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RESEARCH ARTICLE

Exercising for food: bringing the laboratory closer to nature

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ABSTRACT

Traditionally, exercise physiology experiments have borne little resemblance to how animals express physical activity in the wild. In this experiment, 15 adult male rats were divided into three equal-sized groups: exercise contingent (CON), non-exercise contingent (NON) and sedentary (SED). The CON group was placed in a cage with a running wheel, where the acquisition of food was contingent upon the distance required to run. Every 3 days the distance required to run to maintain food intake at free feeding levels was increased by 90% in comparison to the previous 3 days. The NON group was housed identically to the CON group, but food acquisition was not dependent upon running in the wheel. Finally, the SED group was kept in small cages with no opportunity to perform exercise. A two-way ANOVA with repeated measures was used to determine significant differences in responses between the experimental phases and treatment groups, and ANCOVA was used to analyse growth and tissue mass variables with body length and body mass used separately as covariates. A *post hoc* Tukey's test was used to indicate significant differences. A Pearson's correlation was used to test the relationship between the distance travelled by the animal and the distance/food ratio. The level of significance was set at $P<0.05$ for all tests. The CON group showed the hypothesized correlation between distance required to run to obtain food and the mean distance travelled ($P<0.001$), during 45 days in the contingency phase. This group showed a decrease in body mass, rather than an increase as shown by NON and SED groups. The CON group had a significantly lower body temperature ($P<0.05$) and adiposity ($P<0.05$) when compared with the other two groups for the same body size. The present experimental model based on animals choosing the characteristics of their physical exercise to acquire food (i.e. distance travelled, speed and duration) clearly induced physiological effects (body characteristics and internal temperature), which are useful for investigating relevant topics in exercise physiology such as the link between exercise, food and body mass.

KEY WORDS: Feeding behaviour, Motivation, Progressive ratio, Running wheel, Voluntary exercise

INTRODUCTION

Traditionally, experiments in exercise physiology have been different from the conditions in which wild animals would undertake

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

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

In behavioural ecology, one of the fundamental concepts is that animals express behaviour in a manner that provides them with the greatest net return (Krebs et al., 2012). In other words, animals try to maximise the cost–benefit ratio in terms of energy intake, and this concept is the basis of optimal foraging theory, one of the cornerstones of behavioural ecology, which has received considerable empirical support (Krebs et al., 2012). However, animals being forced to run on a treadmill as a result of electric shocks are unable to maximise the cost–benefit ratio, as even basic factors affecting energy consumption such as running speed and duration are out of their control. Thus, this traditional experimental model does not relate closely to the reality of wild animals' lives. It is, after all, the choices that animals make that affect their fitness and upon which natural selection acts (Careau and Garland, 2012;

Swallow et al., 2009). Other experimental models may approach the decisions that animals deal with in the wild by considering some choices that animals make in order to perform the exercise, such as the relationship between energy expenditure and time spent seeking and obtaining a certain amount of food (Rowland et al., 2008).

The theoretical model of energy homeostasis considers the control of body mass because of the balance between the energy cost of the activity performed for obtaining food, basal metabolism and the energy value of the food obtained (Gelegen et al., 2006). Therefore, there is a natural link between physical activity and feeding. This link has been assessed using experimental protocols, which isolate the effects of one factor such as the effect of exercise on caloric intake (Tokuyama et al., 1982; Stubbs et al., 2002) or the effect of energy on the amount of exercise performed (Belke et al., 2004; Kanarek et al., 2009). Nevertheless, in the exercise protocols usually used, exercise is driven by one of the following: threat of electrical stimulation on a treadmill (rodents); survival in water tanks (rodents); or verbal encouragement on a treadmill or exercise bicycle (humans). Even in protocols that utilize voluntary exercise, performed on a running wheel, the exercise is usually not contingent upon the provision of food; therefore, it is observed that some species, including rats, will voluntarily perform physical activities without an apparent function (Sherwin, 1998; Meijer and Robbers, 2014), which alters various aspects of physiology and behaviour, such as their caloric intake (Novak et al., 2012). It is known that rats and mice exhibit abnormal behaviour when subjected to caloric restriction protocols with free access to the wheel [i.e. obsessive wheel running (Sherwin, 1998)], and this behaviour has been associated with anorexia nervosa (Routtenberg and Kuznesof, 1967; Altemus et al., 1993; Burden et al., 1993).

In the laboratory, few studies have linked the provision of food to the amount of physical activity performed. Perrigo and Bronson (Perrigo and Bronson, 1983), for example, pioneered the use of this method; they investigated the effect of increasing the cost of foraging on the reproductive capacity of mice *Mus musculus* (females). Vaanholt et al. (Vaanholt et al., 2007), using the same experimental model in mice, observed that animals selected for high activity levels ran approximately 28% more than the control group when there was no contingency between physical activity and food. When the food was contingent upon exercise, the two groups travelled similar distances (23.2 ± 1.4 versus 23.4 ± 1.4 km day^{-1}, respectively). This result suggests that when animals need to perform exercise to acquire food, the intrinsic differences between individuals mentioned above (such as selection for high levels of activity) may not be decisive in their performance – instead, hunger is the key determinant.

