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Title page

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Running title: Wheel running in female C57BL/6J mice.

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Abstract

Wheel running in female C57BL/6J mice: impact of oestrus and dietary fat, and effects on sleep and body mass.

Objective: To examine the impact of two diets differing in fat content and of wheel-running exercise on body mass.

Methods: 32 female C57BL/6J mice were assigned to either a high fat (HF, 41% of dietary energy as fat) or low fat (LF, 11% of dietary energy as fat) diet (16 per diet, individually housed). 8 mice from each diet group were housed with running wheels. Non-running mice were housed in similar cages, minus wheels. Total cage activity (including non-exercise physical activity (NEPA) + wheel running) and sleep time were also measured using an infra-red sensing device. Oestrus stage of the wheel-running mice was assessed daily for 17d.

Results: After 8 weeks HF mice were significantly heavier than LF mice (P=0.004) but there was no detectable difference in body fat mass. Wheel running mice tended to have a lower body mass than non-running controls (P=0.056). Voluntary cage activity was greater in LF control mice than HF control mice, and in wheel-running compared with non wheel-running mice. HF control mice slept more than LF control mice. Stage of oestrus was significantly correlated with running distance, with mice running farthest in the immediate pre-oestrus phase and least immediately after oestrus.

Conclusion: This study demonstrates that high fat diets in female C57BL6/J mice may increase sleep time similar to the effect of daytime sleepiness observed in obese humans.

Keywords

Body composition, high fat diet, wheel-running, activity, oestrus cycle, sleep.

Introduction.

Obesity is becoming increasingly prevalent among both adults and children. Where life expectancy has been increasing for decades, obesity reverses this trend (1). Health implications of being obese include increased risk of heart disease, high blood pressure, diabetes, osteoarthritis, stroke and cancer (2). The increase in obesity is caused by sustained positive energy balance, as lives have become more sedentary and energy dense foods have become more readily available, facilitating higher energy intake (2).

Obesity can affect sleep patterns; sleep apnoea, caused by excess fat around the chest, abdomen and neck increases the work of breathing, leading to frequent waking and therefore poor sleep quality. Additionally, decreased sleep time in children predicts overweight (3) and is associated with obesity in the elderly (4). Disordered sleep may also be a risk factor for metabolic syndrome in both children and adults (5, 6). However, excessive daytime sleepiness (EDS) is thought to relate to obesity independently of sleep-disordered breathing (7), and recent studies have linked increased body weight in mice with increased sleep (8), particularly during the dark period when mice are usually more active, an observation the authors compare with human daytime sleepiness.

The two major modifiable behaviours in the energy balance equation are dietary intake and energy expenditure in the form of physical activity. In animal models "voluntary" physical activity includes general cage activity such as climbing, rearing, foraging and grooming described as non-exercise physical activity (NEPA), in addition to wheel running and other more structured forms of activity. In elegant early studies, Richter reported that female (but not male) rats displayed a 4d periodicity in voluntary running distance (9). Mice also exhibit sexual dimorphism with respect to both wheel and treadmill running (10-12). De Bono *et al*., (11) alluded to an effect of oestrus cycle on daily running distance and a similar effect was seen in pilot studies in our laboratory (data not shown).

The objectives of this study were to examine the effects of high and low fat diets on body mass, physical activity and time spent sleeping in female C57BL/6J mice. In addition, we examined the effect of the oestrus cycle on running distance and investigated the effects of dietary and exercise treatments on sleep behaviour.

Materials and methods.

Animals. All procedures were undertaken under licence from the UK Home Office. Thirty two 8week old female C57BL/6J mice (Harlan, Oxon, UK) were housed in individual cages and randomised to either high fat (HF) or low fat (LF) diets (16 mice per diet). A 12h light:dark cycle was maintained.

Diets. The formulation of the diets was based on Bell *et al.*, (1997) (13), as summarised in Table 1. Contributions of fat, carbohydrate and protein to total energy were 41, 37 and 22% respectively for the HF diet and 11, 64 and 25% respectively for the LF diet. Mice were offered 6g food daily and food refusals were measured daily for HF-fed mice only over 2 weeks of the study (weeks 4 and 5). Water was freely available.

