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REVIEW

Changes in behaviour and in the circadian rhythms of melatonin and corticosterone in rats subjected to a forced-swimming test

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Summary
We studied the influence of physical activity stress on the circadian rhythms of melatonin and corticosterone in 3-month old male Wistar rats. Every two hours for 24 h around the clock, an animal from the stressed group was first made to swim for two hours, and was then subjected to a further ten minutes of forced swimming using a modification of the apparatus employed in the Porsolt test. The capacity to resume swimming after the exhausting 2-hour swim was measured by the number of swimming movements that were made by the animal in the additional 10-min swimming period. Blood was collected immediately after the trial, and the plasma melatonin and corticosterone levels determined by RIA. Control group blood was collected at 1-h intervals in the periods from 22:00 to 06:00 and from 16:00 to 18:00, and at 2-h intervals during the remaining periods. The control rats presented plasma melatonin and corticosterone circadian rhythms with nocturnal (02:00) and diurnal (17:00) maxima, respectively. The pattern of these rhythms in the stressed rats was flatter, and the animals tested during hours of the night presented greater endurance than those tested during daytime hours. This suggests that, in evaluating an animal's response to stress, it is important to take into account the co-ordination between the time of day when the physical stressing test is applied and the natural sleep/activity periods of the study species.

Keywords: Melatonin – corticosterone – behaviour – physical activity – stress – rat – circadian rhythms

INTRODUCTION

The alternation of day and night in the Earth's cycle is so reliable that it is not surprising that animals, plants, and bacteria adjust their behaviour and physiology accordingly, reflected in natural behavioural changes with a daily or circadian rhythm (Bünning 1967). These changes represent one of the most ubiquitous strategies used by living organisms to adapt to their environment. Underlying the daily changes in behaviour are a multitude of circadian or seasonal endocrine and metabolic rhythms which provide adaptively significant temporal organization within the organism (Turek 1994).

The functional mechanism involved is based on one or more internal biological clocks, one of

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the critical components of which is melatonin, the principal hormone produced by the pineal gland. (Pévet 2000). This hormone displays characteristic daily and seasonal patterns of secretion which result in robust and predictable rhythms that seem to act as strong synchronizers of the circadian clock in photoperiodic species (Simonneaux and Ribelayga 2003) by giving a time-related signal to a number of physiological processes (Arendt 1995), and encoding for the duration of darkness (Reiter 1993), since its production appears to be related to one of an organism's most important functions presenting circadian rhythmicity – the alternation of sleep and wakefulness (Brown 1994, Lavie 2001).

The body's interpretation of melatonin's message is essential for an animal to adapt its physiological functions to environmental conditions, and thereby increase its likelihood of survival (Skwarło-Sońta 1996). It has been observed that stressors – defined as conditions that endanger or that are perceived to endanger the survival of an individual (Van de Kar and Blair 1999) – alter the endogenous profiles of melatonin secretion (Persengiev et al. 1991, Reiter et al. 1992, Barriga et al. 2002).

While physical exercise, which is regarded as one of the main forms of provoking stress episodes in an organism (Simon 1991), has been one of the most widely studied stressors (Buxton et al. 1997, Barriga et al. 2000, Barriga et al. 2001), there is some controversy about the effects of physical activity on the endogenous profile of melatonin secretion. Thus, different workers have reported that melatonin concentrations increase, decrease, and remain unaffected following bouts of exercise (Carr 1981, Theron et al. 1984, Monteleone et al. 1990, Monteleone et al. 1992, Miyazaki et al. 2001). These discrepancies are found in the particular case of experiments in which the stressor was forced swimming (Troiani et al. 1988a, Wu et al. 1988, Tannenbaum et al. 1989, Yaga et al. 1993, Barriga et al. 2001), which is the classical paradigmatic model of physical-activity-induced stress (Ferry et al. 1991, Forner et al. 1995, Ortega et al. 1997, Barriga et al. 2001).

