Physiological and behavioural consequences of long-term moderate treadmill exercise

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Summary

The benefits of long-term moderate exercise for health are widely accepted in humans, but few animal studies have been undertaken to characterize the effects of such activity on emotionality and responsiveness to stress. The present study describes the effects of long-term moderate forced treadmill training (36 weeks) on exploratory activity, anxiety-like behaviour, and the resting or stress levels of some physiological variables, including pituitary-adrenal (PA) hormones. Five-week-old male Sprague-Dawley rats were trained on the treadmill (TM) for 36 weeks, using a more moderate training (12m/min, 30 min/day, 4-5 days/week) than that currently used in the literature. Two groups were used as controls: a non-handled sedentary (SED) group, receiving no manipulation, and a control (CON) group exposed to a stationary treadmill for the same amount of time as the TM group. In accordance with literature data, TM rats showed lower resting levels of glucose, triglycerides and cholesterol than the other two groups. The TM and CON groups both showed higher ambulation than the SED group in some behavioural tests, without evidence for altered anxiety. Resting levels of adrenocorticotropin (ACTH) and corticosterone did not differ among the groups, but a reduced ACTH response to both a novel environment (mild stressor) and an active escape-avoidance task (severe stressor) was observed in TM rats, whereas changes in corticosterone were modest. The results support the view that the physiological consequences of long-term moderate training are beneficial, including reduced PA responsiveness to stress, even though exercise training did not affect anxiety-like behaviour.

Keywords: long-term moderate exercise, treadmill, hole board, elevated plus maze, open field, stress responsiveness, ACTH, corticosterone, metabolism.

1. Introduction

Considerable evidence now supports the idea that an active lifestyle produces benefits for overall health, preventing cardiovascular diseases (Blair and Morris, 2009; Crimi et al., 2009), enhancing cognitive function (Dishman et al., 2006; Kramer et al., 2006; Cotman et al., 2007; van Praag, 2009) and improving mood (Russo-Neustadt et al., 2001; Greenwood et al., 2003; Deslandes et al., 2009; Marais et al., 2009). Exercise has also been associated with a reduced risk of dementia and could help to reduce late-life cognitive impairment (Kramer et al., 1999; Colcombe and Kramer, 2003; Abbott et al., 2004; Weuve et al., 2004), although the oldest age at which starting physical activity can benefit cognition has still not been determined (Bunce and Murden, 2006).

Exercise of moderate intensity for 30 min, five days per week, has been widely recommended for humans (Haskell et al., 2007; Nelson et al., 2007), but there are few studies specifically aimed at characterizing the exercise conditions (intensity and duration) that can produce the most robust effects in both healthy people and those with pathological conditions (Kramer et al., 2006; Roland et al., 2010). It is unclear the extent to which increased levels of exercise can exert additional healthy effects (Blair et al., 2004), but there is evidence that moderate exercise is better than excessive exercise and over-training in terms of improvement of cardiovascular function, upregulation of the immune system or modulation of redox homeostasis (Radak et al., 2008).

It is well accepted that exercise improves cognition in humans (van Praag, 2009) and most data in animals favour this hypothesis, although the effects are not always consistent. For instance, wheel running in rats for 13 months (from 5 to 18 months of age) showed no protective effect on spatial memory (Hansalik et al., 2006). In contrast, in another study, improvement of age-related impairment of spatial learning and

associated cellular mechanisms (long-term potentiation, LTP, in the dentate gyrus) was observed in middle-age rats after 8 months of treadmill training (O'Callaghan et al., 2009). Quite interestingly, in the latter study, the beneficial effect was also observed in the group of animals receiving the same treatment in a stationary treadmill, suggesting a contribution of enriched environmental information acquired by the animals, rather than a specific contribution of exercise.

Less is known about the influence of exercise on emotional reactivity and anxiety-like behaviour in experimental animals. Indeed, to our knowledge, there have been no animal studies about the influence of prolonged periods of exercise (several months) on these two aspects of behaviour. With shorter periods of exercise (1-10 weeks), the results are clearly inconsistent and cannot be explained by the species used (rat or mice), the type of exercise (wheel running versus treadmill), the period of exposure, or the age of the animals (e.g. Dishman et al., 1996; Fulk et al., 2004; Trejo et al., 2008; García-Capdevila et al., 2009; Fuss et al., 2010). There are two possible reasons for these inconsistencies. First, exercise-trained animals are exposed to handling and other manipulations that can reduce the response to such procedures, as compared to non-trained (sedentary) animals that have not been subjected to the same manipulations. Second, although certain training procedures may initially increase anxiety, this effect may progressively disappear over time, thereby, making long-lasting exercise training the most appropriate protocol for discovering any real beneficial effects of exercise on anxiety.

Given the critical role of glucocorticoids on cognitive function and aging-associated neurodegeneration (De Kloet et al., 1999), considerable attention has been paid to the influence of exercise on the hypothalamic-pituitary-adrenal (HPA) axis, one of the key

systems in the response to stress (Armario, 2006). Although it is well documented that acute exercise can activate the HPA axis, less is known about the influence of long-lasting exercise on resting HPA function, and its responsiveness to additional superimposed stressors. Moreover, all studies have used a relatively short period of training (2-10 weeks). The general pattern is that increases in resting levels of corticosterone can be observed during the first week after wheel running, with normalization after 4 or 8 weeks (Fediuc et al., 2006; Campbell et al., 2009). This pattern results in null or small effects of prolonged periods of exercise on resting levels of HPA hormones, either in rats or mice (Watanabe et al., 1991; 1992; Chennaoui et al., 2002; Droste et al., 2003; 2006; 2007). Regarding the PA response to predominantly emotional stressors, normal (Chennaoui et al., 2002; Droste et al., 2006; 2007) or reduced (Watanabe et al., 1992; Droste et al., 2003) response has usually been reported.