Running wheels. 8 mice from each diet group were given free access to a running wheel (designated WH) (internal diameter 14cm) in the cage. A magnet attached to the wheel actuated a revolution counter fixed to the top of the cage and the number of revolutions was recorded daily. Mice without access to wheels were designated control (CON) animals.

Oestrus cycle. The oestrus stage of mice with access to running wheels was assessed at the same time on 17 consecutive days. A fine tip pastette (Alpha Laboratories Ltd, Hants, UK) was placed into the vagina and one drop of sterile saline used to collect the cells. The resulting suspension was placed onto a microscope slide and left to air dry before being stained with 0.1% methylene blue. Cell suspensions were examined under a microscope to determine stage of oestrus (14). Slides were categorised stage 1-4 as follows: 1- (not in oestrus) many leucocytes present, possibly a few epithelial cells; 2- (approaching oestrus) mainly epithelial cells, few leucocytes; 3- (oestrus) no leucocytes, cornified cells only; 4-(post-oestrus) cornified cells and leucocytes.

Body composition. After 8 weeks of study, total anaesthesia was obtained using 3% isofluorane in oxygen and blood was collected by cardiac exsanguination. The amount of fat in the carcass (excluding the heart and liver) was measured. Carcasses were first freeze-dried to remove water and then body fat was extracted following the Soxhlet procedure.

Non-exercise physical activity (NEPA). An infrared-sensing device (“Inframot”, TSE Systems, Bad Homburg, Germany) was used to quantify cage activity by singly

housed mice. Inframot detects movements of 5ms length and greater and stores the data accumulated in set timescales as 'counts'. Each mouse was assessed for 24 hours on two non-consecutive occasions to provide information on total amount of activity and activity by light and dark phases. Activity was accumulated per minute during the first recording and in 10 minute bouts for the second recording. Data from the first recording were then accumulated to 10 minutes. Time spent sleeping was determined according to 3 criteria viz. i) zero movement 'counts' recorded in each 10 minute recording epoch; ii) up to 10 counts of activity per 10min epoch and iii) zero counts of activity per 1 minute epoch (only one recording available).

Statistical analysis. The study was designed as a 2*2 factorial design with 2 levels of each of the main factors viz dietary fat content ("Diet") and wheel running ("Exercise"). The four experimental groups were thus high fat wheel-running (HF WH), high fat control (HF CON), low fat wheel-running (LF WH) and low fat control (LF CON). In compliance with standard practice for the analysis and reporting of such studies (15) we have focussed on the effects of the main factors. For example, the effect of dietary fat content i.e. LF v. HF represents a comparison of concentration of dietary fat averaged across both levels of exercise. Data were analysed using the General Linear Model of ANOVA in Minitab release 14. Non-normal data (assessed using the Anderson-Darling test for normality) were transformed (Log10) before analysis. "Diet" and "exercise" were used as the factors, with interactions between the two factors tested where applicable. Mean and SEM are shown for normally distributed data and geometric mean and 95%CI for transformed data. Data for cage activity and food intake were transformed. The difference between the distances run during each stage of the oestrus cycle was tested using one-way ANOVA with Tukey's 95% simultaneous confidence intervals. P values <0.05 were considered significant.

Statement of ethics. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. Newcastle University Ethics Committee approved the research.

Results.

Wheel-running distances. The average daily distance run per week increased with time to peak in weeks 2-3 and then declined gradually, with little further decrease

over the final 2-3 weeks of the study. The distance run by HF mice peaked at week 2 and at week 3 for LF mice (Figure 1). There was no significant effect of diet on running distance at any week, or on the total distance run after 8 weeks. The average daily distances run were 6.6 and 6.7km for HF and LF mice respectively.

Body mass, body composition and food intake. Due to variation in initial body weights, the weight at week 1 was included in the statistical analysis as a covariate. After 8 weeks dietary exposure, HF mice were 2.1g heavier than LF mice (P=0.004) and CON tended to be heavier than WH (P=0.056, Figure 2). HF mice gained 4.6g compared with 2.5g for LF mice (P=0.004). One HF CON mouse became much heavier than the others (33.2g compared with an average of 21g (0.27) for the rest of the group). After exclusion of this mouse the final body masses of the groups were 21.9g v. 20.1g for HF v. LF (P<0.001) and 20.8g v. 21.1 g for WH v. CON (P=0.481). There was no significant interaction between diet and exercise (P=0.424).

