffected by sex ($t \leq 0.70, p \geq 0.13,$
287 for all) and body mass ($t \leq 1.69, p \geq 0.09,$ for all). While, T_a did significantly affect TAC
288 in *Cycle 2* ($t = -8.26, p < 0.0001$), *4* ($t = -3.86, p = 0.0002$), *5* ($t = -3.43, p = 0.001$) and *6*
289 ($t = -13.1, p < 0.0001$). In each cycle as T_a increased TAC decreased (Figure 8).

290

291 **Discussion**

292 This study aimed to address three fundamental questions concerning the chronobiology
293 of Damaraland mole-rats, an exclusively subterranean mammal that resides in burrow
294 systems without regular exposure to light. Namely, are Damaraland mole-rats nocturnal
295 under constant T_a in a laboratory environment, as described by Oosthuizen and Bennett
296 (2015)? Can the Damaraland mole-rat entrain their LA circadian rhythms to T_a in the
297 absence of light? And finally, which is the strongest entrainer of Damaraland mole-rats
298 LA circadian rhythms, is it light or T_a ?

299

300 Oosthuizen and Bennett (2015) observed that under laboratory conditions (at constant
301 T_a s of 20°C , 25°C and 30°C), Damaraland mole-rats entrain their LA circadian rhythms
302 primarily to light, where most of the activity occurred during the dark phase of the cycle.
303 This was unsurprising, as Němec, Cveková et al. (2008) showed that although the visual
304 system of many members of the family Bathyergidae is severely regressed, the subsystem
305 involved in photoreception is well-developed and comparable to those exhibited by
306 surface-dwelling rodents. This current study challenges the findings of Oosthuizen and
307 Bennett (2015), as Damaraland mole-rats displayed circadian patterns with both diurnal
308 and nocturnal behaviours, as well as arrhythmic patterns. A possible cause for the
309 discrepancy between the two studies may be the use of both males and females in the

310 current study, whereas only females were used in the study by Oosthuizen and Bennett
311 (2015). In the current study, most females were solely nocturnal (four out of the six
312 females), while the remaining two females showed more diurnal activity patterns. No
313 males showed a nocturnal LA pattern and exhibited only diurnal or arrhythmic LA
314 patterns. Similar patterns of increased activity at night in females have been observed in
315 the social-living highveld mole-rat (*Cryptomys hottentotus pretoriae*) (Haupt, Bennett et al.,
316 2017). Contrasting patterns were found in the social-living Mahali mole-rat (*C. b. mahali*),
317 with males being more active during the dark phase (van Jaarsveld, Bennett et al., 2019).
318 Social species of African mole-rats, such as the Damaraland mole-rat, exhibit an increased
319 level of intra-individual and intra-sexual variation in their LA patterns (Riccio & Goldman,
320 2000; Oosthuizen, Cooper et al., 2003). This variation may be necessary to desynchronise
321 activity patterns within colonies, helping in effective foraging, maintaining territories, and
322 protecting colonies against predators throughout the 24 hour day (Šklíba, Lövy et al.,
323 2014). Oosthuizen and Bennett (2015) classified Damaraland mole-rats as primarily
324 nocturnal, but other factors such as sex, body mass, social class and T_a are likely to
325 influence their activity patterns more than light, leading to a spectrum of circadian
326 behaviours. However, larger sample sizes of each sex are required to fully investigate the
327 intra- and inter-sexual variation in the LA patterns of Damaraland mole-rats seen in this
328 study.

329
330 Damaraland mole-rat LA patterns are affected by T_a (Oosthuizen & Bennett, 2015), but
331 whether T_a can entrain LA patterns under constant dark conditions has never been directly
332 tested. Oosthuizen and Bennett (2015) suggested that LA was preferred at a T_a of 25°C,
333 followed by 20°C, and least preferred at 30°C. This finding contrasts with those findings
334 for the Mahali mole-rat (Hart, van Jaarsveld et al., 2021), the highveld mole-rat (Haupt et
335 al. 2017;) and the social Natal mole-rat (*C. b. natalensis*) (Finn, Janse van Vuuren et al.,
336 2022). This is also true for the solitary-living Cape mole-rat (*Georycus capensis*) (Hart, van
337 Jaarsveld et al., 2021) and the Silvery mole-rat (*Heliophobius argenteocinereus*) (Lövy, Šklíba et
338 al., 2013) that all saw increased activity during the coldest times of the 24 hour cycle

339 (<22°C). In contrast to Oosthuizen and Bennett (2015), the present study found that
340 Damaraland mole-rats, similar to other African mole-rat species, prefer to increase their
341 LA during the cooler period (18-23°C) of the 24 hour T_a cycle rather than the warmer
342 period (23-28°C), when under constant dark conditions in the presence of a 24 hour T_a
343 cycle.