The aim of this study was to verify the effect of increasing physical exercise (distance travelled and speed) required to obtain the same amount of food on the anthropometrical (body mass, growth and tissue mass) and physiological parameters (core temperature) of rats *Rattus norvegicus* (Berkenhout 1769).

RESULTS

As hypothesized, the mean (±s.e.m.) daily distance travelled by the exercise contingent (CON) group increased throughout the experiment from 1311 ± 275 m during adaptation (phase 2) to 9037 ± 1033 m on day 42 (phase 3), with an overall mean of 6132 ± 757 m during the experimental phase. This was a 589% increase compared with the spontaneous distance travelled by the animals during the adaptation period (phase 2). As expected, there was a significant correlation between the distance required to obtain food and the distance travelled by the animals ($r=0.86$, $P<0.001$).

For the non-exercise contingent (NON) group, there was no significant difference in spontaneously travelled distance over the time of the experiment (overall mean 1068 ± 95 m). In the last 3 days of the experiment, the distance travelled by the CON group was 5.6 times higher than that of the NON group (mean ± s.e.m. = 9037 ± 1033 versus 1621 ± 782 m, respectively; Fig. 1A).

The sedentary (SED) group and the NON group body masses steadily increased during the experiment [mean ± s.e.m. = 311.4 ± 6.8 versus 296.5 ± 7.3 g on day -16 (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
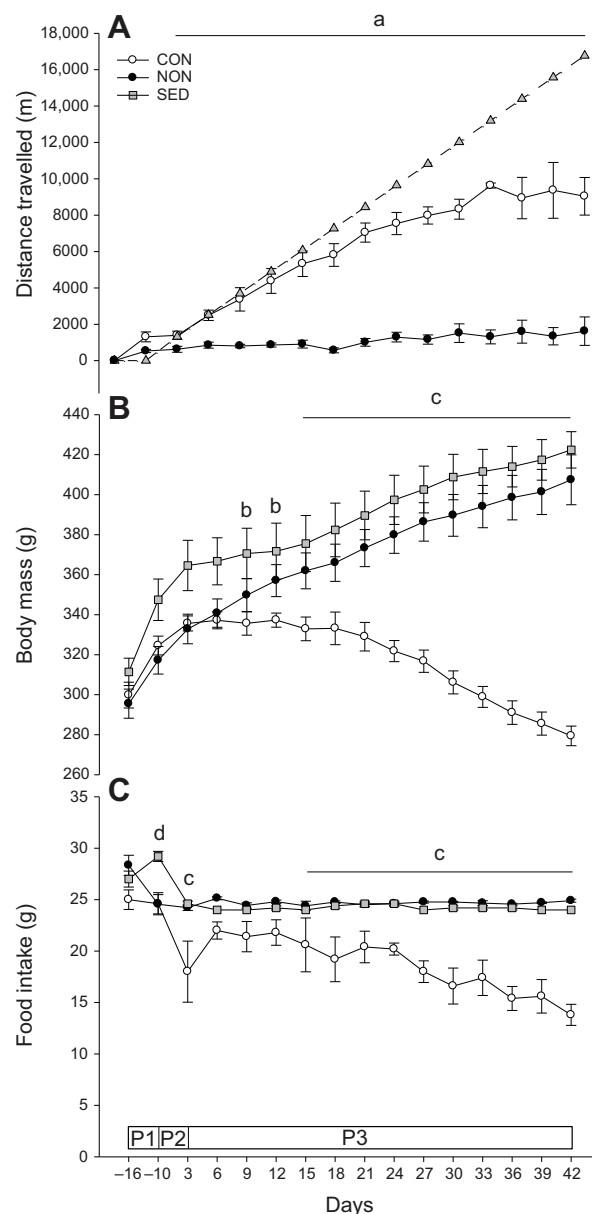

Fig. 1. Measured variables in phase 1 (P1; −16 days), in phase 2 (P2; −10 days) and then every 3 days, in contingent (CON), non-exercise contingent (NON) and sedentary (SED) groups (P3, phase 3). Data are presented as means ± s.e.m. (A) Mean distance travelled (m); (B) mean body mass (g); (C) mean food intake (g). The dashed line represents the distance that must be travelled to obtain *ad libitum* feeding levels (~24 g of food per day). a, difference between CON and NON; b, difference between CON and SED; c, difference between CON and the other groups; d, difference between the SED and the others (*P*<0.05). The horizontal bar at the bottom of the graph indicates the different experimental phases.
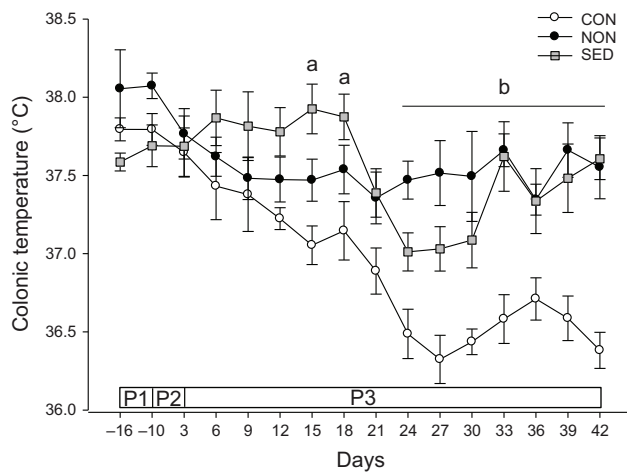
Fig. 2. Colonic temperature (°C) depending on the day of the experiment in contingent (CON), non-exercise contingent (NON) and sedentary (SED) groups. Data are presented as means ± s.e.m. a, difference between CON and SED; b, difference between NON and the other groups ($P<0.05$). The horizontal bar at the bottom of the graph indicates the different experimental phases (1 to 3).