There was no difference in % body fat of HF and LF mice (13.4% v. 11.1%, P=0.107), or for WH compared with (11.5% v. 13.0%, P=0.276). There was no correlation between the distance run in wheels and either final body mass (r = 0.263, P=0.263) or body fat content (r = 0.353, P=0.180).

The amount of high fat food eaten was not different between exercise groups for either week of measurement (3.3 v. 3.1g/mouse for WH and CON respectively, P=0.337 for week 4 and 3.1g for both groups in week 5 (P=0.826), data Log10 transformed). It was not possible to measure consumption of LF diet accurately because of scattering by the mice due to the powdery consistency of this diet.

Running and oestrus. Examination of the day-to-day variation in distance run by individual mice suggested a distinct pattern with a periodicity of approximately 4d (Figure 3a). We tested the hypothesis that this day-to-day variation was associated with stage of the oestrus cycle. We observed that mice ran furthest in stage 2 of the cycle (mean 7.77 km) with significantly (P=0.012) shorter running distances in stages 3 and 4 (5.67 and 5.45 km respectively, Figure 3b).

Activity. There were large inter-mouse differences in cage activity but individual mice had similar activity during both recording periods indicating good repeatability of the measurement (Pearson's r = 0.675, P<0.001, Figure 4). This relationship was evident during both the dark and light phases of the day (Table 2).

As expected, cage activity during the dark phase was much greater than activity during the light phase, with a mean of 12% of total daily cage activity performed during the light phase (Table 3). Light phase activity was very similar for both groups on the HF diet (80 and 91 $x10^3$ counts for WH and CON respectively) and for the WH mice on the LF diet (83 $x10^3$ counts). However, the CON mice on the LF diet recorded more than twice this activity (193$x10^3$ counts) resulting in highly significant diet*exercise interaction (P=0.003). There was no evidence of any effect of diet on cage activity during the dark phase but, as expected, cage activity was much higher for WH than for CON mice (P<0.001). This difference was attenuated in the LF diet group resulting in an apparent diet*exercise interaction that showed a trend towards significance (P=0.075).

As expected, there was a significant (P=0.001) effect of exercise treatment on 24h activity and CON mice recorded 59% of the activity of WH mice (855 $x10^3$ v. 1 439 $x10^3$ counts). There was no correlation between total 24h cage activity and final body weight (r = -0.17, P=0.341) but % body fat showed a trend towards a negative correlation with both dark phase activity (r = -0.340, P=0.057) and total activity (r = -0.328. P=0.067).

Sleep. We considered three criteria for quantifying sleep i) the stringent criterion of zero movement 'counts' recorded in each 10 minute recording epoch; ii) a slightly less stringent criterion of up to 10 counts of activity per 10min epoch and iii) the least stringent criterion of zero counts of activity per 1 minute epoch (only one recording available). The correlation in estimated sleep time between the two activity recording periods (shown in Table 2) was greater when using the second criterion for sleep i.e. up to 10 counts per 10 minute epoch than for the most stringent criterion. Using criterion (i) (the most stringent), the only statistically significant effect was that WH mice slept less during the dark phase (P=0.001) than CON mice. When criterion (ii) (i.e. up to 10 counts per 10 minutes) was applied, a similar pattern was observed. In total, HF CON slept more and LF CON slept less than the WH mice, resulting in a diet*exercise interaction (P=0.046). Again, WH mice slept less during the dark period (P=0.001). Using criterion (iii) (zero counts per minute), mice in both diet groups with access to a running wheel slept less during the dark phase (P<0.001) than did control animals (Table 4) and more during the light phase (P=0.019). This contributed to HF CON mice sleeping more in total than both LF CON (P=0.036) and the other groups

(Table 4). There were no interactions between diet and exercise on any of the sleep variables and no effect on % body fat or final body mass

The average total sleep times using the different criteria were 500 minutes (criterion i), 571 minutes (criterion ii) and 750 minutes (criterion iii).

Discussion.