On the basis of the above studies, the main objective of the present work was to characterize the still untested effects of a long-term regular moderate exercise procedure (36 weeks) on exploratory and anxiety behaviour, HPA function, cholesterol and triglyceride levels in adult male rats. The treadmill model was used to administer the same amount and intensity of exercise to the animals throughout the entire period of training, thereby, avoiding the differences described with voluntary running models (Narath et al., 2001; García-Capdevila et al., 2009). Furthermore, an additional control group of rats, which was exposed to the stationary treadmill, was included to rule out a specific influence on the variables of interest, by daily handling and other procedures associated to treadmill exposure.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats (from the *Servei d'Estabulari*, *Universitat Autònoma de Barcelona*) were used. They were housed 2 per cage in standard macrolon cages (40 cm in length x 23 cm in width x 18 cm in depth) and maintained under 12h/12h light/dark cycle (lights on at 0800h) in standard conditions of temperature (21±1ºC) and humidity (50±10%), with free access to food and water. Animals, which were 5 weeks old at the start of the training, were randomly assigned to three groups, balancing the total body weight before starting the training sessions: sedentary (SED, n=8, 95.85 ± 4.8 g), control (CON, n=8, 102.2 ± 5.2 g) and treadmill (TM: n=11, 99.4 ± 3.7 g). The experimental protocol was approved by the Ethics Committee of the *Universitat Autònoma de Barcelona*, and was carried out following the 'Principles of laboratory animal care', in accordance with the European Communities Council Directive (86/609/EEC).

2.2 Moderate forced treadmill training procedure

Two treadmills were used, each consisting of 3 parallel runways (35 x 8 x 24 cm, Cibertec, Spain; and 45 x 11 x12 cm, Columbus Instruments, USA) without inclination. Training sessions were conducted in the colony room, 4-5 days per week, between 1330h to 1630h, and lasted for 30 min. Subjects were habituated to the treadmill for 30 min (0 m/min) on the first day, to minimize novelty-induced stress. Exercise training began gently the day after, and the intensity of the treadmill speed was gradually increased until reaching a maximum intensity of 12 m/min, which was maintained until the end of the experiment. The treadmill (TM) and control (CON) rats were weighed daily before being placed in the treadmill. The CON rats stayed in a stationary treadmill (0 m/min) for the same number of sessions and the same amount of time as the TM rats.

The SED rats remained in their own cages and were weighed weekly. Neither electrical shock nor physical prodding was used to motivate the animals. During the first sessions, some animals slowed their gait over the session and displaced towards the back wall of the lane; on such occasions they were gently pushed by hand for a few seconds to stay at the front part of the lane. Despite these cautions, 25% of the rats had to be rejected from the experiment because they refused to run. This percentage is similar to that reported by other authors (Dishman et al., 2000). Rats were trained for 36 weeks, receiving a total of 152 sessions. Behavioural tests were intercalated between the last 20 sessions to avoid training interruption and a possible decrease in the effects of exercise.

2.3 Behavioural procedures

All experiments were performed in a room painted black and illuminated by one 40w bulb or two 40w bulbs, in the experiments where the two animals of the same cage were being simultaneously tested on two separate apparatus. The testing battery was administered over five weeks between 0900h to 1400h (except for basal hormones, see physiological variables) as follows: hole board, day 1; basal hormones, day 3; elevated plus maze, day 7; open field, day 14; and the shuttle box stress test for escape-avoidance behaviour, day 31. Apparatus were cleaned with a 20% ethanol solution after each rat.

2.3.1 Hole-board test

The two hole-board (HB) apparatus were beige wooden boxes (66 x 66 x 47 cm), with four equidistant holes (3.7 cm diameter, 18 cm deep) on the floor, which were divided into 12 equal squares with red lines. A score was kept of the number of head dips (HD), the time spent head dipping, and the number of crossings. The two animals housed in the same home cage were tested simultaneously, and were recorded by two cameras; each placed 140 cm above one of the two hole-board apparatus. The rat was placed into

the centre of the arena and allowed to explore it for 15 min and then, immediately afterwards, blood was taken from each rat by tail nick (see below) in a different room.

2.3.2 Elevated plus maze (EPM)

The EPM consisted of four arms made of black Formica, extending from a 10-cm square centre positioned 90º from each other to form the shape of a plus sign. Each arm was 50 cm long and 10 cm wide. Two opposing arms had wooden walls (enclosed arms, 40 cm high), whereas the other two were open arms with a 0.5 cm ridge to provide additional grip. The whole maze was elevated 50 cm above the floor. The rat was placed in the centre of the maze facing a closed arm, and during the 5 min test the following data were recorded: total entries; entries in open and enclosed arms; time in enclosed and open arms, as well as in the central area; latency to enter for the first time into an open arm; and the number of defecations. An entry was defined as "placing all four paws into a given arm".

2.3.3 Open field (OF)

A 10-min OF session was administered. The two animals of the same home cage were tested simultaneously in two identical apparatus, which each consisted of a white open arena (100 x 50 x 45cm) made of white Plexiglas. The animal was placed in the centre of the arena and the distance travelled was measured using video tracking software (ViewPoint S.A.).

2.3.4 Shuttle Box (SB)

Shuttle Box apparatus (Panlab, S.L.) was divided into two equally sized compartments (25 x 25 x 25cm) connected by an opening door (8 cm wide and 10 cm high). A trial consisted of simultaneously presenting a light (7w) and a sound (2400hz at 40db)

stimulus, followed immediately by a scrambled electric shock (0.6mA), which was administered through the metal grid floor of the box. Animals received 30 trials for a period of 30 min, with an inter-trial interval of 15 sec.

2.4 Physiological variables

Plasma levels of some physiological variables were measured under resting conditions or in response to stress. Samples were taken by tail nick (ACTH, corticosterone, glucose) or by decapitation (cholesterol, triglycerides). The tail nick was carried out by gently wrapping the animals with a cloth, making a 2-mm incision at the end of the tail vein and then massaging the tail while collecting, within 2 min, 300 µl of blood into ice-cold EDTA capillary tubes (Sarsted, Granollers, Spain). The two cage-mated animals were sampled simultaneously by two experimenters in a separate room from the colony room and the testing room.

To evaluate the HPA response to a mild stressor, the animals were exposed for 15 min to the HB in a testing room. Immediately afterwards, the animals were transported to another room and sampled. This time point was chosen because 15 min is the minimum time needed for plasma corticosterone to reflect initial ACTH release (Armario, 2006). To evaluate the HPA axis response to a more severe stressor, the animals were previously exposed to the SB. Immediately after the SB session, and again 30 min after its termination (SB30), rats were sampled by tail nick. Basal samples were taken two days after the HB exposure, on a day when neither behavioural testing nor treadmill training was being undertaken to prevent possible interferences with hormonal data. Sampling was carried out, under resting conditions, during the morning (0900h-1000h) and during the evening (1900h-2000h).