344

345 Both male and female Damaraland mole-rats of this study were most active at T_a s below
346 their thermoneutral zone (27 - 31°C (Šumbera, 2019)), similar to the Mahali and Cape
347 mole-rat (Hart, van Jaarsveld et al., 2021). Hart, van Jaarsveld et al. (2021) have suggested
348 that African mole-rats tolerate the energetic demands of increased activity below their
349 thermoneutral zone, thereby avoiding exercise-induced hyperthermia associated with the
350 metabolic heat generated by burrowing through metabolic exertion (McNab, 1974;
351 Speakman & Król, 2010; Jacobs, Bennett et al., 2020; 2020; 2022). A likely cause of this
352 increased susceptibility to exercise-induced hyperthermia is the poor evaporative cooling
353 capabilities present in African mole-rats due to physiological (Luna, Šumbera et al., 2020;
354 Wallace, van Jaarsveld et al., 2021) and environmental (burrow) constraints (Roper,
355 Bennett et al., 2001; Hart, Bennett et al., 2022). Yet, it could be argued that as described
356 by Speakman and Król (2010), the Heat Dissipation Limit theory posits that the energetics
357 of an African mole-rat could be increased through enhanced heat dissipation capacity, the
358 increased activity during the cooler periods may potentially allow for increased energetic
359 output during LA; an important factor to take into account as foraging underground is
360 approximately 360–3400 times more energetically expensive than above-ground
361 movement (Vleck, 1979). As such, in a subterranean environment, which receives no
362 reliable photic cue, the limited heat dissipation and energy constraints during the digging
363 activity experienced by Damaraland mole-rats make T_a a more reliable and consistent
364 "time-keeping" variable.

365

366 This study indicates Damaraland mole-rats are able to synchronise their LA to T_a .
367 However, it is important to note that the current experimental design and presented results

368 prevent conclusive differentiation of entrainment to T_a from that of masking. One of the
369 main determinants that allow us to identify if an animal entrained its LA circadian rhythms
370 to an environmental factor (such as T_a) is the observation of animals when exposed to
371 constant conditions (i.e. *Cycle 3*), they would free-run from the same time point as those
372 in the previous entrained rhythm (i.e. *Cycle 2*) and display a drift (Daan & Aschoff, 2001;
373 Refinetti, 2015). While, when exposed to a phase advance in the proposed environmental
374 factor (i.e. *Cycle 4*) after free-running conditions, it would result in a progressive shift in
375 LA patterns until a new LA pattern is achieved (Daan & Aschoff, 2001; Refinetti, 2015).
376 This would suggest entrainment rather than a masking event. Such strict requirements
377 were not met in this study as the needed progressive shift (Figure 4) was not as marked as
378 those of other studied African mole-rats (Oosthuizen, Cooper et al., 2003; Oosthuizen &
379 Bennett, 2015, Haupt, Bennett et al., 2017, van Jaarsveld, Bennett et al., 2019; Hart, van
380 Jaarsveld et al., 2021) and may be due to confounding biological factors uncontrolled for
381 in this study, but should be considered in future studies. These may include humidity,
382 which is tightly linked to T_a ; humidity has been found to be related to sleep onset and
383 offset times in mammals (Gravett, Bhagwandin et al., 2017). This study did not control
384 for humidity as it was impossible to achieve levels similar to those experienced by mole
385 rats in the wild (Holtze, Braude et al., 2018). Furthermore, the form of activity measured
386 in this study would only form part of the behavioural profile of mole-rats in the wild, as a
387 large portion of their activity in the wild would comprise of digging; excluding such
388 behaviour may be a reason that a less pronounced drift is seen in this study, and as such
389 these factors should be controlled for in future studies. Thus, we cannot make an inference
390 about the role of T_a as a *zeitgeber* of LA at this time.

391
392 When providing a reliable photic cue to Damaraland mole-rats under a 24-hour variable
393 T_a cycle, this study presents the first evidence that T_a affects LA circadian rhythms more
394 strongly than light in an exclusively subterranean environment. Our study clearly shows
395 that the Damaraland mole-rat maintains its activity during the coldest 12 hours (18-28°C)
396 of the 24-hour T_a cycle regardless of a photic cue. Firstly, when the coldest 12 hours

397 occurred in the dark, 80% of Damaraland mole-rats demonstrated nocturnality. However,
398 upon reversing (12 hour phase advance), resulting in the cooler period in the light, 90%
399 of Damaraland mole-rats exhibited diurnal activity patterns. Mammals which have
400 previously been exposed to T_a above what they would experience in nature have been
401 shown to mask nocturnal behaviour (Gall & Shuboni-Mulligan, 2022). However, this is
402 unlikely the case in this study, as Damaraland mole-rats were exposed to a recorded
403 naturally occurring T_a range (Rooper et al., 2001), which was somewhat muted compared
404 to possible natural T_a ranges (Holtze, Braude et al., 2018). However, as stated previously,
405 the current experimental design and presented results prevent conclusive differentiation
406 of entrainment to T_a from that of masking and, ultimately, prevent the determination of
407 whether light or T_a is the stronger entrainer of LA circadian rhythms of Damaraland mole-
408 rats. Future studies should include additional light and T_a profiles, which would include
409 profiles with wider T_a ranges and duplication of sinusoidal natural light exposure with
410 more realistic light intensity (lower lux).