This study was designed to test the hypothesis that the fat content of the diet influences voluntary physical activity with implications for maintenance of energy balance and weight gain. In addition, the effect of the diet on sleep time was assessed using a non-invasive technique. Animal studies of obesity (16) have found that a high fat diet can disrupt the circadian rhythm which, in turn, affects eating and sleeping patterns. Mice with a mutation in the *Clock* circadian gene have a higher body mass than controls, exhibit hyperphagia and have symptoms of the metabolic syndrome (17).

We found that high fat (HF)-fed mice were heavier than low fat (LF)-fed mice ($P=0.004$). However, there were no significant ($P>0.05$) differences in fat mass or running distance between the two diet groups. In contrast, Bell *et al.*, observed a significant decrease in body fat in low fat-fed compared with high fat-fed female Swiss Albino mice, and in exercised compared with non-exercised mice ($P<0.05$) (13). Turner *et al.*, (18) did not find an association between wheel-running distance and body weight in female chow-fed mice after 26 weeks. Rogers *et al.*, (19) reported no difference in body weight or fat mass of wheel-running chow-fed female C57BL/6J mice compared with non-running controls. However, Jenkins *et al,*. (2006) observed that mice fed a high-fat diet not only increased their body weight but also their sleep time (8).

The observation of reduced body fat in the wheel-running mice of Bell *et al.*, (13) but the lack of such a finding in this study could be due to the much greater distances run by the mice in the study by Bell *et al.*, (1997) compared with the present study (15km v. 6.6km).The average daily distance run by HF mice in our study was 6.6km, and by LF mice, 6.7km. For comparison, female C57BL/6J mice in the study of Lightfoot *et al.*, (12) ran approximately 5km/d, and those of Rogers *et al.*, (2008) ran 4.2km/d. The distances run by female mice in the present study compare favourably with distances run by female mice selected and bred for their running ability, which

covered 6.5km/day (20). We did not observe a correlation between cage activity or wheel running distance and body mass, but it should be noted that our female mice did not become very fat. Even so, the reason for a lack of effect of running in mice on body mass is not apparent from this study, and is contrary to the perception that increased physical activity will lead to reduced body mass. More rigorous measurements of both sides of the energy balance equation will be needed to resolve this issue – a limitation of the present study was the lack of information on food intake for mice on the LF diet and the limited recording (weeks 4 and 5 only) of food intake by mice on the HF diet.

The Inframot system which uses infrared technology to quantify animal movements as counts per unit time was used to quantify sleep time. Other studies have measured sleep using implantable telemetry (21) and implantable electrodes (8) to measure rapid eye movement (REM) sleep and non-REM sleep (NREMS). As the Inframot was not designed specifically to measure sleep, we estimated sleep using 3 different criteria based on the amount of movement measured in defined time epochs. We conclude that the Inframot device is a useful non-invasive means of characterising and quantifying sleep behaviour in C57BL/6J mice and that it may be appropriate to designate "sleep time" as periods of 1 minute with no detectable movement. This time frame of no movement as a measure of sleep has been validated using infra-red beam break technology against EEG recordings (22). The total amount of "sleep" time measured in this way compares favourably with other studies using C57BL/6 mice e.g. Pack *et al.*, (2007) (22), as does the diurnal distribution of sleep time (17). As anticipated, with the more stringent criteria for sleep assessment, total sleep time declined and inter-individual variation tended to increase.

In the present study, the total time spent sleeping and the amount of sleep during the dark phase were greater in HF CON compared with LF CON. This increased sleep time with a higher fat diet confirms the findings by Jenkins *et al.*, (2006) (8) who observed an increase in both light-phase and dark-phase NREMS in male C57BL/6J mice fed a 59% fat diet compared with mice fed a regular chow, with a greater effect seen during the dark phase (when mice are usually most active). The time spent in NREMS sleep also increased with duration of exposure to the high fat diet (8). Even when we used the most stringent criterion for sleep i.e. no movement during each 10 minutes epoch, HF CON mice slept longer than the other groups.

LF CON mice exhibited at least twice as much activity during the light phase as any of the other groups including the mice with access to running wheels. A potential explanation for this observation is that, with the lower energy-dense diet, the mice spent more time eating during the light phase, and more research on the nature of the activity undertaken during the light phase, linked with quantitative measures of diurnal eating behaviour, are warranted.

