1   **Exercising for food: bringing the laboratory closer to nature**

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4   Ivana A.T. Fonseca[1]; Renata L.F. Passos[1]; Fernanda A. Araújo[2]; Milene R.M. Lima[1]; Débora R.

5   Lacerda[1]; Washington Pires[1]; Danusa D. Soares[1]; Robert, J. Young[3,4*]; Luiz Oswaldo C.

6   Rodrigues[1]

7

8   [1]Laboratório de Fisiologia do Exercício da Escola de Educação Física Fisioterapia e Terapia

9   Ocupacional da Universidade Federal de Minas Gerais, Campus Pampulha, Belo Horizonte,

10   Minas Gerais 31270-901, Brazil

11   [2]Instituto de Ciências Biomédicas, Área de Ciências Fisiológicas, Universidade Federal de

12   Uberlândia, Av. Pará, 1720, Campus Umuarama, CEP: 38400-678, Uberlândia - MG, Brazil

13   [3]Pontifícia Universidade Católica de Minas Gerais, Postgraduate Program in Zoology,

14   Coração Eucaristico, Belo Horizonte 30610-535, Brazil

15   [4]School of Environment and Life Sciences, Peel Building, University of Salford Manchester,

16   Salford M5 4WT, UK

17

18   *Corresponding author: r.j.young@salford.ac.uk

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20   Running title: Running contingent on food rewards

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22   **SUMMARY**

23   Traditionally, exercise physiology experiments have borne little resemblance to how animals

24   express physical activity in the wild. In this experiment, 15 adult male rats were divided into

25   three equal-sized groups: exercise contingent (CON), non-exercise contingent (NON) and

26   sedentary (SED). The CON group was placed in a cage with a running wheel, where the

27   acquisition of food was contingent upon the distance run. Every three days the distance

28   required to run to maintain food intake at free feeding levels was increased by 90% in

29   comparison to the previous 3 days. The NON group were housed identically to the CON

30   group, but food acquisition was not dependent upon running in the wheel. Finally, the SED

31   group were kept in small cages with no opportunity to perform exercise. A two-way ANOVA

32   with repeated measures was used to determine significant differences in responses

33   between the experimental phases and treatment groups and ANCOVA to analyse growth

34   and tissue mass variables with body length and body mass used separately as covariates. A

35   *post hoc* Tukey's test was used to indicate significant differences. A Pearson's correlation

36   was used to test the relationship between the distance travelled by the animal and the

37   distance/food ratio. The level of significance was set at $p<0.05$ for all tests. The CON group

38   showed the hypothesized correlation between distance required to run to obtain food and

39   their mean distance travelled ($p<0.001$), during 45 days in contingency phase. The CON

40   group showed a decrease in body mass, rather than an increase as shown by NON and SED

41   groups. The CON group had a significantly lower body temperature ($p<0.05$) and adiposity

42   ($p<0.05$) when compared to the other two groups for the same body size. The present

43   experimental model based on animals choosing the characteristics of their physical exercise

44   to acquire food (i.e., distance travelled, speed and duration) clearly induced physiological

45   effects (body characteristics and internal temperature), which are useful for investigating

46   relevant topics in exercise physiology such as the link between exercise, food and body

47   weight.

48

51 **INTRODUCTION**

52   Traditionally experiments in exercise physiology have been different from the
53 conditions in which wild animals would undertake physical activity. For example, most
54 experiments are conducted in highly controlled laboratory environments in which the
55 animals are, often, forced to exercise stimulated by electric shocks on a treadmill
56 (Tokuyama et al., 1982; Lambert et al., 1996; Lima et al., 1998; Soares et al., 2003). Thus,
57 experiments are controlled and convenient for the human experimenter, but they do not
58 allow the simulation of the relationship that a wild animal has between its need for
59 resources and how it acquires those resources. It is possible that being put on a treadmill
60 with the threat of an electric shock, if the animal does not run, is a stressful experience and
61 one in which the animal has no choice, as observed by Fleshner et al. (2007).

62   Psychologists have established that the performance of behaviour is enhanced
63 through the use of positive reinforcement, whereas punishment is not an effective
64 mechanism for modifying behaviour (Mazur, 2002). In the wild, resource acquisition by
65 animals is normally contingent upon them expressing physical activity: a hungry rat must go
66 out and seek food (i.e., express physical activity) (Russel et al., 1987; Overton and Williams,
67 2004; Duffy et al., 1997; Challet et al., 1997; Krebs et al., 2012). Thus, for most wild animals
68 the obtaining of resources is contingent upon the expression of physical activity (i.e.,
69 appetitive behaviour; Young, 1997). Importantly, wild animals have control over when,
70 where and how much physical activity (e.g., duration and speed) they will express in order
71 to obtain desired resources. It is this control that contributes significantly to the well-being
72 of animals both in the wild and in captivity (Young, 2003) and, probably, it is the lack of
73 control (i.e., ability to express appetitive behaviour in a manner that affects their
74 environment) that animals in traditional exercise physiology experiments face. Wild animals
75 live in an operant world (i.e., they operate on the world using their behaviour to obtain
76 resources such as food) and gain control over it by expressing behaviour (Young, 1997). The
77 application of different operant ratio types (i.e., whether it is expressing a certain frequency
78 or duration of behaviour that will result in resource acquisition) can be used to simulate
79 situations in the wild, for example, a progressive ratio can simulate increasing food scarcity;
80 that is, more work required to obtain the same amount of food (Mazur, 2002).