groups, respectively; Fig. 1B]. The CON group, in comparison, had a stable body mass until day 24 (phase 3) (332.5±1.8 g), followed by a progressive reduction until day 42 (279.4±4.9 g), when the animals reached a 16% reduction in body mass in relation to their phase 3

stable body mass (Fig. 2). On the day of euthanasia, the CON group had lower body mass compared with the NON and SED groups (269.2±7.0, 413.4±13.2 and 426.9±7.7 g, respectively). Furthermore, on the same day, there was no difference between groups in body length without or with body mass as a covariate ($P>0.05$ in both cases; 162.5±4.9, 172.4±4.4 and 162.5±4.9 mm for CON, NON and SED, respectively, without body mass as a covariate; 157.4±14.7, 174.7±7.8 and 165.6±9.9 mm with body mass as a covariate).

Only the CON group progressively reduced the amount of food ingested (mean ± s.e.m. = 25.0±0.9 phase 1 versus 13.8±1.0 g in phase 3; equates to –44.8%; Fig. 2C). In the NON and SED groups, there was no change in food intake during the experiment (24.7±0.1 versus 24.2±0.1 g, respectively).

Fig. 3 shows a colonic temperature reduction in the CON group throughout the experiment in relation to the other groups, which occurred mainly from day 24 onwards (mean ± s.d. = 36.5±0.2 versus 37.5±0.1 versus 37.0±0.1°C on day 24 for CON, NON and SED, respectively). This was also seen when the CON group's first colonic temperature (phase 1=37.8±0.1°C) was compared with its last one (phase 3=36.4±0.1°C).

The CON group increased running speed to obtain food (from 18.4±2.1 m min^{-1} on the first progressive ratio to 35.2±3.3 m min^{-1} on the last progressive ratio), and this increase was significantly higher than that of the NON group ($P<0.05$). However, these running speed increases were not enough to compensate for the energy demands, leading to the rats also increasing total exercise time on the wheel (from 71.25 min at the beginning of the experiment to 256.74 min at the end). To maintain a running time of
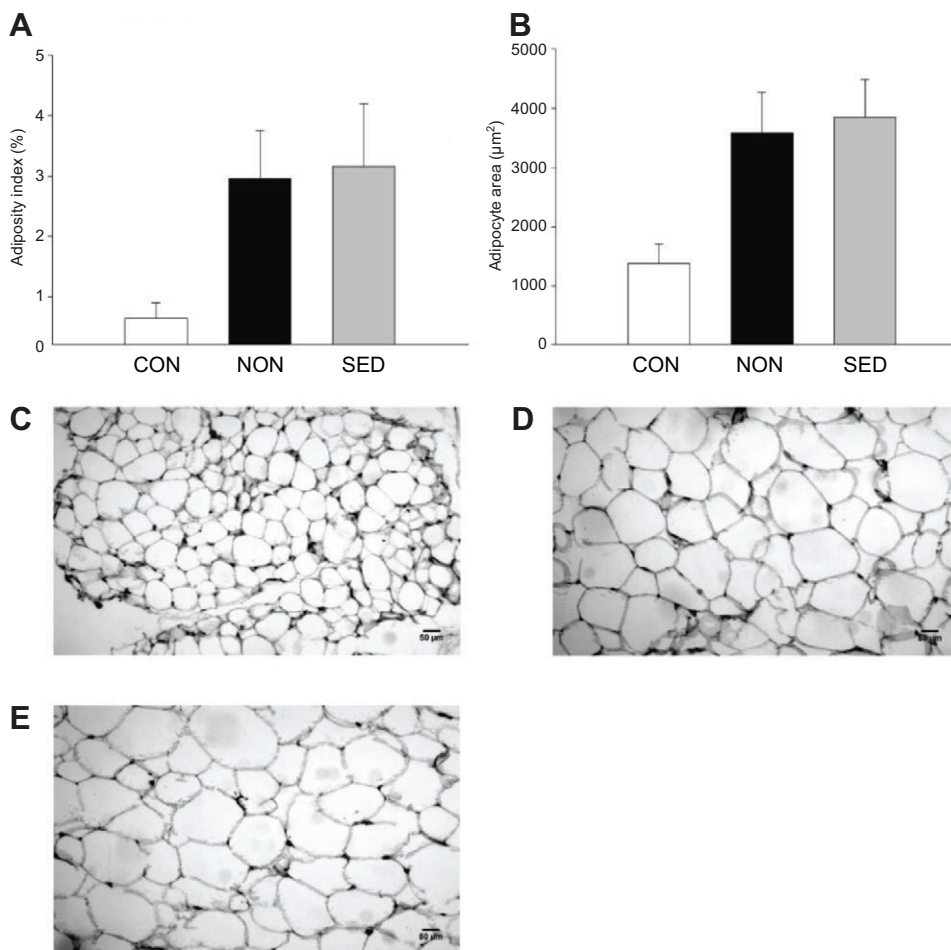