Exposure to hostile conditions also results in a series of co-ordinated responses organized to enhance the probability of survival. These co-ordinated responses – often referred to as "stress responses" – are characterized by the activation of the autonomic nervous system and the hypothalamic-pituitary-adrenocortical axis (HPA) which produces neuroendocrine changes, alterations in behaviour and autonomic function, and the secretion of a multiplicity of hormones, including corticosterone (Van de Kar and Blair 1999) which is also considered to be a regulating chemical pacemaker (Norris 1997). Given this hormone's essential role in survival during emergencies (Van de Kar and Blair 1999), it needs to be studied in investigations involving prolonged stressful episodes.

Most of the many studies on melatonin, corticosterone, and physical activity in rodents have focused on the changes that have taken place in hormone secretion when the exercise has finished. In this sense, the animal behaviour provoked by the physical activity stress has not usually been studied, and indeed the rat "forced-swim test" (FST) has been used only as an experimental model to screen antidepressant drugs (Abel 1993a).

We believed, however, that the apparatus with which the test is performed might be very well suited to studying animal behaviour in relation to forced exercise as a stressor. Given that melatonin and corticosterone are endogenous pacemakers, and that their circadian rhythms are altered in situations of stress, the objective of the present work was to use the physical structure on which the FST is based to evaluate both the animal's response to a single bout of forced swimming exercise (as measured by the number of paddle movements made in the ten minutes following the prior two-hour period of swimming), and the effect of the stressor on the natural rhythms of endogenous melatonin and corticosterone in their plasma.

MATERIAL AND METHODS

Subjects

The trials were performed on male Wistar rats *(Rattus norvegicus)*, aged 12±2 weeks, weighing 450±50g, purchased from the University of Extremadura Animal Service, maintained at a constant temperature (20±2°C), and fed on "Panlab" meal and water *ad libitum*.

The animals were housed, two to a cage, in 500×250×150 mm "Panlab" cages in a 2.86×3.80×2.85 m room, with artificial lighting, indirect ventilation, and at 50% relative humidity. They were kept under a 12/12h light/dark cycle (lights out at 20:00h). They were examined before the trials, and only those with no signs of pathology were used.

The animals were divided into two experimental groups: control group (not subjected to any physical activity, but allowed the exercise voluntarily performed in their cages), and stress group (subjected to forced swimming physical activity stress). The experimental protocol was carried out under the guidelines of the Ethical Committee of the University of Extremadura (Spain) and was in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the European Community Council directives (86/609/EEC).

Acute physical activity procedure

A classical model of physical activity induced stress was employed – forced prolonged swimming. The trials were in two parts. First, an animal was put into a 150×60×60 cm tank full of clean water at 37°C and made to swim continuously for 2h. Second, immediately afterwards it was transferred to another glass tank (50.8cm deep, 15.5cm wide, and 199.4cm in perimeter; Figure 1), whose structure is based on the modified Porsolt test (Porsolt et al. 1978) proposed by Nomura et al. (1982), and subjected to further forced swimming. The tank was filled to a depth of 35cm with clean water maintained at a temperature of 37°C.

In the centre of the tank, a freely rotating drum (length 13.7 cm, diameter 8 cm) fitted with 6 paddles 13.1 cm high and 1.4 cm wide around its circumference offered the animal apparent support, and its rotating movements served to record the swimming activity. Attached to one end of the drum, a small magnet activated a reed relay placed on the outer surface of the enclosure, so that every passage of the magnet produced a closure of the reed relay contacts. As the closure time was fairly irregular in duration, the output of the relay was input to a Schmidt trigger circuit which normalized the pulse duration. In turn, this pulse was used to close the contacts of a second relay connected in parallel with a key in the keyboard of a computer, resulting in a letter appearing on the computer screen. A simple Basic program counted the number of key closures as well as their temporal distribution.

The animal was allowed to move the drum as often as its strength permitted, and when it stopped moving the drum and sank, it was immediately removed from the tank, and the trial was terminated. This two-part test was repeated every two hours over one circadian period. In both parts, the animal swam in clean water because using water previously swum in by another rat has been shown to alter behaviour (Abel 1993b).

In the nocturnal period, both parts of the trial, including the behavioural scoring, were conducted in dim red light, perceived as darkness by albino rats (Reiter et al. 1998, Kelliher et al. 2000).