411

412 The effect of environmental factors, such as light and T_a , on endogenous circadian
413 rhythmicity has arisen to provide intrinsic and extrinsic fitness advantages to all organisms,
414 including mammals. Consequently, it seems counterintuitive that light alone would be the
415 most potent environmental factor for all mammals experiencing vastly different
416 environmental and photic conditions, including subterranean environments. Ambient
417 temperature has been proposed to be a weaker *zeitgeber* of circadian rhythms in mammals
418 due to their predominately homeothermic and endothermic nature (maintaining a constant
419 body temperature through the endogenous production of heat) (Rajaratnam & Redman,
420 1998; Rensing & Ruoff, 2002; Refinetti, 2010). More recently, it has been suggested that
421 like heterothermic mammals (El Allali et al., 2013; Farsi et al., 2020a, b; Finn et al., 2022),
422 homeothermic mammals that possess either poor heat-dissipating abilities and/or live in
423 an environment that reduces heat dissipation, are sensitive to daily T_a changes and can
424 entrain to a 24-hour T_a cycle. In the case of the Damaraland mole-rat, the need to avoid
425 exercise-induced hyperthermia and/or reduce energy requirements is of paramount

426 importance. Damaraland mole-rats can ideally prevent such physiological limitations by
427 increasing their LA activity during the colder periods, regardless of photic stimuli. This
428 study strongly supports Hart, van Jaarsveld et al. (2021) hypothesis that nocturnal, diurnal,
429 or crepuscular activity observed in mammalian species could be an evolutionary artefact
430 of T_a entrainment rather than light entrainment. The effects of T_a and light on the
431 molecular machinery controlling the biological clock are still unknown and thus, highlight
432 the need to disentangle this complex relationship.

433

434 ***Ethical statement***

435 All procedures involving live animals and sample collection described in this manuscript
436 were conducted in accordance with the appropriate national and provincial guidelines,
437 permits and regulations. This study was approved by the animal ethics committee of the
438 University of Pretoria EC038-18.

439

440 **Author contributions**

441 D.W.H. and N.C.B. conceptualised the project. D.W.H. and K.L.G. collected data and
442 performed animal maintenance. D.W.H., P.J.J and K.L.G. analysed the data. N.C.B.
443 provided funding, equipment, and animals for the project. All authors contributed toward
444 the writing of the manuscript and agreed to be held accountable for the content therein.

445

446 ***Data accessibility***

447 All raw data is available upon request.

448

449 ***Competing interests***

450 The authors have no competing interests to declare.

451

452 ***Funding.***

453 This study was funded by SARCHI Chair to NCB (grant no. 64756).

454

455 ***Acknowledgements***

456 The authors would like to thank Barry van Jaarsveld for helping to conceptualise this
457 project. The authors would like to thank the two anonymous reviewers and Prof Roberto
458 Refinetti for their expert opinions and helpful suggestions.

459
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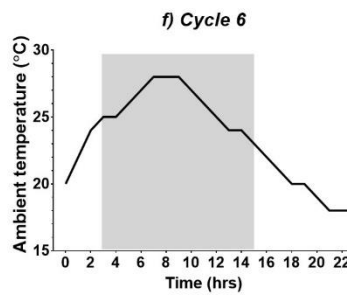
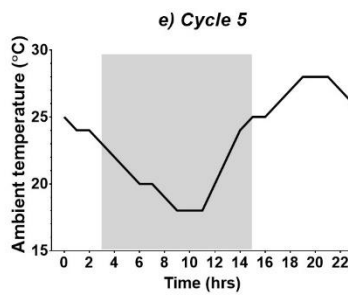
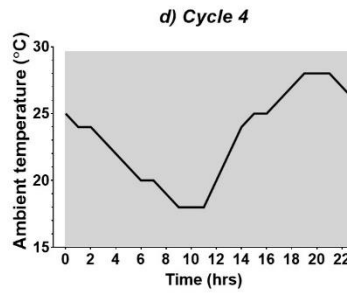
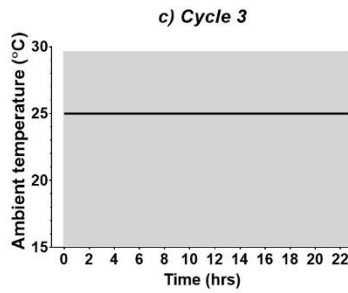
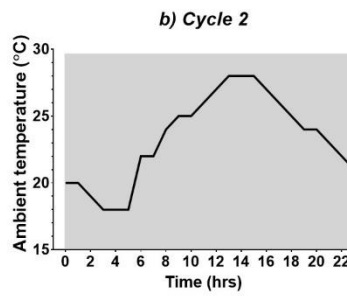
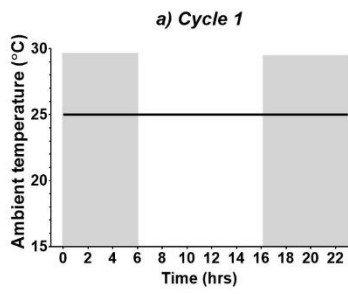
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624