2.5 Radioimmunoassays

Plasma ACTH and corticosterone levels were determined by double-antibody radioimmunoassay (RIA). In brief, ACTH RIA used ^{125}I-ACTH (PerkinElmer Life Science, Boston, USA) as the tracer, rat synthetic ACTH 1–39 (Sigma, Barcelona, Spain) as the standard and an antibody raised against rat ACTH (rb7) kindly provided by Dr. W.C. Engeland (Department of Surgery, University of Minnesota, Minneapolis, USA). The characteristics of the antibody have been described previously (Engeland et al., 1989), and we followed a non-equilibrium procedure. Corticosterone RIA used ^{125}I-corticosterone-carboximethyloxime-tyrosine-methyl ester (ICN-Biolink 2000, Barcelona, Spain), synthetic corticosterone (Sigma, Barcelona, Spain) as the standard, and an antibody raised in rabbits against corticosterone–carboximethyloxime-BSA kindly provided by Dr. G. Makara (Institute of Experimental Medicine, Budapest, Hungary). The characteristics of the antibody and the basic RIA procedure have been described previously (Zelena et al., 2003). All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 3.8 % for ACTH and 7.8 % for corticosterone. The sensitivity of the assays was 12.5 pg/ml for ACTH and 0.1 μg/dl for corticosterone.

2.6 Cholesterol and triglyceride determination

Blood samples for cholesterol and triglyceride determinations were collected in 5% EDTA-tubes at the time of death; plasma was obtained by centrifugation and stored at −80°C until needed. Plasma triglyceride and cholesterol concentrations were measured by using the colorimetric test kits for triglyceride and cholesterol, respectively, from Wako Chemicals GmbH (Neuss, Germany).

2.7 Statistical analysis

The statistical analysis was performed with the 'Statistical Package for Social Sciences' (SPSS, version 17.0), using one-way ANOVA and repeated measures ANOVA. All values are expressed as mean ± standard error for the mean. All post-hoc contrasts were carried out with Bonferroni correction, with $p<0.05$ considered to be significant.

3. Results:

3.1 Body weight

Body weights were analysed by repeated measures ANOVA with treatment as the between-subjects factor and age as the within-subjects factor. The ages included in the analysis were: 4 weeks (pre-exercise), 5 weeks (1 week after treadmill training had started) and 9, 13, 17, 21, 25, 29, and 33 weeks. The analysis indicated (Fig.1) a significant effect of age [$F(8, 160)=1,857.4$; $p<0.0001$], but not for group or interaction [$F(2, 20)=0.64$, ns; age*group: $F(16, 160)=0.54$, ns; respectively].

FIGURE 1

3.2 Hole board

A repeated measure ANOVA was used to analyse exploratory behaviour in the HB, with groups as the between-subjects factor and time (3 time bins of 5 min) as the within-subjects factor. The analysis of the number of head dips did not show any significant main effect or interaction [Fig. 2A; group: $F(2, 20)=0.07$; time: $F(2, 40)=1,79$; group*time: $F(4, 40)= 1.43$]. Time was significant in the analysis of time spent head dipping [Fig. 2B; $F(2, 40)=3.59$; $p<0.05$], but not for group and group*time [$F(2, 20)=0.21$; ns, and $F(4, 40)=1.09$; ns, respectively]. A trend toward significance was found for the effect of group [$F(2, 20)=3.32$; $p<0.06$], whereas the significant effects of time [$F(2, 40)=29.12$; $p<0.0001$] and interaction group*time [$F(4, 40)=6.39$;

$p<0.0001$] appeared for the number of crossings (Fig. 2C). Decomposition of that interaction revealed that groups differed during the first five min [$F(2, 20)=9.12$; $p<0.01$], with the CON group making more crossings than the SED one (Bonferroni, $p<0.01$; Fig. 2C).

FIGURE 2

3.3 Elevated Plus Maze

The one-way ANOVA revealed an overall significant effect for group on the number of total entries, as well as of entries into the closed arms [$F(2, 20)=6.39$; $p<0.01$ and $F(2, 20)=5.23$; $p<0.05$, respectively]. Animals in the SED group showed reduced activity (Bonferroni, $p< 0.05$) in the EPM in comparison with those in the CON (both measures) and TM (closed arms only) groups (Fig. 3). The time spent in the open [$F(2, 20)=1.32$, ns] or closed arms [$F(2, 20)=1.32$, ns], the latency to enter for the first time in an open arm [$F(2, 20)=2.52$, ns], and the defecation rate [$F(2, 20)=1.25$, ns] were not affected by handling and/or exercise (data not shown).

FIGURE 3

3.4 Open Field

A repeated measures ANOVA (between-subjects factor: group; within-subjects factor: 10 time bins of 1 min) was used to analyse the distance travelled in the OF. The analysis indicated no significant effect for group [$F(2, 20)=1.318$, ns], but significant effects for time [$F(9, 180)=45.12$; $p<0.0001$] and the group*time interaction [$F(18, 180)=1.85$; $p<0.05$] (Fig. 4). Decomposition of that interaction showed that the CON group was more active than SED group during the first minute [$F(2, 20)=4.98$, $p<0.05$; post-hoc test (Bonferroni): $p=0.017$] (Fig. 4).

FIGURE 4

3.5 Physiological variables

3.5.1 Baseline levels

A repeated measures ANOVA (between-subjects factor: group; within-subjects factor: time of the day: morning or evening) was applied to analyse baseline levels of HPA hormones and glucose. A significant time effect was observed for: ACTH [$F(1, 20)=18.34$; $p<0.0001$]; corticosterone $F(1, 20)=73.57$; $p<0.0001$]; and glucose [$F(1, 19)=24.48$; $p<0.0001$]; with higher levels for the three variables in the evening (Fig. 5A-C). A significant effect for group was found for glucose [$F(2, 19)=4.664$; $p<0.05$; Fig. 5C), but not for ACTH or corticosterone [$F(2, 20)=0.669$; ns, $F(2, 20)=1.694$; ns, respectively]. Further comparisons showed that overall glucose levels of the TM group were significantly lower than those of the SED ($p=0.025$) and CON ($p=0.011$) groups. Group*time interactions were not significant.