Fig. 3. The effect of treatments on adiposity index and adipocyte area of epididymal adipose tissue. Adiposity index (A) and adipocyte area (B) of epididymal adipose tissue for contingent (CON), non-exercise contingent (NON) and sedentary (SED) groups. Data are presented as means ± s.d. *Difference between NON and SED groups ($P<0.05$). (C–E) Epididymal adipose tissue histology analysis (haematoxylin and eosin) in the (C) CON, (D) NON and (E) SED groups. Scale bars, 50 μm.

Table 1. Maximum speed and duration of progressive tests, undertaken by the experimental rats, before (pre) and after (post) the experimental phase (phase 3)

	Maximal speed (m min⁻¹)		Duration (min)	
	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*

Data are presented as means ± s.d. CON, contingent group; NON, non-exercise contingent group; SED, sedentary group. *Significant difference between pre and post conditions ($P<0.05$).

71.25 min, rats in the CON group would have needed to run at 126.8 m min⁻¹ during the last progressive ratio, which would be faster than their spontaneous maximum-recorded running speed. Interestingly, the maximum spontaneous running speeds recorded were similar for the CON (mean ± s.e.m. = 81.6±1.7 m min⁻¹) and NON groups (99.5±1.2 m min⁻¹) during phase 3.

Intrinsic differences in running speed between groups were not observed, because speed and exercise time in the progressive conventional treadmill tests showed no differences among groups both in pre- and post-testing ($P>0.05$; Table 1).

Food contingent on exercise (i.e. the CON group) did not affect the final body length of the animals (mean ± s.d. = 163±10 versus 172±10 mm versus 163±9 mm for CON, NON and SED, respectively; $P>0.05$) nor the mass of femurs ($P>0.05$; Table 2). The length of the right femur was significantly shorter in the CON group compared with the other groups ($P<0.05$; Table 2). There was no influence of body length on mass of femurs, but when body length was accounted for as a covariate, the length of the right femur of the CON group was shorter compared only with the SED group (Table 2).

The CON group showed smaller masses of brown adipose tissue, liver, heart and right gastrocnemius compared with the NON and SED groups. The right adrenal gland was heavier in the NON group compared with the other groups. There was no difference between groups in the mass of the soleus (Table 2). When body length was

considered as a covariate, liver and heart showed smaller masses in the CON group compared with the NON and SED groups. The right gastrocnemius was heavier in the NON group compared with the CON and SED groups, and the right adrenal gland was heavier in the NON group compared with the CON group. There was no difference between groups in the mass of the brown adipose tissue and soleus (Table 2, adjusted means).

No difference between the groups in growth or tissue mass data was found when body mass was considerate as covariate, except for the adiposity index (mean ± s.d. = 0.64±0.25 versus 2.96±0.78 versus 3.15±1.03% for CON, NON and SED, respectively; Fig. 3A) and adipocyte area (1376.5±331.0 versus 3588.6±685.2 versus 3850.1±630.6 μm² for CON, NON and SED, respectively, Fig. 3B–E). In other words, adiposity was lower in the CON group compared with the NON and SED groups ($P<0.05$).

DISCUSSION

These results demonstrate that rats will travel increasingly longer distances to acquire food, as is expected to happen under natural conditions, during periods of decreasing food availability. Vaanholt et al. (Vaanholt et al., 2007) observed similar results in mice selectively bred for high activity levels on running wheels. Foraging is a complex behaviour that depends on factors such as food availability, presence of predators, time and energy expenditure involved in seeking and obtaining food, animal age and species, among others. In nature all these factors may be present, either singly or combined, and the animal's behaviour is the result of the costs and benefits generated by this behaviour (Houston and McNamara, 1989; Lemon and Barth, 1992; Shamoun-Baranes and van Loon, 2006; Young and Lawrence, 1996), as well as the possible intrinsic motivation to be physically active (e.g. see Meijer and Robbers, 2014, and references therein). The data recorded indicate that the present contingency method allows the experimenter to precisely control the daily distance to be travelled by the animal through manipulating the relationship between the amounts of food given as a function of distance required to obtain it. Furthermore, it was found that: (1) the amount of exercise performed voluntarily by the animal is five times higher when