Physical parameters

The number of drum movements (paddle movements) made by each animal during a 10-min period was logged at 1-min intervals.

Plasma collection

The animals were killed by decapitation and blood from the neck blood vessels was collected in EDTA tubes and centrifuged at room temperature for 15 min at 1100g in order to separate the plasma, which was stored frozen (–20°C) until assay. The control group plasma collection was performed at 1-h intervals in the periods from 22:00 to 06:00 and from 16:00 to 18:00, and at 2-h intervals for the rest of the 24-h cycle.

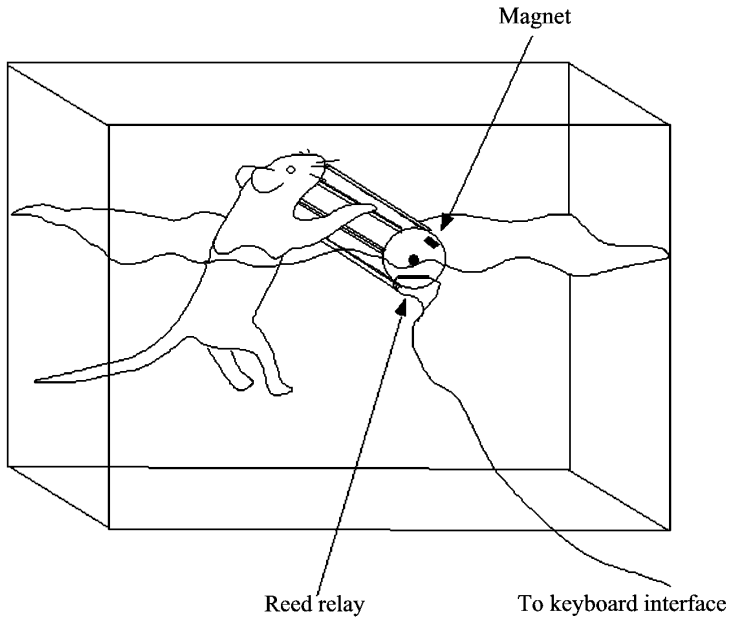

Fig. 1. **Diagram of the apparatus**

The more exhaustive analysis of the former two periods was designed to establish precisely the natural endogenous secretion patterns of the two hormones being studied, since their maxima may occur within those intervals of the day (Płytycz and Seljelid 1997, Urbanski 2000). In the stressed group, the animals were sacrificed immediately after finishing the exercise test, so that there was a plasma collection at 2-h intervals throughout the 24-h cycle. In both the control and the stress group the number of animals used for each time point was 10 (n=10).

Determination of plasma haemoglobin
Plasma haemoglobin was quantified using a haematological autoanalyser (Belchman Coulter, Fullerton, USA).

Determination of plasma corticosterone
Plasma corticosterone levels were determined by means of a radioimmunoassay (RIA) kit (DRG Diagnostics International Inc., USA), using ^{125}I-corticosterone (<3 μCi). The results are expressed in ng/mL.

Determination of plasma melatonin
Plasma melatonin levels were determined by means of an RIA kit (IBL Immuno-Biological Laboratories, Hamburg, Germany), using ^{125}I-melatonin (=140 kBq 5.5 mL). The results are expressed in pg/mL.

Determination of the interference from haemoglobin present in the plasma in the RIA of the plasma melatonin
In melatonin RIA, false values may arise due to interference from the haemoglobin present in the plasma. Therefore, prior to the melatonin assay, we first determined the haemoglobin concentration in the plasma. Samples found to have haemoglobin concentrations >1.5 mg/mL were discarded from further consideration because of the excessively high false signal they would have given in the melatonin assay. We then performed a melatonin RIA using a haemoglobin standard (BioRad Laboratories, California, USA) at the different concentrations found in the first step. Finally, these "false" melatonin values were subtracted from the corresponding final plasma melatonin RIA values.

Statistical analysis
All data are expressed as mean ± SD. Variables were tested for normality, and the Scheffe ANOVA parametric F-test was used for comparison between groups. Student's parametric t-test was used to analyse the paired data. The level of significance was taken to be $2\alpha=0.05$.