81   In behavioural ecology, one of the fundamental concepts is that animals express
82   behaviour in a manner that provides them with the greatest net return (Krebs et al., 2012).
83   In other words, animals try to maximise the cost-benefit ratio in terms of energy intake, and
84   this concept is the basis of optimal foraging theory, one of the corner stones of behavioural
85   ecology, which has received considerable empirical support (Krebs et al., 2012). However,
86   animals being forced to run on a treadmill due to electric shocks are unable to maximise the
87   cost-benefit ratio, as even basic factors affecting energy consumption such as running speed
88   and duration are out of their control. Thus, this traditional experimental model does not
89   relate closely to the reality of wild animals' lives. It is, after all, the choices that animals
90   make, which affects their fitness and upon which natural selection acts (Careau and Garland,
91   2012; Swallow et al., 2009). Other experimental models may approach the decisions that
92   animals deal with in the wild by considering some choices of animals to perform the
93   exercise, such as the relationship between energy expenditure and time spent seeking and
94   obtaining a certain amount of food (Rowland et al., 2008).

95   The theoretical model of energy homeostasis considers the control of body mass
96   because of the balance between energy cost of activity performed for obtaining food, basal
97   metabolism and energy value of the food obtained (Gelegen et al., 2006). Therefore, there is
98   a natural link between physical activity and feeding. This link has been assessed using
99   experimental protocols, which isolate the effects of one factor such as the effect of exercise
100   on caloric intake (Tokuyama et al., 1982; Stubbs et al., 2002) or the effect of energy on the
101   amount of exercise performed (Belke et al., 2004; Kanarek et al., 2009). Nevertheless, in the
102   exercise protocols usually used exercise is driven by: threat of electrical stimulation on a
103   treadmill (rodents); survival in water tanks (rodents); or verbal encouragement on a
104   treadmill or exercise bicycle (humans). Even protocols that utilize voluntary exercise,
105   performed on a running wheel, the exercise is usually not contingent upon the provision of
106   food and, therefore, it is observed that some species, including rats, will voluntarily perform
107   physical activities without an apparent function (Sherwin, 1998; Meijer and Robbers, 2014),
108   which alters various aspects of physiology and behaviour, such as their caloric intake (Novak
109   et al., 2012). It is known that rats and mice exhibit abnormal behaviour when subjected to
110   caloric restriction protocols with free access to the wheel (i.e., obsessive wheel running;

111   Sherwin, 1998); and this behaviour has been associated with anorexia nervosa (Routtenberg

112   and Kuznesof 1967, Altemus et al., 1993, Burden et al., 1993).

113   In the laboratory, few studies have linked the provision of food to the amount of

114   physical activity performed. Perrigo and Bronson (1983), for example, pioneered the use of

115   this method; they investigated the effect of increasing the cost of foraging on the

116   reproductive capacity of mice *Mus musculus* (females). Vaanholt et al. (2007) using the

117   same experimental model in mice, observed that animals selected for high activity levels ran

118   about 28% more than the control group when there was no contingency between physical

119   activity and food. When the food was contingent upon exercise, the two groups travelled

120   similar distances (23.2 ± 1.4 versus 23.4 ± 1.4 km/day, respectively). This result suggests that

121   when animals need to perform exercise to acquire food, the intrinsic differences between

122   individuals mentioned above (such as selection for high levels of activity) may not be

123   decisive in their performance – instead hunger is the key determinant.

124   The aim of this study was to verify the effect of increasing physical exercise (distance

125   travelled and speed) required to obtain the same amount of food on the anthropometrical

126   (body mass, growth, tissue mass) and physiological parameters (core temperature) of rats

127   *Rattus norvegicus.*

128   **RESULTS**

129   As hypothesized, the mean daily distance travelled by the CON group increased

130   throughout the experiment from 1311±275 m during adaptation (phase 2) to 9037±1033 m

131   on day 42 (phase 3), and an overall mean of 6132±757 m during the experimental phase.

132   This was a 589% increase compared to the spontaneously distance travelled by the animals

133   during the adaptation period (phase 2). As expected, there was a correlation between the

134   distance required to obtain food and the distance travelled by the animals (r = 0.86;

135   p<0.001).

136   For the NON group there was no significant difference in spontaneously travelled

137   distance over the time of the experiment (overall mean 1068±95 m). In the last three days

138   of the experiment the distance travelled by the CON group was 5.6 times higher than NON

139   group (9037±1033 m versus 1621±782 m, respectively) (Figure 1a; mean±SE).

140   It can be seen in Figure 1b (mean±SE) that the SED group and the NON group body

141   masses steadily increased during the experiment (311.4±6.8 versus 296.5±7.3 g on day -16

142   (phase 1) to 422.4±9.1 versus 407.4±12.5 g on the last day of phase 3, for the SED and NON

143   groups, respectively). The CON group, in comparison, had a stable body mass until day 24

144   (phase 3) (332.5±1.8 g) followed by a progressive reduction until day 42 (279.4±4.9 g), when

145   the animals reached a 16% reduction in body mass in relation to their phase 3 stable body

146   mass (Figure 2; mean±SE). On day of euthanasia, the CON group had lower body mass

147   compared to NON and SED (269.2±7.0, 413.4±13.2, and 426.9±7.7 g, respectively,

148   mean±SE). Furthermore, on the same day, there was no difference between groups in body

149   length without or with body mass as a covariate (P>0.05 in both cases; 162.5±4.9,

150   172.4±4.4, and 162.5±4.9 mm, for CON, NON and SED, respectively, without body mass as a

151   covariate or 157.4±14.7, 174.7±7.8 and 165.6±9.9 mm with body mass as a covariate)

152   (mean±SE).