Table 2. Wet tissue masses and length of right femur of the experimental rats and adjusted means from ANCOVA using body length as a covariate

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

Data are presented as means ± s.d. Data for brown adipose tissue, liver, heart, right gastrocnemius, soleus and adrenal gland were multiplied by 1000. CON, contingent group; NON, non-exercise contingent; SED, sedentary group. Different letters denote significant differences between groups ($P<0.05$).

obtaining food is contingent on physical activity; (2) the motivation to perform physical activity, as demonstrated by greater daily distance travelled, is greater in animals subject to food-exercise contingency; and (3) animals submitted to a food-exercise contingency reached a stable body mass, whereas those without showed progressive increase. The use of the present experimental model may change our understanding of the impact of exercise in various relevant issues to exercise physiology such as control of body mass and physical activity; thermoregulation and exercise; pregnancy and physical activity; and sleep and physical activity, amongst others.

Another important observation of this study was that using a progressive ratio of the distance to be travelled by the animals to obtain food (for 42 days) reduced the variability of distance travelled. Several studies have shown that when there is no contingency between physical activity and food, the distances travelled by rodents may exhibit high coefficients of variation (Chappell et al., 2004; Chappell et al., 2007). Lambert and Noakes (Lambert and Noakes, 1990), for example, observed that the distance travelled by rats ranged widely between 3.3 and 76.0 km week^{-1}. In the present work, the variability in distance run by the CON group was reduced by 49% when compared with the period of adaptation in activity cages (phase 2). The coefficient of variation was also lower in the CON group compared with the NON group (24% versus 59%, during phase 3). This reduction in the variability of distance run suggests that animals of the same species travel similar distances when they are motivated to run for food, and that this behaviour can be independent of age-related differences (Mondon et al., 1985), sex (Garland et al., 2011) and aerobic capacity (Vaanholt et al., 2007), whose effects are observed when animals perform exercise without it being rewarded with food. Thus, our results show that the contingent method decreased the interference of intrinsic and extrinsic factors on the exercise performance variability by the animals under the present restricted and controlled experimental situation, which could be useful for drug experimental studies, for example. However, in the wild, in the case of food shortage, other factors such as predation risk (or other environmental challenges) or minimal differences in age, sex and the intrinsic physical ability of the animal may be crucial for survival.

In relation to the traditionally used method to study physical exercise in animals (i.e. forced exercise on a treadmill), it is interesting to compare the coefficient of variation of the total exercise time in this type of exercise with the mean distance travelled in the present study. In previous studies from our laboratory, we found a coefficient of variation of 42% in total time of forced exercise on a treadmill at 18 m min^{-1} (Pires et al., 2007), which is a moderate level of exercise for rats, compared with the coefficient of ~20% for the total distance travelled in the CON group of the present study. It is therefore reasonable to assume that there is less variability in animal motivation when exercise was linked to obtaining food [i.e. positive reinforcers compared with electrical stimuli (i.e. punishment)].

In the laboratory, when the animals had free access to the running wheel, the NON group performed some physical activity without apparent reason. This behaviour was also observed in this investigation in the CON and NON groups, and its mechanism is unknown. Perhaps it is due to the intrinsic exploratory behaviour of each animal (Inglis et al., 2001); some studies have shown that certain genetic variants in some species are more active than others (Dewsbury, 1980; Swallow et al., 2009; Garland et al., 2011).

Our results showed that exercise linked to food caused changes in the rats' body composition. The CON group adiposity index was ~80% lower when compared with the NON and SED groups.

Similar results were found by Vaanholt et al. (Vaanholt et al., 2007), who observed a reduction in adipose tissue of ~70% in mice that ran for food (contingency was fixed at 90% of maximum) compared with the group that had free access to food and a running wheel. The effect of the food-exercise contingency on adipose tissue observed in the present study and that of Vaanholt et al. (Vaanholt et al., 2007) is higher when compared with the effects of training conducted on a running wheel without such a contingency (Tokuyama et al., 1982; Pitts, 1984; Cortright et al., 1997), on a treadmill (Lacerda, 2012; Barcellos, 2011; Pitts, 1984) or in restriction food protocols (Hill et al., 1985). The contingency of exercise to obtain food in the present study simulated a natural condition and caused a pronounced reduction in adipose tissue not observed in traditional protocols of exercise/training. Some of these results could be associated with higher daily energy expenditure for animals that have to run on wheels to obtain food as compared with those forced to run (even to exhaustion) on a motorized treadmill, even daily. Because of the caloric content of feed and the period of stability in body mass between days 3 and 24 (phase 3 in CON group), it was possible to estimate the daily energy expenditure of the animals of the CON group, which was 73.6 kcal day^{-1}. It has been reported that rats with free access to the wheel (without contingency), spend, on average, 13% of this in ingested food energy per day to run on the wheel (Afonso and Eikelboom, 2003). In this case, the animals of the present study consumed 9.56 kcal to travel approximately 4673 m (average distance travelled by the animals during the period described above), or 2.04 kcal km^{-1}. This value is lower than the 2.26 kcal km^{-1} observed by Afonso and Eikelboom (Afonso and Eikelboom, 2003), but is in agreement with values reported for Wistar rats during constant exercise on a treadmill at 18 m min^{-1} for ~50 min (2.77 kcal km^{-1}) (Soares et al., 2003). These results suggest that per kilometre there were no differences in energy expenditure, but the distance travelled by the CON group was much greater than that observed in spontaneous-wheel-running rats or rats undergoing forced exercise on a treadmill. Thus, there may be important, long-term physiological changes in food-contingent rats not found in the aforementioned protocols. This issue needs more investigation, but suggests that the natural link between exercise and food must be considered when the aim is to study body mass control, obesity and associated diseases.