Among CON animals, we observed that LF mice were more active than HF mice. This is in accord with reported greater voluntary cage activity of LF compared with HF fed male C57BL/6 mice by Funkat et al., (23) and Almind and Kahn (24). A similar effect has been observed in rats (25).

Although previously demonstrated in rats (9), to our knowledge a novel discovery from this study was the direct link between oestrus and running distance, which had not been reported previously for laboratory mice. Earlier studies have shown that the female golden hamster runs further during pre-oestrus and oestrus than post-oestrus (26) whilst female rats run most before oestrus and least immediately after (9, 27). We observed that running distance was greatest in Stage 2 (pre-oestrus) and then declined significantly ($P=0.021$) in stages 3 and 4 (oestrus and post-oestrus), which is in accord with the findings from the rat. The early studies by Richter showed that bilateral ovariectomy reduced overall running distance by female rats and ablated the 4d cycle in distance run. These observations were confirmed by Gorzek at al. (28) who demonstrated that ovariectomized mice ran 80% less than control mice and that administration of estradiol restored the activity pattern. Richter showed that the 4d running cycle in female rats did not appear until puberty and disappeared during pregnancy and lactation (9). These results highlight the importance of monitoring wheel running behaviour in female mice for at least 4 days to avoid bias in estimates of running behaviour because of the influence of the stage of the oestrus cycle.

Excessive daytime sleepiness (EDS) in humans is associated with obesity and high fat diets (7), and mice fed high fat diets slept more during the dark phase when they would be expected to be awake (8). The greater sleep time of WH animals during the light period (when the mice are expected to sleep) may parallel the better sleep quality (29) and reduction in EDS reported by obese individuals who exercise (7). This leads to the possibility of using the mouse model to test treatments for EDS.

In summary, female C57BL/6J mice fed a high fat diet were heavier (P=0.004) than their low fat fed counterparts, but did not have a greater fat mass. Access to a wheel tended to lower body mass (P=0.056) but there was no interaction between dietary fat and wheel running. In the absence of an exercise wheel, high fat-fed mice exhibited lower levels of voluntary cage activity (NEPA) than low fat mice, and slept more. Voluntary wheel-running distance increased significantly in the pre-oestrus stage of the cycle. We provide preliminary evidence that an infra-red sensing device may be useful in quantifying sleep behaviour as well as physical activity, and may provide a non-invasive, low-cost alternative to EEG for sleep phenotyping in mice.

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Tables

Table 1. Composition of experimental diets

Ingredient (g/kg)	High fat (HF)	Low fat (LF)	Supplier
Cornstarch	253	500	Sigma
Casein	241	200	Sigma
Sugar	181	150	Co-op
Beef fat	145	0	Co-op
Corn oil	60	50	Co-op
Cellulose (Alphacel)	60	50	MP Biomedicals
Mineral mix AIN-93-G-MX	42	35	MP Biomedicals
Vitamins AIN-93-VX	12	10	MP Biomedicals
Methionine	4	3	MP Biomedicals
Choline	2	2	MP Biomedicals
Butylated hydroxytoluene	0.04	0.01	Sigma

Table 2. Correlation between 24h recording periods 1 and 2 for cage activities during different phases of the day

Criterion	Phase of day	Pearson's r	Probability
Activity	24h (total amount of activity)	0.675	<0.001
	Light period	0.634	<0.001
	Dark period	0.707	<0.001
Sleep criterion (i) (0 counts per 10min)	Total sleep minutes	0.319	0.075
	Light period (minutes)	0.240	0.185
	Dark period (minutes)	0.462	0.008
Sleep criterion (ii) (up to 10 counts per 10 min)	Total sleep minutes	0.486	0.005
	~~Sleep (bouts)~~	~~0.160~~	~~0.383~~
	Light period (minutes)	0.326	0.069
	Dark period (minutes)	0.538	0.001

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Table 3. Total cage activity of mice by each treatment group (arbitrary units $x10^3$)

Phase of day	High fat				Low fat				Probability of effect		
	Wheel		Control		Wheel		Control		Diet	Exercise	Diet*exercise
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI			
All day	1718	1346-2192	761	596-971	1258	986-1605	1016	796-1297	0.945	0.001	0.023
Light-phase	80	64-99	91	73-113	83	67-103	194	156-241	0.015	0.002	0.003
Dark-phase	1634	1242-2150	664	505-874	1167	887-1535	795	604-1045	0.672	<0.001	0.076
Light activity (as % total activity)	4.7	3.5-6.5	12.4	9.1-17.1	6.9	5.0-9.4	18.9	13.8-26.0	0.107	<0.001	0.881

n = 8 for each group. Data Log10 transformed.