FIGURE 5

One-way ANOVA analysis showed significant differences for measurements of plasma cholesterol and triglycerides ($F(2, 15)=4.246$, $p<0.05$ and $F(2, 17)=3.983$, $p<0.05$, respectively; Fig. 6A-B). The TM group showed lower levels of triglycerides than the SED group (Bonferroni, $p<0.05$). A trend toward significance was found in cholesterol levels of the TM group, as compared to the SED and the CON groups ($p=0.065$; $p=0.085$, respectively).

FIGURE 6

3.5.2 Response to stressors

Plasma glucose was not studied for the HB response, because glucose response is low or null after mild stressors. Analysis of the ACTH and corticosterone responses indicated a significant effect of group for ACTH [$F(2, 20)=3.59$; $p<0.05$] (Fig. 7A), but not for

corticosterone (Fig. 7B). Further comparisons of ACTH indicated that TM animals showed lower ACTH levels than CON ones (Bonferroni, p=0.05), but they did not differ from SED animals (Fig. 7A).

FIGURE 7

Behavioural data regarding escape-avoidance behaviour in the SB could not be recorded due to technical problems, but the available data indicate that the number of escapes was similar in the three groups (SED 26.38±1.2, CON 27.5±0.9 and TM 25.5±2.3, p=0.67), suggesting that the amount of shocks received were also similar. A repeated measures ANOVA (between-subjects factor: group; within-subjects factor: time SB, SB30) was conducted to analyse the ACTH response to the SB (Fig. 8A), and showed significant effects for time [$F(1, 20)=237.23$; $p<0.0001$] and group [$F(2, 20)=4.63$; $p<0.05$], but not for interaction group*time [$F(2, 20)=0.59$; ns]. Contrast comparisons indicated lower ACTH levels in the TM group, as compared with the SED (p=0.07) and the CON groups (p=0.063) (Fig. 8A). The analysis of corticosterone showed a significant effect for group*time interaction [$F(2, 20)=4.47$; p=0.025], but no significant effects for group or time [$F(2, 20)<0.8$; ns] (Fig. 8B). Decomposition of the interaction revealed that the TM group showed a decrease in corticosterone levels, during recovery after shuttle-box stress, whereas the SED and the CON groups did not (Fig. 8B). This decrease was also confirmed by analysis of the difference between SB30 and SB levels for each group (SB30-SB: SED: 4.76±0.87, CON: 3.06±1.24, TM: -4.47±3.96) [$F(2, 20)=4.47$, $p<0.05$]; post-hoc test (Bonferroni, p=0.021) TM < SED. The analyses of glucose showed a significant effect for time [$F(1, 20)=134.78$, $p<0.0001$], which reflected the decrease in glucose levels during the post-SB period (Fig. 8C). There were no significant effects for group or the group*time interaction [$F(2, 20)=1.17$; ns, $F(2, 20)=1.58$, ns, respectively].

FIGURE 8

Finally, the ANOVA revealed a significant effect for group on relative whole adrenal weight (Fig. 9; $F(2, 20)=3.63$; $p<0.05$), but comparison of groups (post-hoc Bonferroni test) only revealed marginally significant effects: $p=0.095$ (TM > SED) and $p=0.077$ (TM > CON).

FIGURE 9

4. Discussion

The present study is, to our knowledge, one of the first to expose young rats to long-term moderate treadmill exercise (36 weeks) to find out the influence of this exposure on exploratory and anxiety-like behaviour, as well as on the resting and stress levels of some physiological parameters. Two groups of control rats were included, one sedentary (SED) group that always remained in their home cages, and another control (CON) one, exposed to the very same procedure as the treadmill (TM) trained rats, except that they could not run on the treadmill. In this way, a non-specific effect could be ruled out for all the procedures associated to TM regarding anxiety and responsiveness to stress. Hence, our study demonstrates for the first time that long-term moderate training has beneficial physiological effects and reduces the ACTH response to stress. Yet, such beneficial effects are not explained by all procedures associated to training.

The moderate training protocol used in the present study (12 m/min, 30 min/d, 5d/w) did not reduce body weight over the entire period of study. Using the treadmill procedure, body weight reduction has been related to the intensity of training (e.g. Watanabe et al., 1992; Chennaoui et al., 2002; Albeck et al., 2006; Hansalik et al.,

2006), and our procedure is milder than all those previously described. Therefore, it appears that treadmill running exercise must be above a certain level of intensity to reduce body weight in rats. Despite the lack of changes in body weight, TM rats showed lower resting levels of glucose, cholesterol and triglycerides than CON and SED groups, a result consistent with previous data obtained in rats (Pels et al. 1985; Suzuki and Machida 1995) and humans (Martí et al 1990). As lower levels of these variables have beneficial effects on metabolic and cardiovascular diseases (Eriksson et al 1997), the present data strongly support the efficacy of our moderate exercise programme.

The behavioural data in the three novel environments (HB, EPM and OF) revealed that, in general, both TM and CON rats increased horizontal activity as compared with SED rats. There were no evident differences between rats in the TM and CON groups. Furthermore, no group differences were observed in variables more specifically related to exploration (head dipping in the HB test) or anxiety-like behaviour (e.g. entries and time in the open arms of the EPM). Hence, our results strongly indicate that there is no specific effect of moderate exercise on activity, exploration or anxiety-like behaviour, and that increased activity may be related to the procedures associated to daily exposure to the treadmill, rather than to exercise per se. Throughout the 36 weeks of training, CON animals were picked up from the home cage, weighed, placed in the treadmill for 30 min and returned to the home cage when the session finished, whereas SED animals were only weighed once per week. The animals of the CON group remained in the apparatus without running, sniffed the surrounding, reared, moved forward and backward in the line, and after some time, sat downward and fell asleep. Thus, CON animals were exposed to some kind of enriched rearing condition, and it has been repeatedly reported that even less intensive and brief handling procedures, as well as other environmental rearing conditions involving increased stimulation, are able to

modify activity, exploration or anxiety profiles (Fernandez-Teruel et al., 2002; Leal-Galicia et al., 2008; Peña et al., 2009; Simpson and Kelly, 2011). Moreover, the behavioural similarities between CON and TM rats observed in the present experiments agree with previous studies, which also reported a similar performance by both groups in hippocampal neurotrophin expression, spatial learning, anxiety and social behaviour (Burghardt et al., 2004; O'Callaghan et al., 2009; Spangenberg et al., 2009). In line with this, we have recently reported that both TM and CON rats showed increased serum levels of IGF-1 and brain activation of sirtuin 1 pathway, as compared to SED rats, although the increase was higher in the TM group (Bayod et al., 2011).