153   Figure 2c (mean±SE) shows that only the CON group progressively reduced the

154   amount of food ingested (25.0±0.9 phase 1 versus 13.8±1.0 g in phase 3; which is minus

155   44.8%). In groups NON and SED there was no change in food intake during the experiment

156   (24.7±0.1 versus 24.2±0.1 g, respectively).

157   Figure 3 (mean±SD) shows a colonic temperature reduction in the CON group

158   throughout the experiment in relation to the other groups, which occurred mainly from day

159   24 onwards (36.5±0.2 versus 37.5±0.1 versus 37.0±0.1 °C on day 24 for CON, NON and SED,

160   respectively). This was also seen when the CON group's first colonic temperature (phase 1 =

161   37.8±0.1 °C) was compared to its last one (phase 3 = 36.4±0.1 °C).

162   The CON group increased running speed to obtain food (from 18.4±2.1m/min on the

163   first progressive ratio to 35.2±3.3m/min on the last progressive ratio), which was higher

164   than NON group (p<0.05). However, these running speed increases were not enough to

165   compensate the energy demands, leading to the rats also to increase total exercise time on

166   the wheel (from 71.25 min at the beginning to 256.74 min at the end of the experiment). To

167   maintain a running time of 71.25 min rats in the CON group would have needed to run at

168   126.8 m/min during the last progressive ratio, which would be faster than their spontaneous

169   maximum-recorded running speed. Interestingly, the maximum spontaneous running

170   speeds recorded were similar for CON (81.6±1.7m/min) and NON groups (99.5±1.2m/min)

171   during phase 3 (data are mean±SE).

172   Intrinsic differences in running speed between groups were not observed, because

173   speed and exercise time in the progressive conventional treadmill tests showed no

174   differences among groups both in the pre and post testing (p>0.05; Table 1; mean±SD).

175   Food contingent on exercise (i.e., the CON group) did not affect the final body length

176   of the animals (p>0.05) (163±10 versus 172±10 mm versus 163±9 mm for groups CON, NON

177   and SED, respectively, mean±SD) nor the mass of femurs (p>0.05) (Table 2; mean±SD). The

178   length of the right femur was significantly shorter in the CON group compared to the others

179   (p<0.05) (Table 2; mean±SD). There was no influence of body length for mass of femurs, but

180   when body length was accounted for as a covariate, the length of right femur of the CON

181   group was shorter compared only to SED group (Table 2; mean±SD).

182   The CON group showed smaller masses of brown adipose tissue, liver, heart and

183   right gastrocnemius compared to NON and SED groups. The right adrenal gland was heavier

184   in the NON compared to the other groups. There was no difference between groups in the

185   mass of the soleus (Table 2; mean±SD). When body length was considered as covariate, liver

186   and heart showed smaller masses in CON group compared to NON and SED groups. The

187   right gastrocnemius was heavier in NON group compared to CON and SED groups, and the

188   right adrenal gland was heavier in the NON compared to the CON group. There was no

189   difference between groups in the mass of the brown adipose tissue and soleus (Table 2,

190   adjusted means; mean±SD).

191   No difference between the groups in growth or tissue mass data was found when

192   body mass was considerate as covariate, except for the adiposity index (0.64±0.25 versus

193   2.96±0.78 versus 3.15±1.03% for the groups CON, NON and SED, respectively; Figure 3a;

194   mean±SD) and adipocyte area (1376.5±331.0 versus 3588.6±685.2 versus 3850.1±630.6 μm^2

195   for the groups CON, NON and SED, respectively, Figures 3b, c, d and e). In other words,

196   adiposity was lower in the CON group compared to NON and SED groups (p<0.05;

197   mean±SD).

198

199   **DISCUSSION**

200   These results demonstrate that rats will travel increasingly longer distances to
201   acquire food, as is expected to happen under natural conditions, during conditions of
202   decreasing food availability. Vaanholt et al. (2007) observed similar results in mice
203   selectively bred for high activity levels on running wheels. Foraging is a complex behaviour
204   that depends on factors such as food availability, presence of predators, time and energy
205   expenditure involved in seeking and obtaining food, animal age, species, among others. In
206   nature all these factors may be present, either singly or combined, and the animal's
207   behaviour is the result of the costs and benefits generated by this behaviour (Houston and
208   McNamara, 1989; Lemon; Barth, 1992; Shamoun-Baranes; van Loon, 2006; Young and
209   Lawrence, 1996), as well as possible intrinsic motivation to be physically active (e.g., see
210   Meijer and Robbers, 2014 and references therein). The data recorded indicate that the
211   present contingency method allows the experimenter to precisely control the daily distance
212   to be travelled by the animal through manipulating the relationship between the amounts
213   of food given as a function of distance required to obtain it. Furthermore, it was found that:
214   (1) the amount of exercise performed voluntarily by the animal is five times higher when
215   obtaining food is contingent on physical activity; (2) the motivation to perform physical
216   activity, as demonstrated by greater daily distance travelled, is greater in animals subject to
217   food-exercise contingency; and (3) animals submitted to a food-exercise contingency
218   reached a stable body mass, whereas those without showed progressive increase. The use
219   of the present experimental model may change our understanding of the impact of exercise
220   in various relevant issues to exercise physiology such as control of body weight and physical
221   activity; thermoregulation and exercise; pregnancy and physical activity; sleep and physical
222   activity, amongst others.