Additionally, the initial stable body mass phase of CON rats may have been due to the use of stored fat reserves at the beginning of the experiment, when the cost of the activity was still small. The mass reduction at the end of the experiment (which was not due to changes in body length), in turn, indicates the amount of energy obtained was not sufficient to maintain the body mass of rats. The linear relationship between distance ran and required to run to obtain *ad libitum* feeding level (Fig. 1A) suggests that, if the progressive ratio had continued beyond the maximal daily distance (day 42), rats would have gone into an increasing energy deficit situation.

Lower body masses because of smaller fat deposits may be an advantage for survival in nature, especially for those species that depend on the realization of physical activity to avoid predators or to obtain food. Investigations into the effects of domestication on the body composition of rats (Castle, 1947) and mice (Dohm et al., 1994) have reported that wild animals are smaller and perform better on a treadmill and running wheel when compared with domesticated animals. Although the performance of the animals in the CON group was not different from the other groups when subjected to a progressive treadmill test in present study, the observed alterations in body mass and the mass of the heart, liver, gastrocnemius and adrenal CON group suggest that the link between providing food

and exercise can be a method that approximates the characteristics of laboratory animals from those observed in animals in nature.

The caloric intakes and body masses were not different when comparing the groups NON and SED. These results suggest that spontaneous physical activity of rats in the NON group was not sufficient to cause changes in body mass. Afonso and Eikelboom (Afonso and Eikelboom, 2003) found similar results. These authors found daily distances ranging between 841 and 3303 m in rats with free access to a running wheel without any contingency on food, distances similar to those observed in the NON group (mean 1068 ± 95 m day^{-1}) in the present study. A continuous increase in body mass has been observed in mice that underwent training on a treadmill (Lacerda, 2012; Barcellos, 2011), on running wheels without food-exercise contingency and those without access to exercise (Cortright et al., 1997), as was observed in the NON and SED groups of the present study.

The data relating to the final length of the rats in the present study suggest that there was no effect of the contingency on the growth of rats. Cortright et al. (Cortright et al., 1997) found similar results when they measured the effect of 9 weeks of free access to a running wheel, with no contingency to the food supply. Rezende et al. (Rezende et al., 2006) observed a reduction in body length of animals that were selected for high levels of activity on running wheels in both males and females. There animals were artificially selected for 35 generations and the changes in body size may have represented an effect of exercise-associated selection in relation to running wheels. The results observed in animals that have not been artificially selected for high levels of activity suggest that the increase in mass of the animals according to age is due to increased deposits of fat and not body growth as a whole.

The early days of running wheel access caused a reduction in food intake by the CON and NON groups. This suppression in caloric intake has been well documented in the literature in voluntary (Collier, 1970; Goodrick et al., 1983; Afonso and Eikelboom, 2003; Lattanzio and Eikelboom, 2003) and forced exercise (Richard and Rivest, 1989; Stevenson et al., 1966). Indeed, this behaviour appears to be independent of novelty (wheel presence) (O'Connor and Eikelboom, 2000), sex (Tokuyama et al., 1982), age (Looy and Eikelboom, 1989) or food availability in rodents (Routtenberg and Kuznesof, 1967; Boakes and Dwyer, 1997). Considering the model of energy homeostasis, it is possible that this behaviour is related to an adjustment of body mass (Richard and Rivest, 1989), but at present, the underlying mechanism is subject to speculation (Lattanzio and Eikelboom, 2003).

The decrease in body temperatures observed at the beginning of the experiment in the CON and NON groups may have been caused by the exercise itself, independent of the contingency, especially as no changes were observed in colonic temperatures of the SED group during this period. Although in this study metabolic rate was not measured, the reduction of colonic temperature as the progressive ratio increased in the CON group may have occurred to maintain a strategic body temperature at the expense of scarce amounts of energy, as happens during certain periods in nature, e.g. in winter (Burns et al., 2006). The reduction in internal temperature as a strategy to reduce the basal metabolism during periods of low food availability has been observed in non-human primates (Lane et al., 1996), and in several species of rodents subjected to various protocols of food restriction (Duffy et al., 1989; Severinsen and Munch, 1999; Speakman and Mitchell, 2011). Another factor associated with the availability of food, and which may also have contributed to the reduction in colonic temperature of the CON group, was the reduction in body mass, which probably changed the relationship between body surface area and body mass, thereby facilitating heat loss (Herrington, 1940). The reductions in both body mass and internal temperature are strategies to reduce basal metabolism and increase the chance of survival of the animal in periods with lower food availability and cold environments.