Although the research reported in the literature used relatively shorter periods of exercise (1-8 weeks) and more intense training than in the present experiments (when the treadmill was used, allowing comparisons), the effects of exercise on activity in novel environments and on anxiety-like behaviour are clearly inconsistent after treadmill exercise, as well as after voluntary wheel running (e.g. Dishman et al 1996; Burghardt et al., 2004; Fulk et al., 2004; Leasure and Jones, 2008; García-Capdevila et al., 2009; Grace et al., 2009; Hopkins and Bucci, 2010). More consistent beneficial effects of exercise have been reported when anxiety was enhanced by pharmacological interventions or psychological stress. Wheel running reduced conditioned freezing and reversed the shuttle-box escape deficit produced by uncontrollable stress (Greenwood et al. 2003; 2007), whereas treadmill exercise reversed the increase of anxiety-like behaviour induced by acute sleep deprivation (Vollert et al. 2011), and by a drug that increased oxidative stress markers (L-buthionine-(S,R)-sulphoximine (BSO); Salim et al. 2010). Therefore, future research about the possible beneficial effects of exercise on anxiety may benefit from protocols that assess resilience to stress-provoking stimuli.

The analysis of basal hormones showed that TM animals did not differ from the SED and CON groups in plasma ACTH and corticosterone levels, measured at two points of the circadian cycle. It cannot be ruled out that transient changes appeared during the initial phases of training, which progressively normalized over the long-term period of training (36 weeks). For instance, increases in resting levels of corticosterone during the first week after wheel running, with normalization after 4 or 8 weeks, have been reported (Fediuc et al., 2006; Campbell et al., 2009). Our results concur with those generally reported in the literature, and indicate only null or small effects of exercise on resting levels of HPA hormones, either in rats or mice (Chennaoui et al., 2002; Droste et al., 2003, 2006, 2007; Watanabe et al., 1991, 1992).

To our knowledge, the present study demonstrates for the first time an important impact of long-lasting moderate levels of exercise on PA response to stress. TM animals showed a reduced ACTH response to either a mild stressor (exposure to a novel environment), or a severe stressor (escape-avoidance training using foot shock as the aversive stimulus, SB). That is, the TM group presented a lower ACTH response to stress than both the SED and CON groups. This reduced ACTH response to the escape-avoidance task cannot apparently be explained by a reduced number of shocks related to better learning of the task, as the number of escapes was similar in all groups.

Corticosterone response followed a partially different pattern. No group differences were observed in response to the novel environment. In response to the escape-avoidance task, slightly higher levels were found in TM rats, as compared to SED or CON rats immediately after the task, whereas the post-task decline was greater in TM animals, in accordance with the greater post-task decline in ACTH in these animals. Discrepancies between the ACTH and corticosterone responses can at least be explained

in part by enhanced adrenal responsiveness to circulating ACTH in TM rats. These TM rats showed a trend toward a higher relative adrenal weight, and this parameter correlated with maximal adrenocortical responsiveness to ACTH (Marquez et al., 2004).

In general, previous studies on the influence of exercise on PA responsiveness to stress have used much shorter periods of exercise (2-10 weeks), and the results showed normal (Chennaoui et al., 2002; Droste et al., 2006, 2007) or reduced (Droste et al., 2006; Watanabe et al., 1992) ACTH response to predominantly emotional stressors. With these relatively short periods of exercise, it is important to consider the dynamic changes in the HPA axis to adapt to exercise. For instance, Campbell et al. (2009) reported that, after 2 weeks of exercise, rats showed reduced ACTH but higher corticosterone response to restraint stress, whereas a response similar to that of sedentary groups was observed after 8 weeks of exercise. The dissociation between ACTH and corticosterone response after 2 weeks was explained by the observation of enhanced adrenocortical responsiveness to ACTH. Dissociation between both hormones has also been observed in mice exposed to wheel running (Droste et al., 2003, 2006, 2007), suggesting some ACTH-independent specific effect of exercise on adrenocortical function. Moreover, the positive effects of exercise on HPA responsiveness to a stressor may extend beyond the acute response to stressors, as Sasse et al. (2008) observed a normal corticosterone response to noise stress, but a significantly improved habituation to daily repeated noise in exercised animals.

In TM rats, there was no reduced glucose response parallel to the reduction observed in the ACTH response. As stress-induced hyperglycaemia is a reflection of adrenaline release (Marti and Armario, 1998), it can be tentatively assumed that long-lasting moderate training did not substantially modify medullo-adrenal responsiveness to

predominantly emotional stressors. However, there are other alternative possibilities. First, exercise-induced metabolic changes may alter glucose metabolism, independently of circulating levels of adrenaline. Second, a reduced ACTH response to stress in TM rats may be the consequence of an altered regulation of the HPA axis in the pituitary, the PVN or above, rather than a reflection of a generalized lower response to stressors. The present results concur with previous reports that 8-10 weeks of treadmill training do not have effects on the noradrenaline and adrenaline response to noise stress (Overton et al., 1991), although it is possible that exercise can be beneficial for reducing noradrenaline and adrenaline response to stress in certain vulnerable animals, such as borderline hypertensive rats (Cox, 1991).

In conclusion, moderate forced exercise practiced from early ages has an important long-term impact in adulthood. Exercise, but not handling, reduced plasma levels of glucose, cholesterol and triglycerides, suggesting beneficial metabolic consequences. The two components involved in the treadmill training procedure, handling and exercise, can affect behaviour and responsiveness to stress in a different manner. Treadmill handling increased horizontal activity, but did not affect anxiety. Although any treatment altered basal hormone levels, the present study is probably the first to demonstrate that long-term moderate treadmill exercise, but not the handling procedures, reduces the ACTH response to both mild and severe stress challenges.

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Legends

Figure 1. Mean (± SEM) of the body weight changed over the weeks of the training period. Groups were: sedentary (SED), control (CON) and treadmill (TM), (n=7-8/group). No significant differences were observed between groups.

Figure 2. Control group (CON) made more crossings (C) than the sedentary group (SED), during the first 5 min of the hole-board test. Mean ± SEM of A) the number of head dips, B) the time spent head dipping, and C) the number of crossings are shown over 3 time bins of 5 min in the hole-board test. TM, treadmill group (n=7-8/group). ** $p<0.01$ *vs.* SED (Bonferroni).