223   Another important observation of this study was that using a progressive ratio of the
224   distance to be travelled by the animals to obtain food (for 42 days) reduced the variability of
225   distance travelled. Several studies have shown that when there is no contingency between
226   physical activity and food, the distances travelled by rodents may exhibit high coefficients of
227   variation (Chappell et al., 2004, 2007). Lambert and Noakes (1990), for example, observed
228   that the distance travelled by rats ranged widely between 3.3 and 76.0 km/week. In the
229   present work, the variability in CON group distance ran was reduced by 49% when

230   compared to the period of adaptation in activity cages (phase 2). The coefficient of variation

231   was also lower in the CON group compared to the NON group (24% vs 59%, during phase 3).

232   This reduction in the variability of distance ran suggests that animals of the same species

233   travel similar distances when they are motivated to run for food, and that this behaviour

234   can be independent of age-related differences (Mondon et al., 1985), sex (Garland et al.,

235   2011) and aerobic capacity (Vaanholt et al., 2007), whose effects are observed when

236   animals perform exercise without it being rewarded with food. Thus, our results show that

237   the contingent method decreased the interference of intrinsic and extrinsic factors on the

238   exercise performance variability by the animals under the present restricted and controlled

239   experimental situation, which could be useful for drug experimental studies, for example.

240   However, in the wild, in the case of food shortage, other factors such as predation risk (or

241   other environmental challenges) or minimal differences in age, sex and the intrinsic physical

242   ability of the animal may be crucial for survival.

243   In relation to the traditionally used method to study physical exercise in animals (i.e.,

244   forced exercise on a treadmill), it is interesting to compare the coefficient of variation of the

245   total exercise time in this type of exercise with the mean distance travelled in the present

246   study. In previous studies from our laboratory, we found a coefficient of variation of 42% in

247   total time of forced exercise on a treadmill at 18m/min (Pires et al., 2007), which is a

248   moderate exercise for rats, compared to the coefficient of approximately 20% for the total

249   distance travelled in the CON group of the present study. It is therefore reasonable to

250   assume that there is less variability in animal motivation when exercise was linked to

251   obtaining food (i.e., positive reinforcers compared to electrical stimuli (i.e., punishment)).

252   In the laboratory, when the animals had free access to the running wheel, the NON

253   group performed some physical activity without apparent reason. This behaviour was also

254   observed in this investigation in the CON and NON groups, and its mechanism is unknown.

255   Perhaps it is due to intrinsic exploratory behaviour of each animal (Inglis et al. 2001) and

256   there are studies showing that certain genetic variants in some species are more active than

257   others (Dewsbury, 1980; Swallow et al., 2009; Garland et al., 2011).

258   Our results showed that exercise linked to food caused changes in rats' body

259   composition. The CON group adiposity index was approximately 80% lower when compared

260 to the NON and SED groups. Similar results were found by Vaanholt et al. (2007) who

261 observed a reduction in adipose tissue of approximately 70% in mice that ran for food

262 (contingency was fixed at 90% of maximum) compared to the group that had free access to

263 food and a running wheel. The effect of the food-exercise contingency on adipose tissue

264 observed in this study and that of Vaanholt et al. (2007) are higher when compared to the

265 effects of training conducted on a running wheel without such contingency (Tokuyama et

266 al., 1982, Pitts, 1984; Cortright et al., 1997), on a treadmill (Lacerda, 2012; Barcellos, 2011;

267 Pitts, 1984) or in restriction food protocols (Hill et al., 1985). The contingency of exercise to

268 obtain food in the present study simulated a natural condition and caused a pronounced

269 reduction in adipose tissue not observed in traditional protocols of exercise/training. Some

270 of these results could be associated to higher daily energy expenditure for animals that have

271 to run on wheels to get food as compared with those forced to run (even to exhaustion) on

272 a motorized treadmill, even daily. Due to the caloric content of feed and the period of

273 stability in body mass between days 3 and 24 (phase 3 in CON group), was possible to

274 estimate the daily energy expenditure of the animals of the CON group, which was 73.6

275 kcal/day. It has been reported that rats with free access to the wheel (without contingency),

276 spend, on average, 13 % of this in ingested food energy per day to run on the wheel

277 (Afonso; Eikelboom, 2003). In this case, the animals of the present study consumed 9.56 kcal

278 to traveled about 4673 m (average distance traveled by the animals during the period

279 described above), or 2.04 kcal/km. This value is lower than 2.26 kcal/km observed by Afonso

280 and Eikelboom (2003), but is in agreement with values reported for Wistar rats during a

281 constant exercise on a treadmill at 18m/min for approximately 50 min (2.77 kcal/km)

282 (Soares et al. 2003). These results suggest that per kilometre there were no differences in

283 energy expenditure, but the distance travelled by the CON group was much greater than

284 that observed in spontaneous wheel running rats or rats undergoing forced exercise on a

285 treadmill.   Thus, there may be important, long-term physiological changes in food

286 contingent rats not found in the aforementioned protocols.   This issue needs more

287 investigation, but suggests that the natural link between exercise and food must be

288 considered when the aim is to study body mass control, obesity and associated diseases.

289   Additionally, the initial stable body mass phase of CON rats may have been due to

290 the use of stored fat reserves at the beginning of the experiment, when the cost of the

291   activity was still small. The weight reduction at the end of the experiment (which was not

292   due to changes in body length), in turn, indicates the amount of energy obtained was not

293   sufficient to maintain the body mass of rats. The linear relationship between distance ran

294   and required to run to obtain *ad libitum* feeding level (figure 1, panel A) suggests that, if the

295   progressive ratio had continued beyond DD_{max} (day 42) that rats would have gone into an

296   increasing energy deficit situation.