Table 4. Sleep time* of mice in each treatment group

Sleep	High fat				Low fat				Probability of effect		
	Wheel		Control		Wheel		Control		Diet	Exercise	Diet*exercise
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Total (min)	729	24.4	828	24.2	700	24.2	744	24.2	0.045	0.010	0.262
Dark phase (min)	154	24.0	272	24.0	132	24.0	226	24.0	0.277	<0.001	0.612
Light phase (min)	576	13.5	556	13.5	567	13.5	518	13.5	0.126	0.019	0.264

*** Sleep was defined as no detectable activity in 1 minute epochs throughout the day.**

Figure captions and figures

Figure 1. Daily distance run each week by mice fed high and low fat diets (n = 8 per group, mean and SE)

Figure 2. Final body masses (mean and SE) of mice fed high fat (HF) and low fat (LF) diets with and without access to a running wheel

Figure 3a. Variability in running distance by one mouse over 15 sequential days.

Figure 3b. Distances (mean and SE) run during each stage of the oestrus cycle

Figure 4. Relationship between total cage activity during each 24h recording period with fitted regression line.

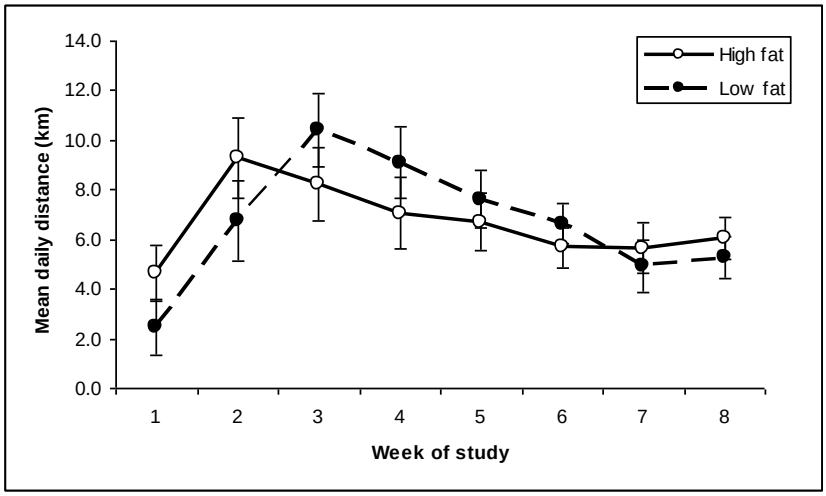

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Mean daily distance (km)
1
2
3
4
5
6
7
8
Week of study
High fat
Low fat

Figure 1.