Figure 3. Animals in the sedentary (SED) group showed a reduced number for enclosed and total entries in the elevated plus maze. This figure shows the mean ± SEM of number of total entries and entries in both open and closed arms. CON, control group; TM, treadmill group (n=7-8/group). * $p<0.05$ (Bonferroni) between the indicated groups.

Figure 4. Control (CON) group was more active than the sedentary (SED) group during the first minute of the Open field test. This figure shows the mean ± SEM of the distance travelled over the 10 min of exposure to the open field test. TM, treadmill group (n=7-8/group). * $p<0.05$ *vs.* SED group (Bonferroni).

Figure 5. Overall evening levels of ACTH (A), corticosterone (B) and glucose (C) were higher than morning levels. Treadmill group (TM) presented less glucose than sedentary (SED) and control (CON) groups. This figure shows the mean ± SEM of hormones and glucose levels under resting conditions during the morning and the evening (n=7-8/group). +++ $p<0.001$ overall time of day effect; ▽ $p<0.0025$, # $p=0.011$ *vs.* SED and CON groups, respectively (regardless of time of day).

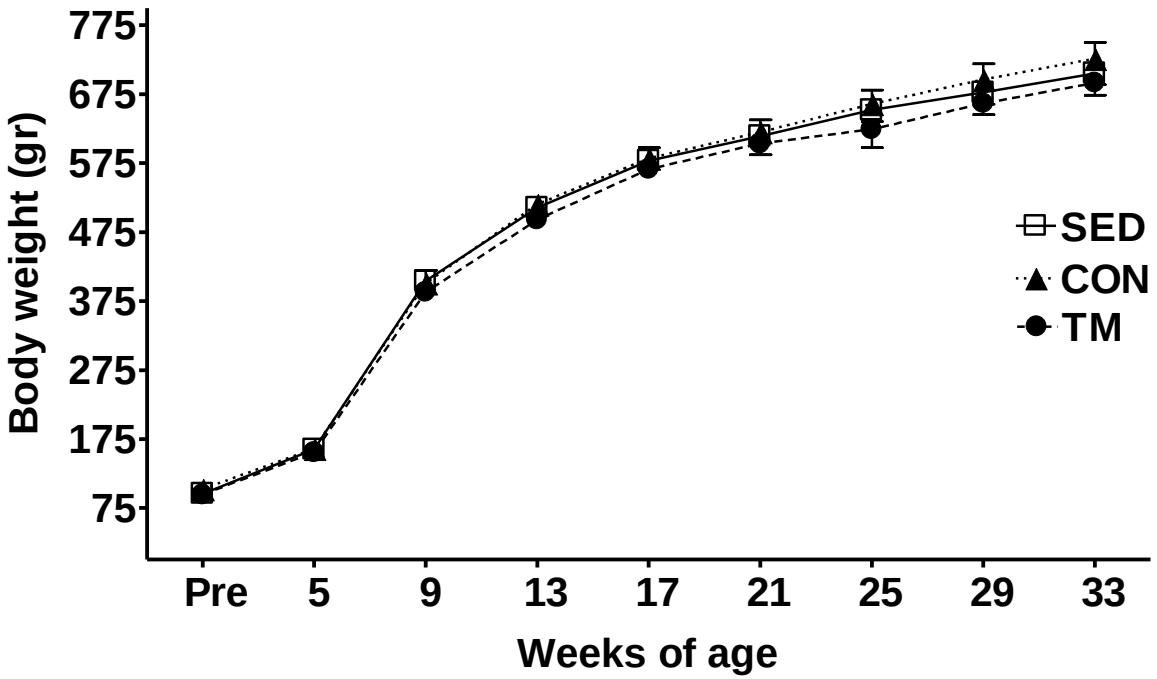
Figure 6. Mean ± SEM of A) triglycerides and B) cholesterol are shown, both were reduced in the treadmill (TM) group, as compared with the sedentary (SED) and control (CON) groups (n=5-6/group). * $p<0.05$ (Bonferroni) between the indicated groups; λ $p=0.065$ *vs.* SED and $p=0.085$ *vs.* CON.

Figure 7. Treadmill (TM) animals presented reduced ACTH levels (A), but not reduced corticosterone levels (B), than the control (CON) group, immediately after exposure to the hole-board test. Means ± SEM are shown; sedentary (SED) group, (n=7-8/group). λ $p=0.05$ (Bonferroni).

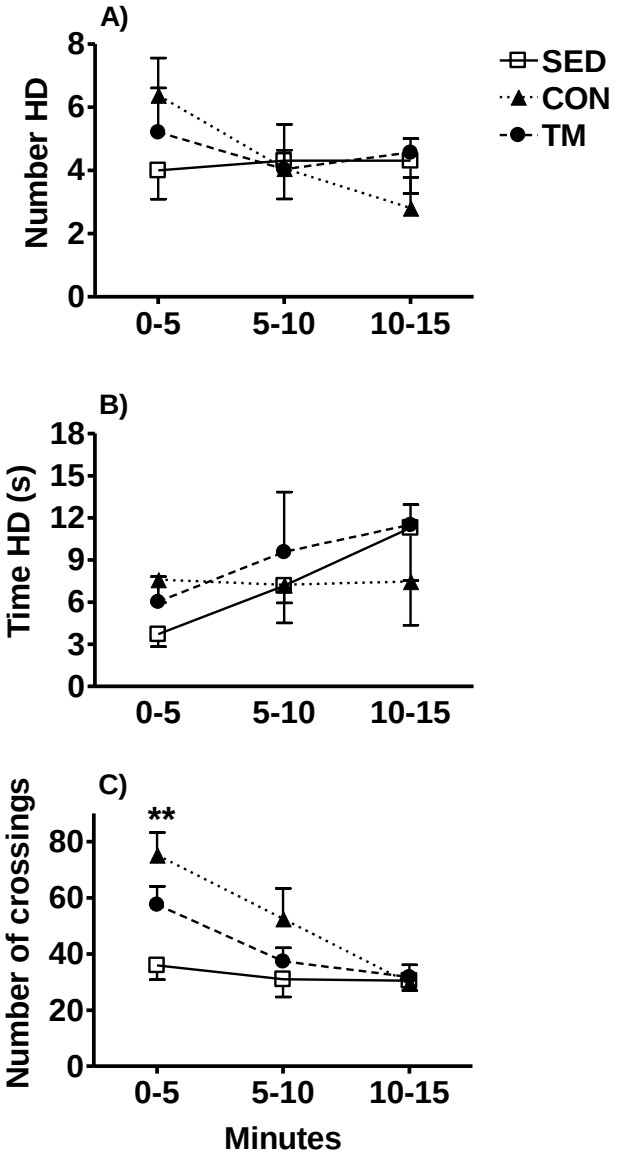
Figure 8. Lower ACTH levels (A) were found in the TM animals, as compared to the sedentary (SED) and control (CON) groups, immediately after the shuttle-box stress session (SB) and after 30 min of recovery (SB30). Corticosterone (B) decreased only in the TM group during the post-shuttle period, and overall levels of glucose (C) decreased after recovery. Mean ± SEM is shown, (n=7-8/group). +++ $p<0.001$ (general effect of sampling time); ^ $p<0.05$ between the indicated groups; ▽$p=0.07$ *vs.* SED and λ $p=0.063$ *vs.* CON group (regardless of sampling time).