625 **Figure legends**



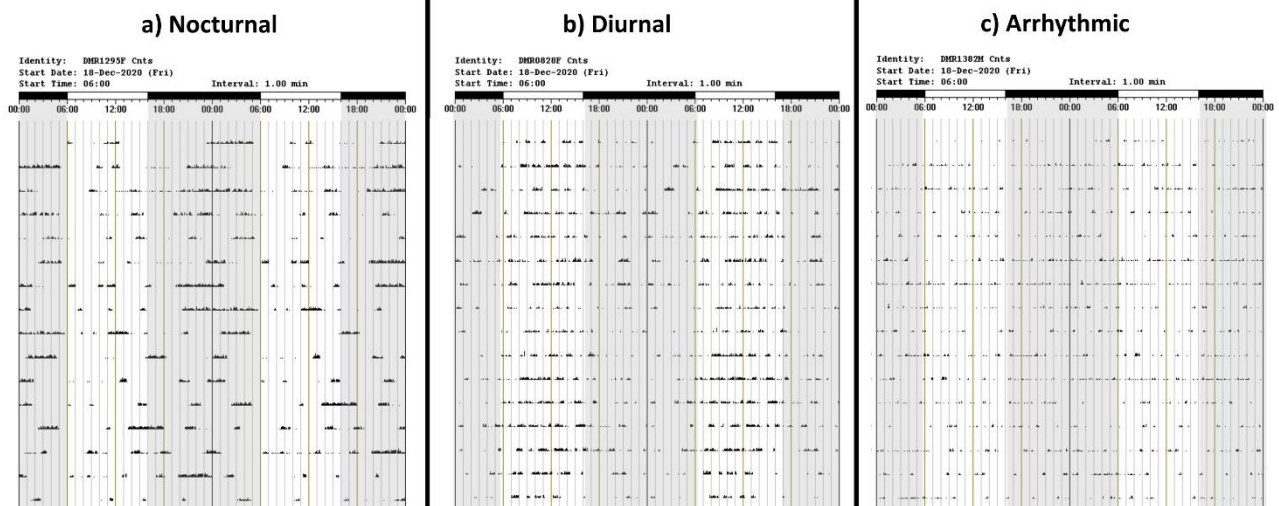
626

627 **Figure 1. Experimental conditions during the a) *Cycle 1*, b) *Cycle 2*, c) *Cycle 3*, d)**

628 ***Cycle 4*, e) *Cycle 5* and f) *Cycle 6*.** The solid line indicates the temperature profile,

629 whereas grey and white bars indicate light's absence or presence.

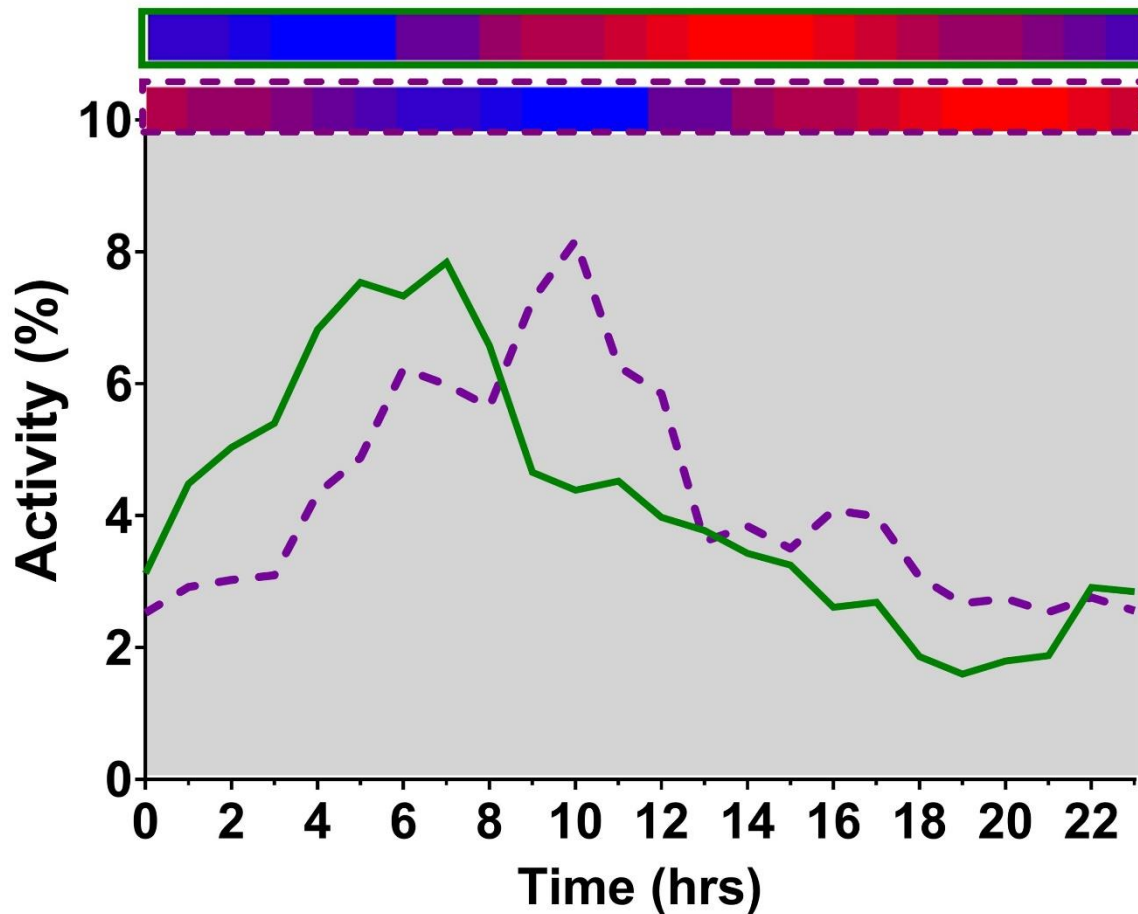
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631

632 **Figure 2: Representative actograms of locomotor activity for Damaraland mole-rat**
 633 **(*Fukomys damarensis*) individuals in *Cycle 1*, showing three activity profiles: a)**
 634 **nocturnal, b) diurnal and c) arrhythmic.** Actograms are double-plotted with time on
 635 the x-axis. During *Cycle 1*, the air temperature was 25°C, and the lights went on at 06:00 h
 636 and off at 16:00 h. Grey and white shading indicate the absence or presence of light,
 637 respectively. Representative actograms were from two female [a) and b)] and one male [c)]
 638 Damaraland mole-rat.