Aerobic capacity, inferred by the maximum speed achieved in the progressive test, was not different between the CON, NON and SED groups and was lower in the period after the experiment, including in the CON group, despite these rats having travelled long distances daily. These data suggest that there is no relationship between the performance achieved by the animals on a running wheel (with or without food contingency) and that achieved with the progressive test (Lambert et al., 1996). Other investigators have described differences in energy cost and performance in experiments on a treadmill or a running wheel (Girard et al., 2001; Chappell et al., 2004).

Animals in nature may choose to move at various speeds and durations to increase the efficiency of their physical activity (Kenagy and Hoyt, 1989; Blumstein, 1992; Weinstein, 1995; Christian et al., 1997; Irschick and Jayne, 1999). Furthermore, unlike what happens in forced protocol exercise, several species, including humans, exhibit an intermittent locomotion, such as eastern chipmunks, *Tamias* (*Tamias*) *striatus* (Kramer and McLaughlin, 2001; Girard et al., 2001). These rodents spend, intermittently, approximately 41% of their time foraging with short breaks that may help in the identification of prey, avoiding predation and improving physical fitness (McAdam and Kramer, 1998).

All of the above results suggest that the effects caused by the present contingent method in the study of energy homeostasis are very different from those involved in forced exercise protocols traditionally used and, therefore, the present protocol should be included as another option in exercise physiology studies. Food acquisition contingent on physical activity is a laboratory method capable of simulating conditions more similar to those faced by animals in the wild, resulting in reduced body mass and thermoregulatory adjustments in a few days of an experiment to cope with food shortages. Our study showed that animals performed less exercise when there was no contingency between the behaviour they were expressing and obtaining food rewards.

MATERIALS AND METHODS
Ethical note
The research herein reported was approved by the Ethics Committee on Animal Experimentation (CETEA protocol 180/10) of the Federal University of Minas Gerais, Brazil.

Animals and housing conditions
Fifteen male Wistar rats from the vivarium of the Institute of Biological Sciences, Federal University of Minas Gerais/Brazil (mean ± s.d. body mass $= \sim287.4\pm13.1$ g at 5–7 weeks of age at the beginning of the experiment), were used as the experimental subjects; they were divided into three equal-sized groups: food-exercise contingent (CON), non-exercise contingent (NON) and sedentary (SED). During all phases of the experiment, rats had *ad libitum* access to water and food (note that in one group food was contingent upon performing physical activity). Throughout the whole experiment, rats were fed with Presence/Purina rodent pellets (gross energy 359.8 kcal g^{-1}, protein 23.8%, fat 5.2%, carbohydrate 54.6% and ash 6.2%). During the study, room temperature was maintained between 24 and 26°C. Rats were maintained on a 14 h:10 h light:dark cycle (lights on at 05:00 h).

The animals in the CON and NON groups were kept in individual activity cages (Gaustec LTDA, Belo Horizonte, Brazil; Fig. 4) with internal dimensions of $400\times400\times400$ mm and external dimensions of $600\times600\times400$ mm (height × length × width). The cages had an electronic interface for programming and storage of data (distance, mean speed and
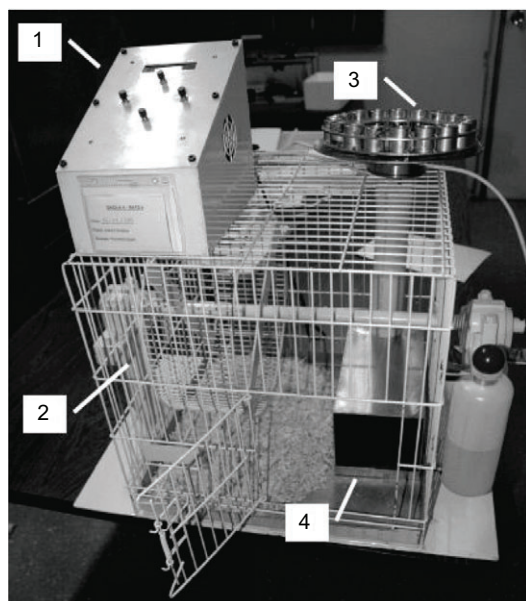
Fig. 4. Photograph of the activity cage that allowed a contingency between the exercise performed and the amount of food earned. 1, Electronic interface for programming and storage of data [distance (m), mean speed (m min^{-1}), maximum speed (m min^{-1}), maximum feed pellets available and number of pellets delivered]; 2, running wheel; 3, electronic food dispenser; 4, feeding box.

amount of food delivered per hour). A dispenser supplied food (see no. 3 in Fig. 4). The dispenser was connected to a stainless steel box in which the animal had access to the food. This box had a false bottom to collect unconsumed food, which allowed the quantification of food consumed by the animal. The running wheel was 300 mm in diameter, 942 mm in circumference and had a mean (±s.d.) torque of 0.02±0.004 Nm (3B Scientific Dynamometer U20032, precision 1 N). The mean torque was measured before each phase. A motion sensor (encoder) attached to the wheel generated the values of distance and mean velocity, independent of the direction of wheel movement. The system was calibrated before all experiments and checked for drift after all experiments.