Figure 9. Mean ± SEM of the relative adrenal weight of sedentary (SED), control (CON) and treadmill (TM) groups (n=7-8/group). λ p=0.077 *vs.* SED and p=0.095 *vs.* CON groups.

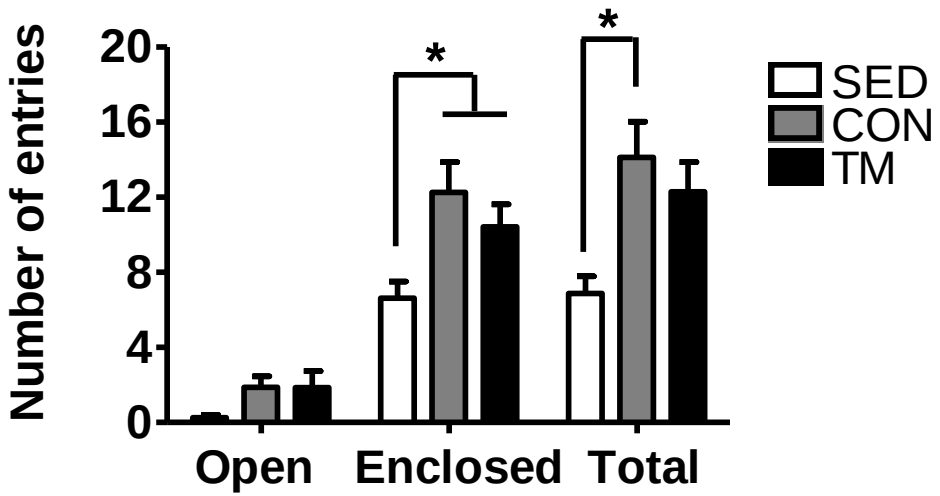
JFL_ Figure 1


775
675
575
475
375
275
175
75
Body weight (gr)
Pre
5
9
13
17
21
25
29
33
Weeks of age
SED
CON
TM

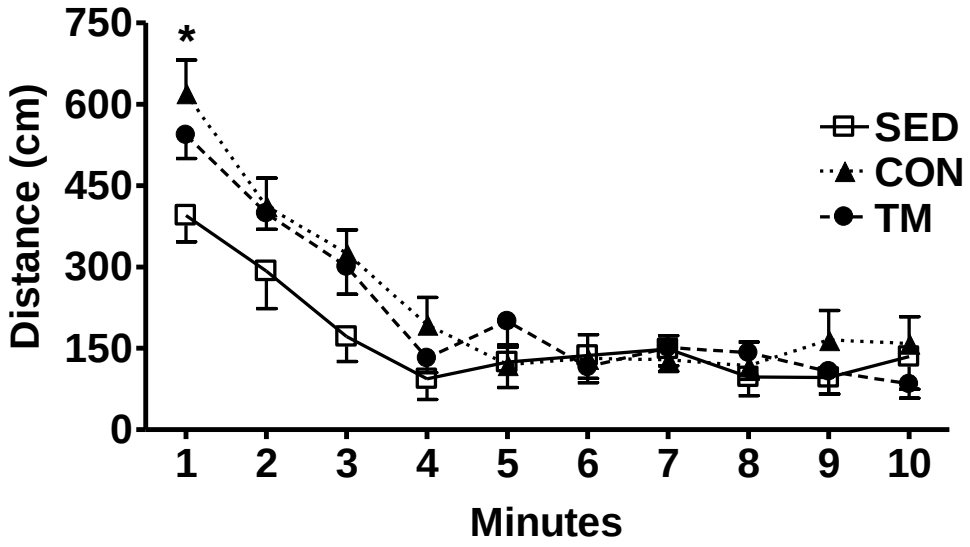
JFL_Figure 2


A)
Number HD
8
6
4
2
0
0-5
5-10
10-15
SED
CON
TM
B)
Time HD (s)
18
15
12
9
6
3
0
0-5
5-10
10-15
C)
Number of crossings
**
80
60
40
20
0
0-5
5-10
10-15
Minutes