639



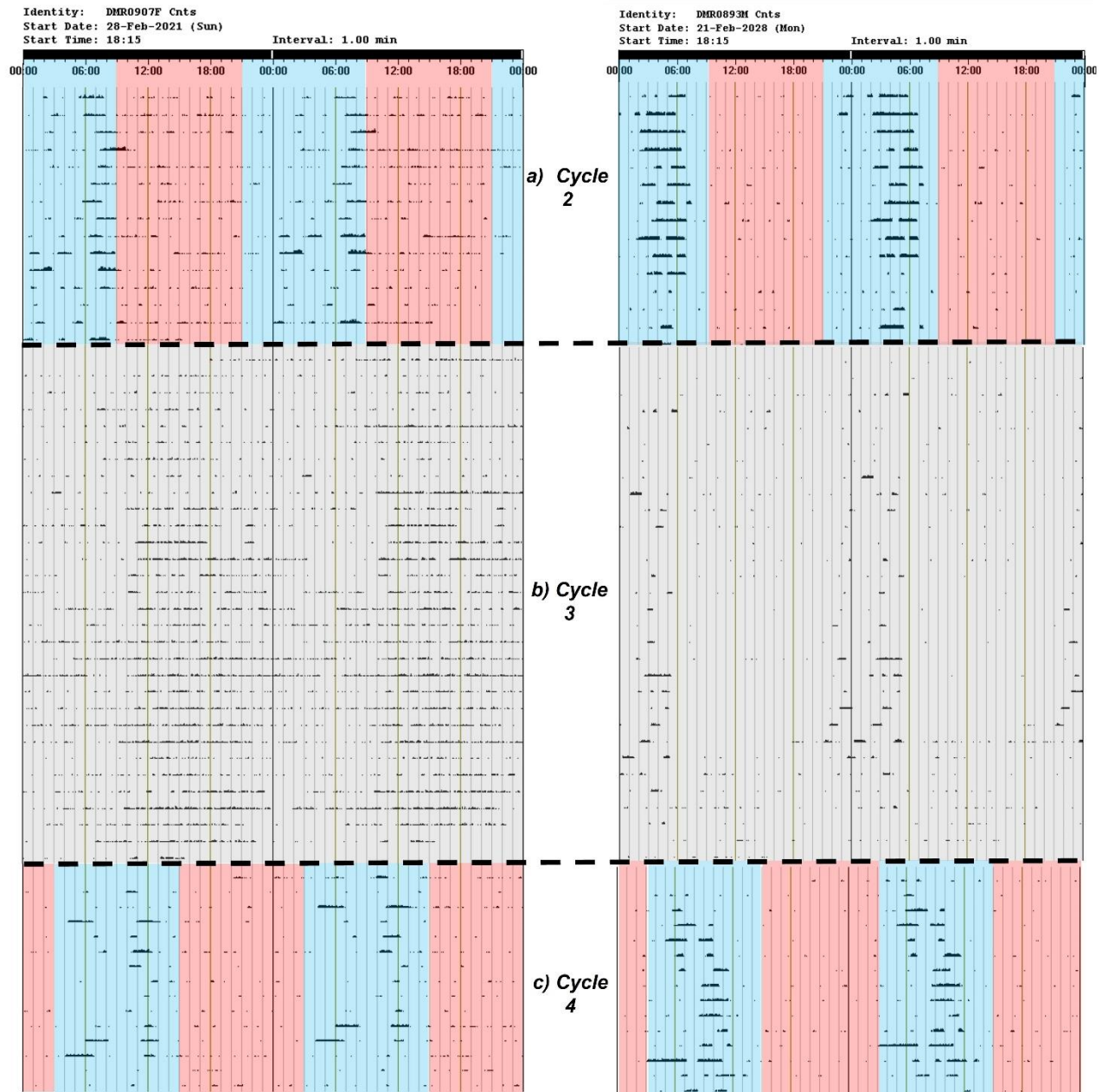
640

641 **Figure 3: Percentage (%) of daily locomotor activity expressed per hour for 10**
 642 **Damaraland mole-rats (*Fukomys damarensis*).** *Cycle 2* (green solid line) consisted of
 643 constant darkness (DD) and a natural temperature increase, with a nadir at 03:00 h. *Cycle*
 644 *4* (purple dashed line) consisted of DD and a 6-hour shift in the natural temperature
 645 increase, with a nadir at 09:00 h. The colour bar indicates the temperature gradient, with
 646 bright red representing the hottest period (28°C) and bright blue representing the coolest
 647 period (18°C).