297   Lower body masses, due to smaller fat deposits may be an advantage for survival in

298   nature, especially for those species that depend on the realization of physical activity to

299   avoid predators or to obtain food. Investigations into the effects of domestication on the

300   body composition of rats (Castle, 1947) and mice (Dohm et al., 1994) have reported that

301   wild animals are smaller and perform better on a treadmill and running wheel when

302   compared to domesticated animals. Although, the performance of the animals in the CON

303   group was not different from the other groups when subjected to progressive treadmill test

304   in present study, the observed alterations in body mass and the mass of the heart, liver,

305   gastrocnemius and adrenal CON group suggest that the link between providing food and

306   exercise can be a method that approximates the characteristics of laboratory animals from

307   those observed in animals in nature.

308   The caloric intakes and body masses were not different when comparing the groups NON

309   and SED. These results suggest that spontaneous physical activity of rats in NON group was

310   not sufficient to cause changes in body mass. Afonso and Eikelboom (2003) found similar

311   results. These authors found daily distances ranging between 841-3303 m in rats with free

312   access to a running wheel without any contingency on food, distances similar to those

313   observed in the NON group (mean 1068±95 m per day) in the present study. A continuous

314   increase in body mass has been observed in mice that underwent training on a treadmill

315   (Lacerda, 2012; Barcellos, 2011), in running wheels without food-exercise contingency and

316   those without access to exercise (Cortright et al., 1997) as was observed in the NON and SED

317   groups of the present study.

318   The data relating to the final length of the rats in this study, suggest that there was

319   no effect of the contingency on the growth of rats. Cortright et al. (1997) found similar

320   results when they measured the effect of nine weeks of free access to a running wheel, with

321 no contingency to the food supply. Rezende et al. (2006) observed a reduction in body

322 length of animals that were selected for high levels of activity on running wheels in both

323 males and females. There animals were artificially selected for 35 generations and the

324 changes in body size may have represented an effect of exercise associated selection in

325 relation to running wheels. The results observed in animals that have not been artificially

326 selected for high levels of activity suggest that the increase in mass of the animals according

327 to age is due to increased deposits of fat and not body growth as a whole.

328   The early days of running wheel access caused a reduction in food intake by the CON

329 and NON groups. This suppression in caloric intake has been well documented in the

330 literature in voluntary (Collier, 1970; Goodrick et al., 1983; Afonso and Eikelboom, 2003;

331 Lattanzio and Eikelboom, 2003) and forced exercise (Richard and Rivest, 1989; Stevenson et

332 al., 1966). Indeed, this behaviour appears to be independent of novelty (wheel presence)

333 (O'Connor and Eikelboom, 2000), sex of animals (Tokuyama et al., 1982), age (Looy and

334 Eikelboom, 1989) or food availability in rodents (Routtenberg and Kuznesof, 1967; Boakes

335 and Dwyer, 1997). Considering the model of energy homeostasis, it is possible that this

336 behaviour is related to an adjustment of body mass (Richard and Rivest, 1989), but at

337 present, the underlying mechanism is subject to speculation (Lattanzio and Eikelboom,

338 2003).

339   The decrease in body temperatures observed at the beginning of the experiment in

340 CON and NON groups may have been caused by the exercise itself, independent of the

341 contingency, especially as no changes were observed in colonic temperatures of the SED

342 group during this period. Although in this study rate metabolic was not measured, the

343 reducing of colonic temperature as the progressive ratio increasing observed in CON group

344 may have occurred to maintain a strategic body temperature at the expense of scarce

345 amounts of energy, as happens during certain periods in nature, in winter, for example

346 (Burns et al., 2006). The reduction in internal temperature as a strategy to reduce the basal

347 metabolism during periods of low food availability has been observed in non-human

348 primates (Lane et al., 1996), and several species of rodents subjected to various protocols of

349 food restriction (Duffy et al., 1989; Severinsen and Munch, 1999; Speakman and Mitchell,

350 2011). Another factor associated with the availability of food, and which may also have

351 contributed to the reduction in colonic temperature of the CON group, was the reduction in

352   body mass, which probably changed the relationship between body surface area/body mass

353   ratio, thereby facilitating heat loss (Herrington, 1940). Both the reduction in body mass and

354   the reduction of internal temperature are strategies to reduce basal metabolism and

355   increase the chance of survival of the animal in periods with lower food availability and cold

356   environments.

357   Aerobic capacity inferred by the maximum speed achieved in progressive test was

358   not different between groups CON, NON and SED and was lower in the period after the

359   experiment, including in the CON group, despite them having travelled long distances daily.

360   These data suggest that there is no relationship between the performance achieved by the

361   animals on a running wheel (with or without food contingency), and the progressive test

362   (Lambert et al., 1996). Other investigators have described differences in energy cost and

363   performance on a treadmill or a running wheel (Girard et al., 2001; Chappell et al. 2004).

364   Animals in nature may choose to move at various speeds and durations to increase

365   the efficiency of their physical activity (Kenagy and Hoyt, 1989; Blumstein, 1992; Weinstein,

366   1995; Christian et al., 1997; Irschick and Jayne, 1999). Furthermore, unlike what happens in

367   forced protocol exercise, several species, including humans, exhibit an intermittent

368   locomotion, such as eastern chipmunks, *Tamias (Tamias) striatus* (Kramer and McLaughlin,

369   2001; Girard et al., 2001). These rodents spend, intermittently, around 41% of their time

370   foraging with short breaks that may help in the identification of prey, avoiding predation

371   and improving physical fitness (McAdam and Kramer, 1998).