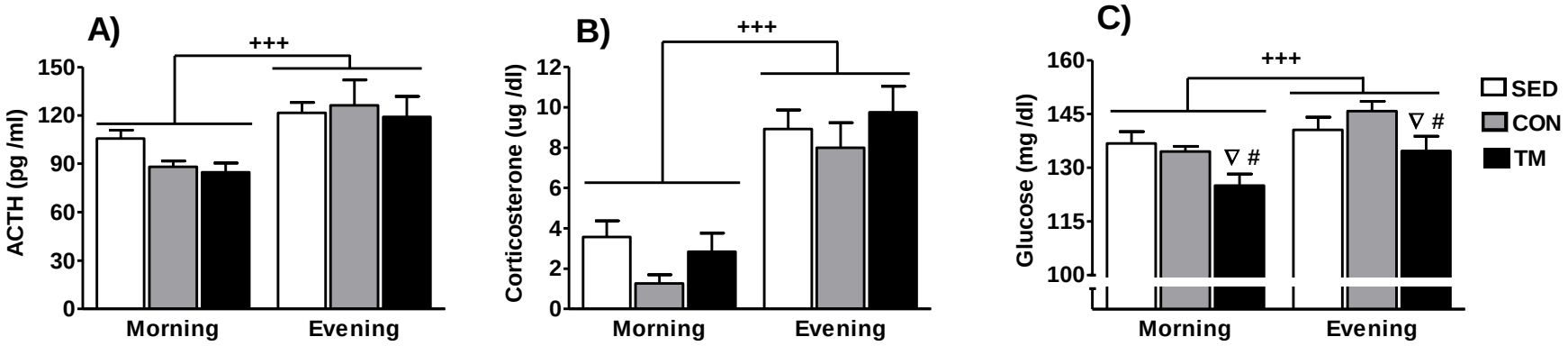
JFL_Figure 3



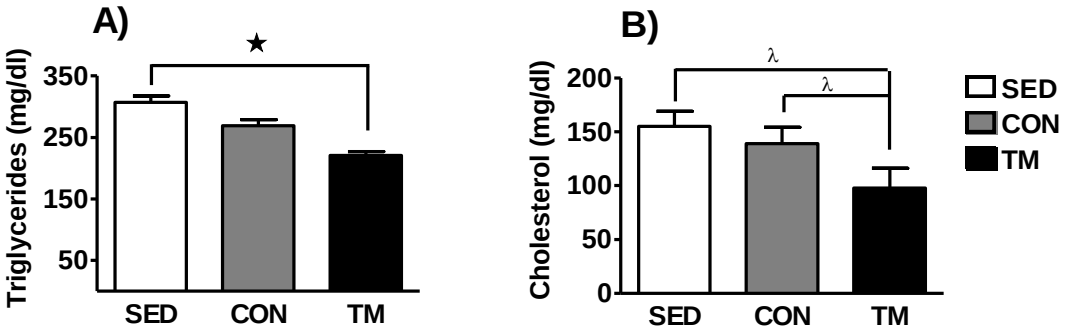
JFL_Figure 4


750
600
450
300
150
0
Distance (cm)
*
1
2
3
4
5
6
7
8
9
10
Minutes
SED
CON
TM

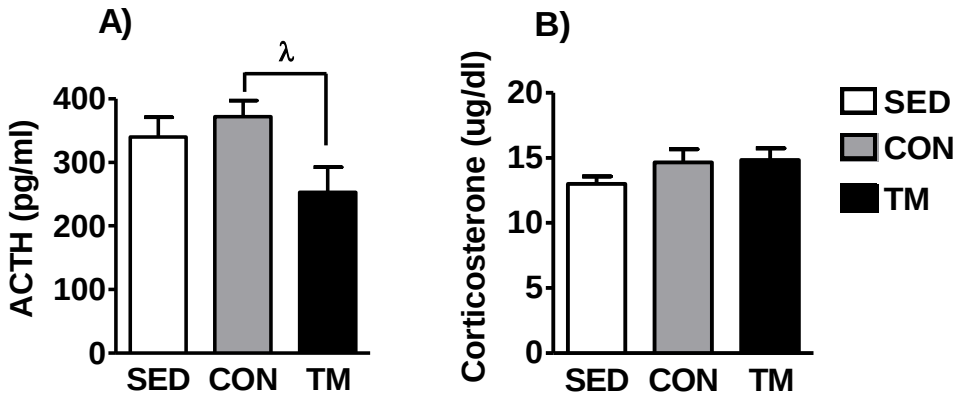
JFL_Figure 5


A)
+++
ACTH (pg /ml)
150
120
90
60
30
0
Morning
Evening
B)
+++
Corticosterone (ug /dl)
12
10
8
6
4
2
0
Morning
Evening
C)
+++
Glucose (mg /dl)
160
145
130
115
100
∇ #
∇ #
Morning
Evening
SED
CON
TM

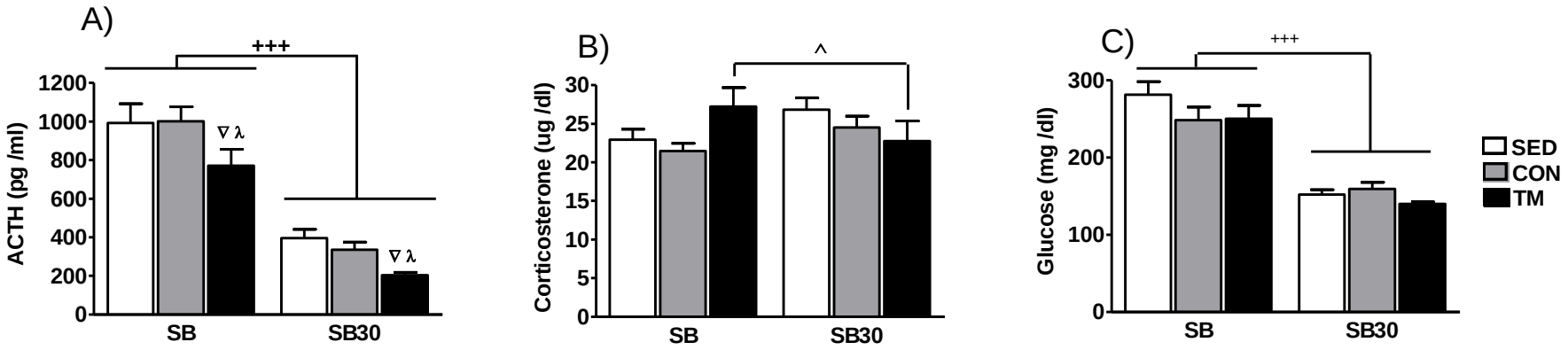
JFL_Figure 6


A)
Triglycerides (mg/dl)
350
250
150
50
★
SED
CON
TM
B)
Cholesterol (mg/dl)
200
150
100
50
0
λ
λ
SED
CON
TM

JFL_Figure 7


A)
λ
ACTH (pg/ml)
400
300
200
100
0
SED
CON
TM
B)
Corticosterone (ug/dl)
20
15
10
5
0
SED
CON
TM
SED
CON
TM

JFL_Figure 8


A)
+++
ACTH (pg /ml)
1200
1000
800
600
400
200
0
∇ λ
∇ λ
SB
SB30
B)
^
Corticosterone (ug /dl)
30
25
20
15
10
5
0
SB
SB30
C)
+++
Glucose (mg /dl)
300
200
100
0
SB
SB30
SED
CON
TM

JFL_Figure 9