648

Female

Male

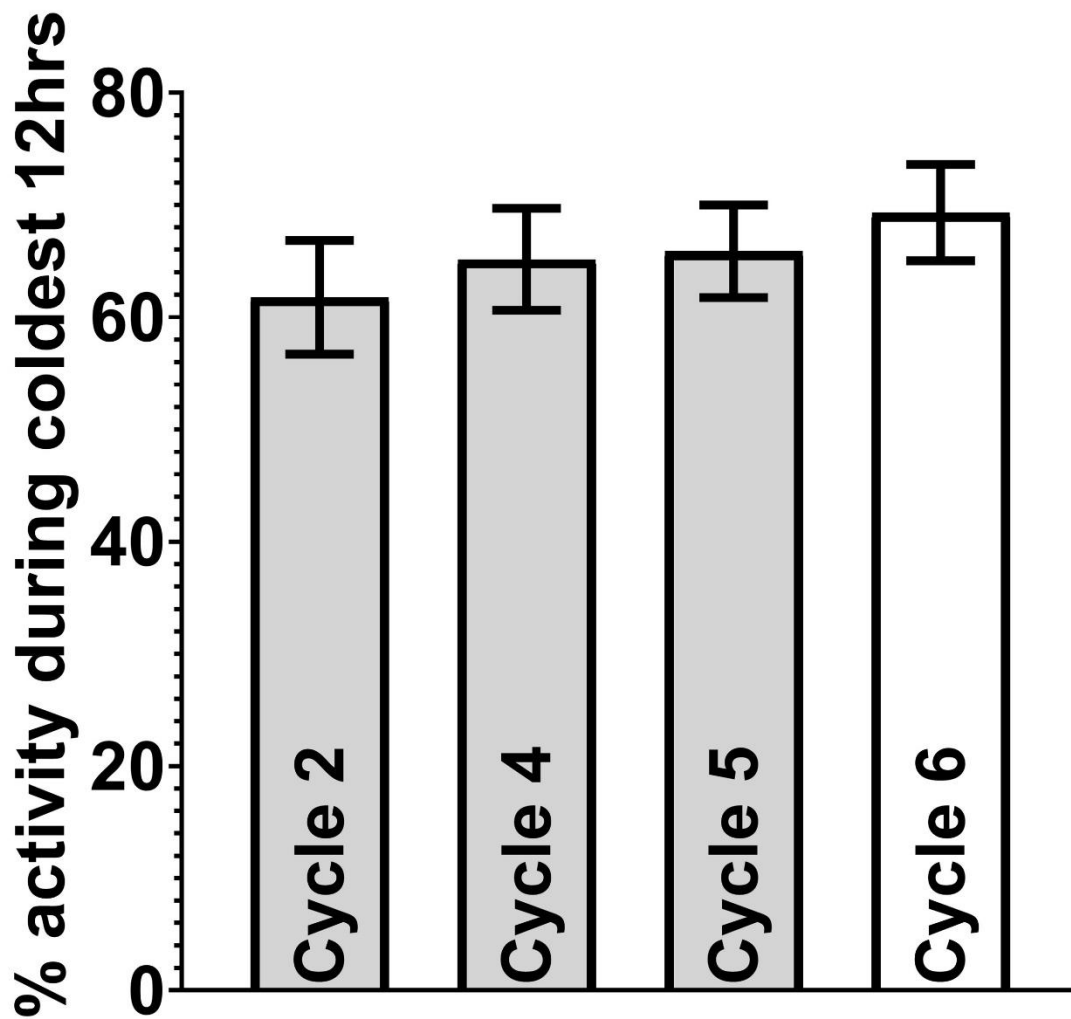


649

650 **Figure 4: Representative actograms of locomotor activity for Damaraland mole-rat**
651 **(*Fukomys damarensis*) individuals.** Actograms are double-plotted with time on the x-
652 axis. a) *Cycle 2* (only the last seven days of the cycle shown) consisted of constant darkness
653 (DD) and a natural temperature ramp (18-28°C), with a nadir at 03:00 h. During b) *Cycle*
654 *3* (all days of the cycle shown), animals were subject to DD and 25°C. c) *Cycle 4* (only the
655 first seven days of the cycle shown) consisted of DD and a 6-hour shift from *Cycle 2* in
656 the natural temperature ramp, with a nadir at 09:00 h. A dashed line indicates a change in

657 cycle. Colour shading indicates temperature gradient, with red representing the hottest 12-
658 hour period (24-28°C) and blue representing the coolest 12-hour period (18-23°C). Grey
659 shading indicates the absence of light, respectively. Representative actograms were from
660 the same female and male Damaraland mole-rat.