372   All the above results suggest that the effects caused by the present contingent

373   method in energy homeostasis are very different from those involved in forced exercise

374   protocols traditionally used and, therefore, the present protocol should be included as

375   another option in exercise physiology studies.

376   Food acquisition being contingent on physical activity is a laboratory method capable

377   of simulating conditions more similar to those faced by animals in the wild, resulting in

378   reduced body mass and thermoregulatory adjustments in a few days of an experiment to

379   cope with food shortages. Our study showed that animals performed less exercise when

380   there was no contingency between the behaviour they were expressing and the obtaining of

381   food rewards.

382 **MATERIALS AND METHODS**

383 **Ethical note**

384 The research herein reported was approved by the Ethics Committee on Animal
385 Experimentation (CETEA - Protocol 180/10) of the Federal University of Minas Gerais, Brazil.

386 **Animals and housing conditions**

387 Fifteen male Wistar rats from the vivarium of the Institute of Biological Sciences, of
388 Federal University of Minas Gerais/Brazil, with approximately 287.4±13.1 g (5 to 7 weeks of
389 age; mean ± SD) at beginning of experiment were used as the experimental subjects; they
390 were divided into three equal-sized groups: food-exercise contingent (CON), non-exercise
391 contingent (NON) and sedentary (SED). During all phases of the experiment, rats had *ad*
392 *libitum* access to water and food (note that in one group food was contingent upon
393 performing physical activity). Throughout the whole experiment, rats were fed with
394 Presence/Purina rodent pellets (gross energy 359.8kcal/gm, protein 23.8%, fat 5.2%,
395 carbohydrate 54.6% and ash 6.2%). During the study, room temperature was maintained
396 between 24 and 26°C. Rats were maintained on a 14-10 hour light-dark cycle (lights on at 05
397 hours).

398 The animals of CON and NON groups were kept in individual activity cages (Gaustec
399 LTDA, Belo Horizonte, Brazil - Figure 4) with internal dimensions 400 x 400 x 400 mm and
400 external dimensions of 600 x 600 x 400 mm (height, length and width, respectively). The
401 cages had an electronic interface for programming and storage of data (distance, mean
402 speed and amount of food delivered per hour). A dispenser supplied food (see No. 3 in
403 Figure 4). The dispenser was connected to a stainless steel box in which the animal had
404 access to the food. This box had a false bottom to collect unconsumed food, which allowed
405 the quantification of food consumed by the animal. The running wheel has 300 mm in
406 diameter, 942 mm in circumference and mean torque of 0.02±0.004 Nm (mean±SD) (3B
407 Scientific Dynamometer U20032, precision 1N). The mean torque was measured before
408 each phase. A motion sensor (encoder) attached to the wheel generated the values of
409 distance and mean velocity, independent of the direction of wheel movement. The system
410 was calibrated before all experiments and checked for drift after all experiments.

411   The SED group of rats were always kept in individual acrylic cages (length 290 mm,
412   width 180 mm and height 120 mm of internal dimensions) without access to exercise
413   throughout the experiment.

414   **Experimental design**

415   The experiment was divided into three phases: (1) all animals were maintained in
416   individual cages, without access to exercise and food *ad libitum*, to provide measurements
417   of food intake, colonic temperature and body mass in a baseline condition, during four to six
418   days; (2) an adaptation phase to the use of the running wheel for groups CON and NON,
419   with free access to exercise and food, during ten days; and (3) experimental conditions
420   where the CON group was exposed to a progressive ratio (i.e., the required distance run to
421   obtain the same amount of food was progressively increased) in terms of distance ran to
422   obtain food; the NON group had free access to food and exercise. This phase lasted 42 to 45
423   days.

424   Five days before phase 1, a standard incremental-speed exercise test on a treadmill
425   was used to characterize the animals. Therefore, all rats underwent familiarization and
426   incremental running tests on a treadmill (Gaustec, Belo Horizonte, Brazil). First, the animals
427   were subjected to a period of 5-8 consecutive days of familiarization on the treadmill. The
428   rats remained 5 minutes with the treadmill off and another 5 minutes running at a speed of
429   18m/min with a 5% incline. The exercise was motivated by a mild electrical current (0.5 mA)
430   from a grid at the back of the treadmill; this level of stimulation has previously been shown
431   to motivate rats to run (Lima et al., 1998, Soares et al., 2003, 2004, Pires et al., 2007;
432   Wanner et al., 2007). Second, a progressive test was performed after this familiarization
433   period to determining each rat's maximum running speed. The initial test speed was 10
434   m/min, increased by 1 m/min every 2 minutes (Koch and Britton, 2001) until the animal
435   remained touching the electrical grid for 10 consecutive seconds (normally the rats avoid
436   being close to the electrical grid). This criterion has been used in previous studies from our
437   laboratory (Lima et al., 1998, Soares et al., 2003, 2004, Pires et al., 2007; Wanner et al.,
438   2007). The rats were matched for maximum speed achieved during the progressive test on a
439   treadmill and divided into the three experimental groups aforementioned.

Phase 1 (-16 days): all animals were familiarized with colonic temperature measurement for a period of 4 to 6 days. Colonic temperature was measured using disposable probes (Yellow Spring Instruments, disposable rectal probe series USA 4400; accuracy 0.01°C) connected to a tele-thermometer (Yellow Spring Instruments). Before insertion of the probe, faeces were removed by means of a gentle massage; the external probe was lubricated with Vaseline gel and inserted 6 cm beyond the anal sphincter. The temperature was recorded for two minutes after insertion of the probe. Also carried out in this period, were measurements of daily food intake and body mass using weighing scales (accurate to 0.5 g; MF3 Filizola scale, São Paulo). The *ad libitum* food intake of the rats was calculated from five days of individual free access to the aforementioned diet, which was approximately 23.70 ± 0.28 g/day or about 6 pellets/day (i.e., 24g/day).