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

Experimental design

The experiment was divided into three phases: (1) all animals were maintained in individual cages, without access to exercise but with access to food *ad libitum*, to provide measurements of food intake, colonic temperature and body mass in a baseline condition, during 4–6 days; (2) an adaptation phase to the use of the running wheel for groups CON and NON, with free access to exercise and food, during 10 days; and (3) experimental conditions in which the CON group was exposed to a progressive ratio (i.e. the distance required to run to obtain the same amount of food was progressively increased) in terms of distance run to obtain food; the NON group had free access to food and exercise. This last phase lasted 42 to 45 days.

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

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 and inserted 6 cm beyond the anal sphincter. The temperature was recorded for 2 min 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 5 days of individual free access to the aforementioned diet, which was ~23.70±0.28 g day^{-1} or approximately six pellets per day (i.e. 24 g day^{-1}).

Phase 2 (−10 days)
After phase 1, the animals in the 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 the previously established *ad libitum* feeding level (i.e. 24 g day^{-1}) was 1320 m and this was the starting level for the progressive ratio in the CON group [for details on operant conditioning and progression ratios, see McSweeney and Murphy (McSweeney and Murphy, 2014)]. To generate the progressive ratio, distances were increased on the previous level by 90% every 3 days (i.e. 1320, 2520, 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 mass (Perrigo and Bronson, 1983; Vaanholt et al., 2007). This limit to the reduction in body mass was chosen according to the results of Ferguson and Paule (Ferguson and Paule, 1997), which showed no change in food-seeking motivation when a reduction of 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 5 days, and progressive test was performed again (for details, see above). This test was used again at the end of all experiments to provide baseline data to compare the impact of the treatments on the rats running abilities and to verify whether the results in performance using this progressive protocol are coherent with performance of rats in wheels.

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

Animal management
The animals were removed daily from their activity (CON and NON) or regular (SED) cages between 09:00 and 12:00 h and placed in individual acrylic cages (29 cm long, 18 cm wide, 12 cm high) for weighing, taking colonic temperature, reading exercise data and to clean cages.

Euthanasia
The animals were killed by decapitation using a guillotine (Insight Equipment, Ribeirão Preto, São Paulo, Brazil) 2 days after the last progressive test. After euthanasia, we measured the length and mass of the right femur, and the final length of the animal. These measurements were used to infer the growth of the animal. To measure the length of femur, a calliper accurate to 0.05 mm (Mitutoyo, Kawasaki, Japan) was used and the measurement was performed between the greater trochanter and the lateral

and medial condyles. For weighing femurs an analytical balance was used (Mettler Toledo AL 204, accurate to 0.0001 g). The final length of the animals was measured from the first thoracic vertebra to the first vertebra of the tail.

The epididymal adipose tissue (EAT), the retroperitoneal adipose tissue (RAT) and the mesenteric adipose tissue (MAT) were collected and weighed separately to calculate the index of adiposity (Taylor and Phillips, 1996), according to the equation: adiposity index (%) = (EAT + RAT + MAT) × 100/body mass

Immediately after euthanasia, samples of the epididymal adipose tissue were fixed in a PBS-buffered formaldehyde solution for 24 h prior to histological analysis. These samples were incubated in 70% ethanol prior to processing via routine paraffin embedding. Five-micrometer-long sections of the tissue were stained with haematoxylin and eosin. Images of the adipose tissue sections were captured using a digital camera coupled to a microscope, and the area was measured under ×10 magnification using Image Pro-Plus software (Media Cybernetics, USA). An area of 50 cells was measured in each animal using Image Pro-Plus software (Media Cybernetics), and ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to calculate mean adipocyte area, according to Oliveira et al. (Oliveira et al., 2013).

Statistics

Data were found to meet the requirements for parametric statistics (Zar, 2010). Thus, we used two-way ANOVA with repeated measures to determine significant differences in responses between the experimental phases and treatment groups. For comparison of progressive tests performed before and after phase 3 and between groups, we used ANOVA with two factors (groups and the pre/post phases). For variables related to growth and mass of tissue, ANCOVA was used, considering the body mass or total length of the animal as a covariate. A *post hoc* Tukey's test was used to indicate significant differences. In the CON group, to verify the relationship between the distance travelled by the animal and the distance/food ratio, a Pearson's correlation test was used. Data are presented as means ± s.e.m. or means ± s.d. The level of significance was set at $P<0.05$ for all tests.

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