661

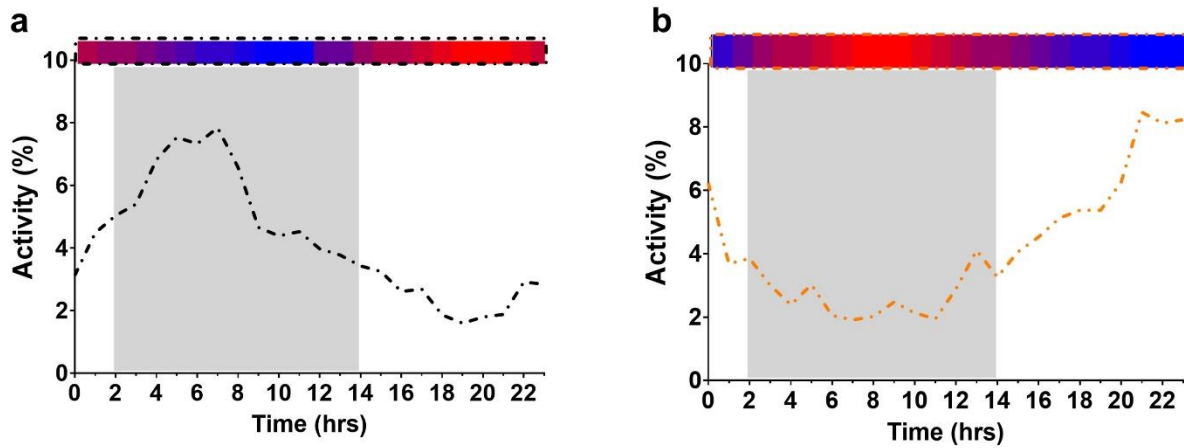


662

663 **Figure 5: The combined percentage (%) of daily locomotor activity expressed per**
664 **hour for 10 Damaraland mole-rat (*Fukomys damarensis*) during the coolest 12**
665 **hours (hours, 18-23°C) of each respective cycle. *Cycle 2* consisted of constant darkness**
666 **(DD) and a natural temperature ramp, with a nadir at 0300 h. *Cycle 4* and *5* consisted of a**
667 **6-hour shift in the natural temperature ramp, with a nadir at 0900 h, with all animals in**
668 ***Cycle 4* being exposed to DD. While in *Cycle 5*, lights were on during the hottest 12 hours**
669 **of the T_a cycle. *Cycle 6* consisted of a 12-hour shift in the natural temperature ramp to *Cycle***

670 5. Grey and white shading indicate the absence or presence of light, respectively. Error
671 bars are presented as mean \pm SE.

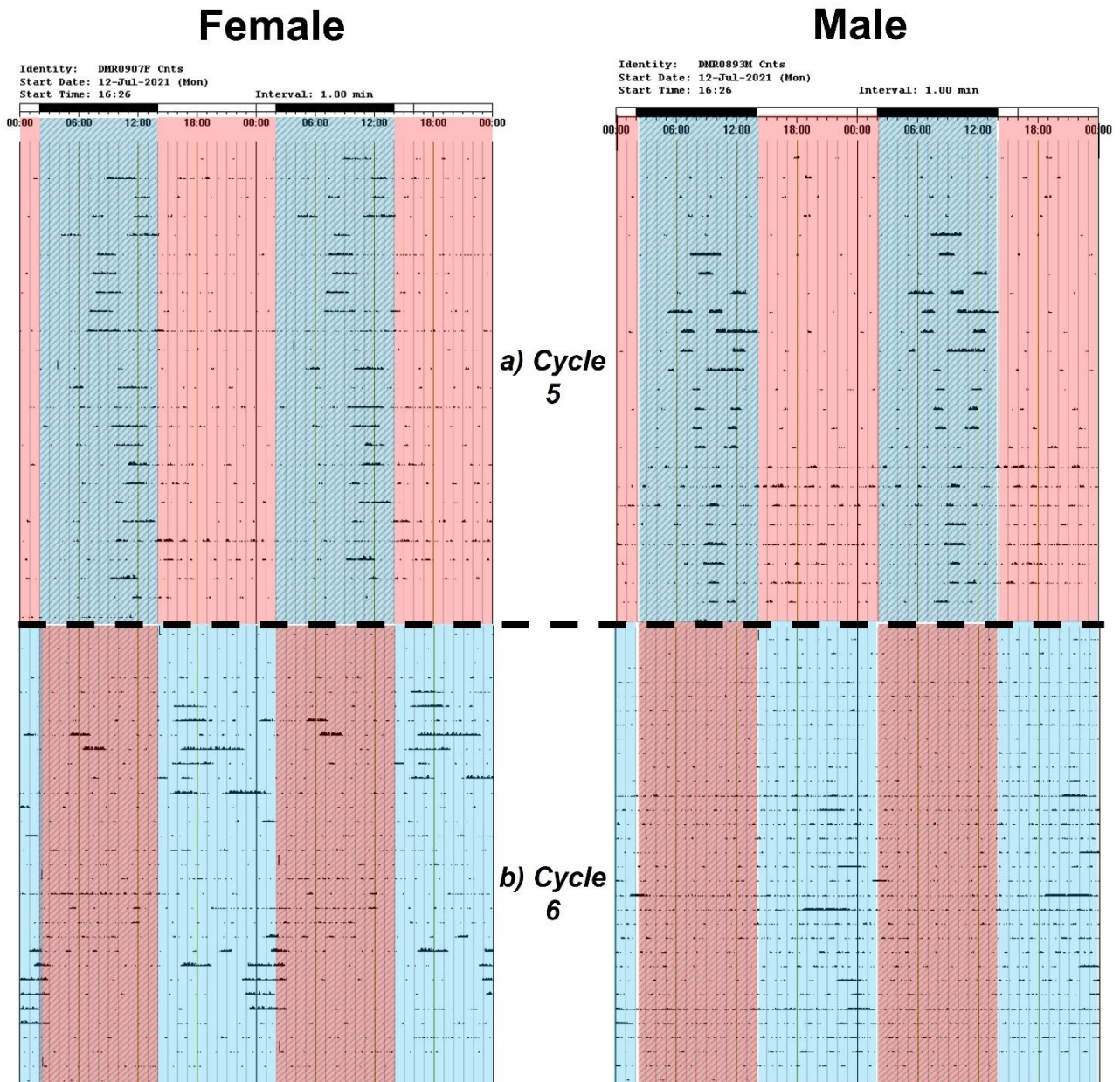
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673

674 **Figure 6: Percentage (%) of daily locomotor activity expressed per hour for 10**
675 **Damaraland mole-rat (*Fukomys damarensis*).** a) *Cycle 5* (black single dotted dashed
676 line) consisted of a natural temperature ramp, with a nadir at 09:00 h with all animals
677 exposed to light during the hottest 12 hours (24-28°C) of the T_a cycle. b) *Cycle 6* (orange
678 double dotted dashed line) consisted of a 12-hour shift (nadir at 21:00 h) in the natural
679 temperature ramp to *Cycle 5*, with all animals exposed to light during the coldest 12 hours
680 (18-23°C). Grey and white shading indicate the absence or presence of light, respectively.