Phase 2 (-10 days): after phase 1, the animals in groups CON and NON were maintained for 10 days in activity cages with free access to the running wheel, without its use being contingent upon food rewards. This familiarized them with the environment of the cage, running on a wheel and the feeding box.

Phase 3 (0 to 45 days): the distance travelled to obtain previously established *ad libitum* feeding level (i.e., 24g/day) was 1320 m and this was the starting level for the progressive ratio in the CON group (details on operant conditioning and progression ratios see McSweeney and Murphy, 2014). To generate the progressive ratio distances were increased on the previous level by 90% every three days (i.e., 1320 m, 2520 m, 3696 m, etc) until the animal reached the maximal daily distance (DD_{max}) in the CON group. The criterion for determining DD_{max} was a reduction of 15% to 20% in body weight (Perrigo and Bronson, 1983; Vaanholt et al., 2007). This limit to the reduction in body weight was chosen according to the results of Ferguson and Paule (1997), which showed no change in food seeking motivation when a reduction between 15 and 20% of body mass was applied.

After achieving the DD_{max} (in 42 to 45 days depending on the individual), animals of all groups were again subjected to a period of familiarization on the treadmill for five days, and progressive test was performed again (to details, see above). This test was used again at the end of all experiments to provide baseline data to compare the impact of the

469 treatments on the rats running abilities and to verify if the results in performance using this
470 progressive protocol are coherent with performance of rats in wheels.

471 The total duration of the experimental procedures was 75 days for all groups.

Animal management

473 The animals were removed daily from their activity (CON and NON) or regular (SED)
474 cages between 0900 and 1200 hrs and placed in individual acrylic cages (29 cm long, 18 cm
475 wide and 12 cm high) for weighing, taking colonic temperature, reading exercise data and to
476 clean cages.

Euthanasia

478 The animals were killed by decapitation using a guillotine (Insight Equipment,
479 Ribeirão Preto, São Paulo, Brazil) two days after the last progressive test. After euthanasia,
480 we measured the length and mass of the right femur, and the final length of the animal.
481 These measures were used to infer the growth of the animal. To measure the length of
482 femurs a calliper accurate to 0.05 mm (Mitutoyo, Kawasaki, Japan) was used and the
483 measurement was performed between the greater trochanter and the lateral and medial
484 condyles. For weighing femurs an analytical balance was used (Mettler Toledo AL 204,
485 accurate to 0.0001 g). The final length of the animals was measured from the first thoracic
486 vertebra to the first vertebra of the tail.

487 The epididymal adipose tissue (EAT), the retroperitoneal adipose tissue (RAT) and
488 the mesenteric adipose tissue (MAT) were collected and weighed separately to calculate the
489 index of adiposity (Taylor and Phillips, 1996), according to the equation:

490 Adiposity index (%) = (EAT + RAT + MAT) x 100/body mass

491 Immediately after euthanasia, samples of the epididymal adipose tissue were fixed in
492 a PBS-buffered formaldehyde solution for 24 h prior to histological analysis. These samples
493 were incubated in 70% ethanol prior to processing via routine paraffin embedding. Five -
494 micrometer-long sections of the tissue were stained with haematoxylin and eosin. Images of
495 the adipose tissue sections were captured using a digital camera coupled to a microscope
496 and the area was measured under 10 x magnification using Image Pro-Plus software (Media

497   Cybernetics, USA). The area of 50 cells was measured in each animal using Image Pro-Plus

498   software (Media Cybernetics, USA) and ImageJ (National Institutes of Health, Bethesda,

499   Maryland, USA) was used to calculate mean adipocyte area, according Oliveira et al. (2013).

500   **Statistics**

501   Data were found to meet the requirements for parametric statistics (Zar, 2010).

502   Thus, we used two-way ANOVA with repeated measures to determine significant

503   differences in responses between the experimental phases and treatment groups. For

504   comparison of progressive tests performed before and after phase 3 and between groups,

505   we used analysis of variance with two factors (groups and the pre/post phases). For

506   variables related to growth and mass of tissue, analysis of covariance was used, considering

507   the body mass or total length of the animal as a covariate. A *post hoc* Tukey's test was used

508   to indicate significant differences. To verify the relationship between the distance travelled

509   by the animal and the distance/food ratio a Pearson's correlation test was used for group

510   CON. Data were presented as mean ± standard error or mean ± standard deviation. The

511   level of significance was set at $p < 0.05$ for all tests.

515   **AUTHOR CONTRIBUTIONS**

516   I.A.T.F., L.O.C.R. and R.J.Y. designed the experiment, wrote the manuscript and

517   analysed the data. I.A.T.F., R.L.F.P, F.A.A., M.R.M.L., D.R.L., W.P. and D.D.S. collected the

518   data, conducted the experiments and all contributed to both statistical analyses and

519   reviewing of the manuscript.