440

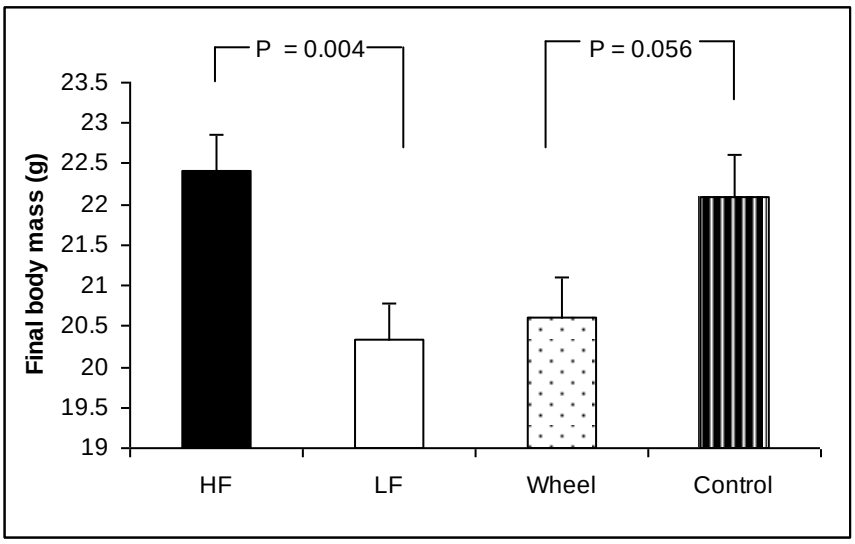
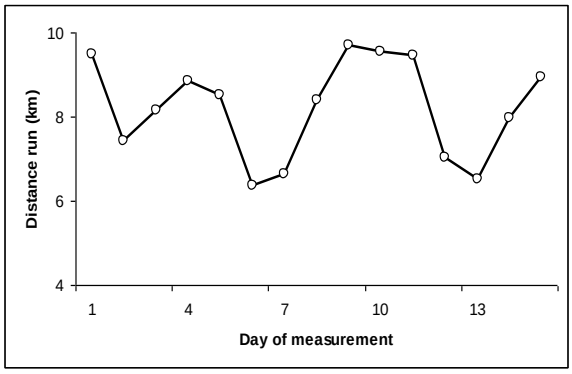
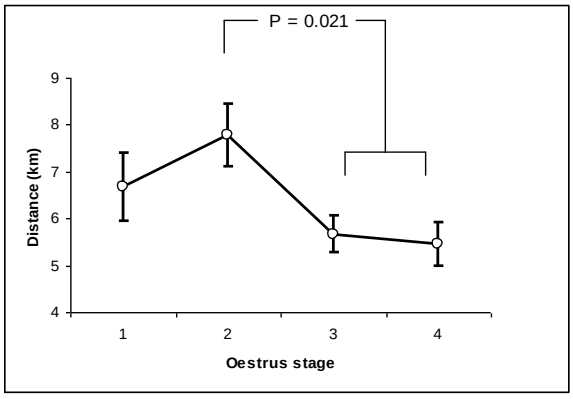

P = 0.004
P = 0.056
Final body mass (g)
23.5
23
22.5
22
21.5
21
20.5
20
19.5
19
HF
LF
Wheel
Control

Figure 2.

445

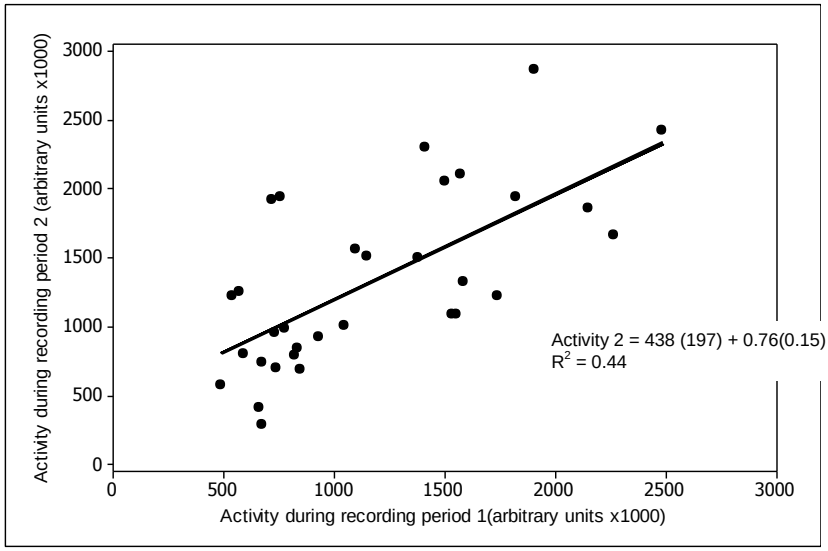


a



b

Figure 3.


Activity during recording period 2 (arbitrary units x1000)
3000
2500
2000
1500
1000
500
0
0
500
1000
1500
2000
2500
3000
Activity during recording period 1(arbitrary units x1000)
Activity 2 = 438 (197) + 0.76(0.15) activity 1
R2 = 0.44

450

Figure 4.