681

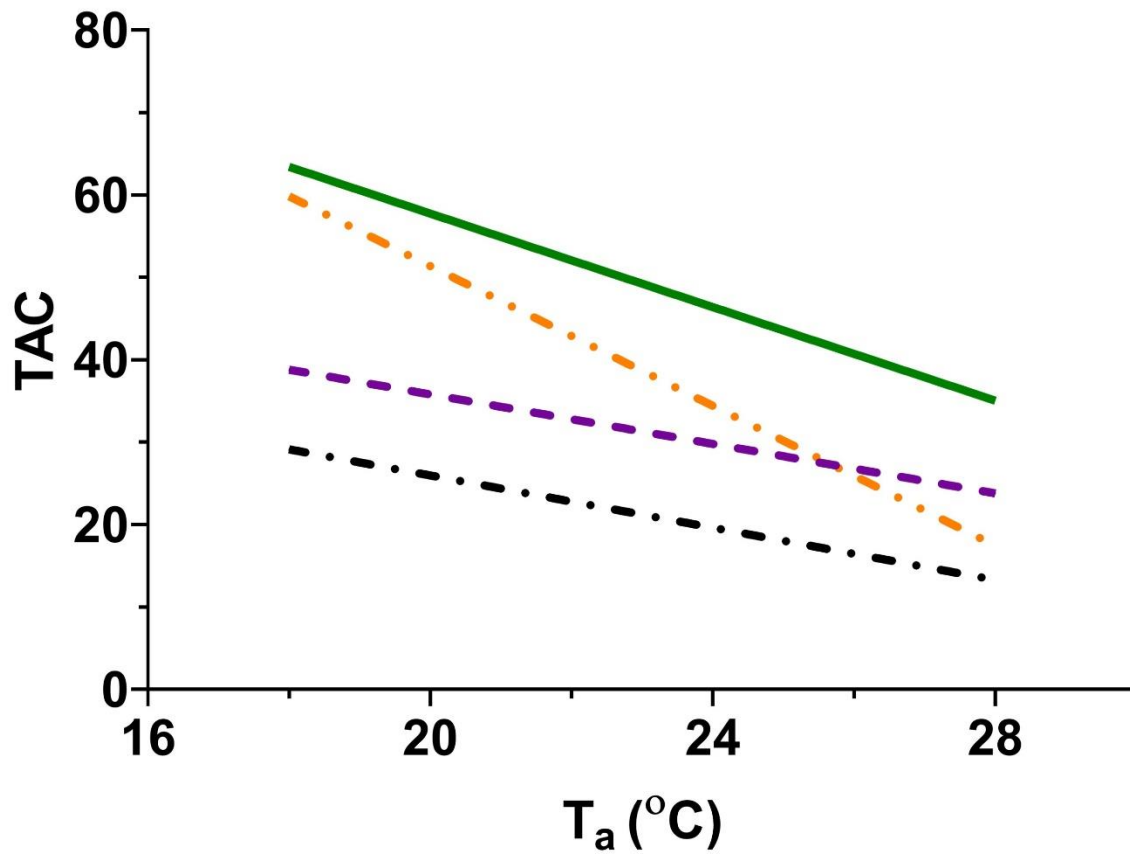


682

683 **Figure 7: Representative actograms of locomotor activity for Damaraland mole-rat**
 684 **(*Fukomys damarensis*) individuals, using a female Damaraland mole-rat, in *Cycle***
 685 ***5* and *Cycle 6*.** Actograms are double-plotted with time on the x-axis. a) *Cycle 5* consisted
 686 of a natural temperature ramp, with a nadir at 09:00 h, with all animals exposed to light
 687 during the hottest 12 hours (24-28°C) of the T_a cycle. b) *Cycle 6* consisted of a 12-hour
 688 shift (nadir at 21:00 h) in the natural temperature ramp to *Cycle 5*, with all animals exposed
 689 to light during the coldest 12 hours (18-23°C). A dashed line indicates a change in cycle.
 690 Colour shading indicates temperature gradient, with red representing the hottest 12-hour
 691 period (24-28°C) and blue representing the coolest 12-hour period (18-23°C).

692 diagonal lines indicate the absence of light. Representative actograms were from the same
693 female Damaraland mole-rat.

694



695

696 **Figure 8:** The negative relationship between total activity counts per day (TACs)
697 and ambient temperature (T_a °C) for *Cycle 2* (green solid line), *Cycle 4* (purple
698 dashed line), *Cycle 5* (black single dotted dashed line) and *Cycle 6* (orange double
699 dotted dashed line).