520   **COMPETING INTERESTS**

521   No competing interest declared.

526

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720 **LEGENDS**

721

722 **Fig. 1. Measured variables in phase 1 (P1; -16 days), in phase 2 (P2; -10 days) and then**
723 **every three days, in contingent (CON), non-exercise contingent (NON) and sedentary**
724 **(SED) groups (P3 – phase 3).** Data are presented as mean ± standard error. 2a) Mean
725 distance travelled (m); 2b) Mean body mass (g); and 2c) Mean food intake (g). The dotted
726 line in the graph represents the distance that must be travelled to obtain *ad libitum* feeding
727 levels (approximately 24g of food per day). The letter "a" indicates the difference between
728 the groups with and without contingency, letter "b" indicates difference between groups
729 contingency and sedentary, the letter "c" indicates the difference between the running-food
730 contingent group (CON) and the other groups, the letter "d 'indicates the difference
731 between the sedentary group and the others (p <0.05). The horizontal bar at the bottom of
732 the graph indicates the different experimental phases.

733 **Fig. 2. Colonic temperature (°C) depending on the day of the experiment in contingent**
734 **(CON), non-exercise contingent (NON) and sedentary (SED) groups.** Data are presented as
735 mean ± standard error. "a" indicates difference between groups food-exercise contingent
736 and sedentary groups. The letter "b" indicates the difference between the food-exercise
737 contingent (NON) and the other groups (p<0.05). The horizontal bar at the bottom of the
738 graph indicates the different experimental phases (1 to 3).

739

740 **Fig. 3. Adiposity index (A) and adipocyte area (B) of epididymal adipose tissue for**
741 **contingent (CON), non-exercise contingent (NON) and sedentary (SED) groups**. Epididymal
742 adipose tissue histology analysis - HE: Bar: 50µm. C: contingent group; D: non-exercise
743 contingent group; E: sedentary group. Data are presented as mean ± standard deviation.
744 *indicates difference between NON and SED groups (p<0.05).

745 **Fig. 4. Photograph of the activity cage that allowed a contingency between the exercise**
746 **performed and the amount of food earned**. 1: electronic interface for programming and
747 storage of data (distance (m), mean speed (m/min), maximum speed (m/min), maximum
748 feed pellets available and number of pellets delivered); 2: running wheel; 3: electronic food
749 dispenser; 4: feeding box.

750

751   Table 1. Maximum speed (m/min) and duration (min) of progressive tests, undertaken by

752   the experimental rats, before (PRE) and after (POST) the experimental phase (phase 3)

	MAXIMAL SPEED		DURATION	
	PRE	POST	PRE	POST
CON	31.0±3.9	16.6±5.9*	42.0±7.9	14.2±11.2*
NON	29.0±4.1	22.4±10.1*	38.8±7.8	25.8±20.4*
SED	27.3±8.3	25.6±18.4*	34.5±16.6	31.8±36.2*

753   Data are presented as mean ± standard deviation. CON: contingent group; NON: non-

754   exercise contingent group   and SED: sedentary group. *Denotes a significant difference

755   between the pre and post conditions (p<0.05).

756 Table 2. Wet tissue masses (g) and length of right femur (mm) of the experimental rats and

757 adjusted means from ANCOVA using body length as covariate.

	CON	NON	SED
Body length	162.50 ± 4.90	172.40 ± 4.40	162.50 ± 4.90
Brown adipose tissue	0.19 ± 0.02^a	0.32 ± 0.11^b	0.35 ± 0.09^b
Adjusted means	0.20 ± 0.04^a	0.31 ± 0.04^a	0.33 ± 0.04^a
Liver	7.50 ± 1.73^a	12.52 ± 1.08^b	12.10 ± 0.89^b
Adjusted means	7.88 ± 0.56^a	12.82 ± 0.53^b	12.02 ± 0.56^b
Heart	0.94 ± 0.10^a	1.34 ± 0.13^b	1.22 ± 0.11^b
Adjusted means	0.90 ± 0.06^a	1.33 ± 0.05^b	1.18 ± 0.06^b
Right gastrocnemius	1.66 ± 0.16^a	2.57 ± 0.16^b	2.25 ± 0.07^c
Adjusted means	1.67 ± 0.08^a	2.58 ± 0.08^b	2.25 ± 0.08^c
Right Soleus	0.15 ± 0.02^a	0.19 ± 0.03^a	0.16 ± 0.04^a
Adjusted means	0.16 ± 0.02^a	0.18 ± 0.02^a	0.16 ± 0.02^a
Adrenal Gland	0.02 ± 0.01^a	0.03 ± 0.01^b	0.02 ± 0.01^a
Adjusted means	0.016 ± 0.003^a	0.028 ± 0.003^b	0.020 ± 0.003^{ab}
Mass of right femur	1.57 ± 0.25^a	1.46 ± 0.30^a	1.66 ± 0.30^a
Adjusted means	1.60 ± 0.16^a	1.47 ± 0.16^a	1.69 ± 0.16^a
Length of right femur	37.60 ± 0.50^a	39.20 ± 0.70^b	39.90 ± 1.00^b
Adjusted means	38.00 ± 0.40^a	38.90 ± 0.40^{ab}	40.20 ± 0.40^b
Body mass	269.20 ± 7.00^a	413.40 ± 13.20^b	426.90 ± 7.70^b
Adjusted means	272.70 ± 12.20^a	415.10 ± 11.60^b	428.10 ± 12.20^b

758 Data presented as mean ± standard deviation. Data of brown adipose tissue, liver,

759 heart, right gastrocnemius, soleus and adrenal gland were multiplied by 1000.

760 CON: contingent group; NON: non-exercise contingent and SED: sedentary group

761 Different letters denote significant differences between groups ($p < 0.05$).

762

A

Adiposity index (%)

- contingent group
- non-exercise contingent group
- sedentary group

*

B

Adipocyte area (μm^2)

*

C

50 µm

D

50 µm

E

50 µm