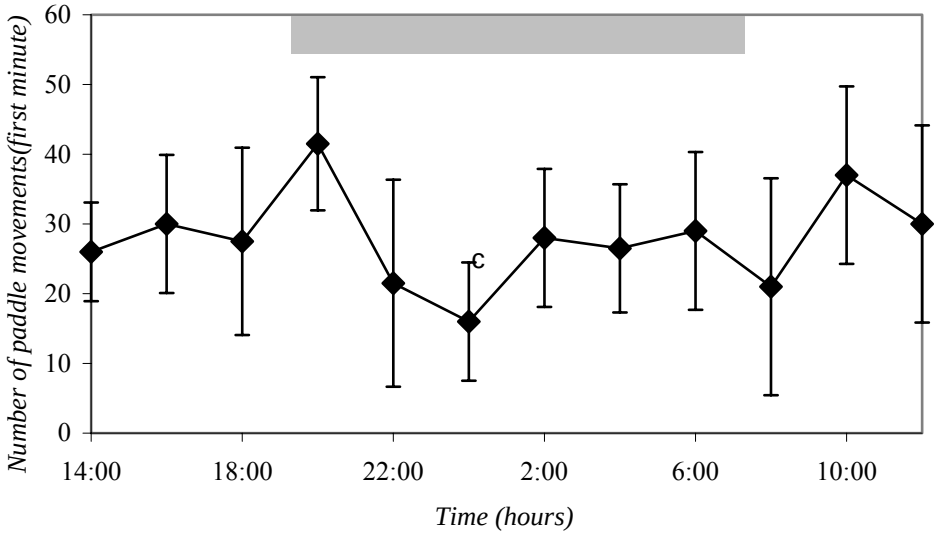

Fig. 2. **The paddle movements made during the first minute of the test by three-month-old male Wistar rats.** Each value represents the mean ± SD of ten determinations. The dark band represents the determinations carried out during the period of darkness.

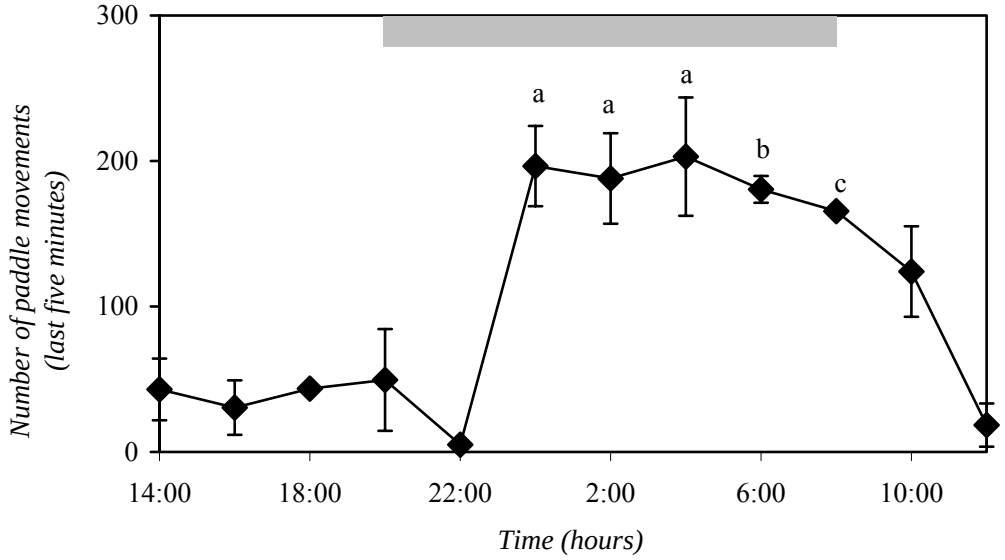

Fig. 3. **The paddle movements made from minute 5 until minute 10 of the test by three-month-old male Wistar rats.** Each value represents the mean ± SD of ten determinations. The dark band represents the determinations carried out during the period of darkness. a) statistically significant with respect to the values obtained at 12:00, 14:00, 16:00, 18:00, 20:00, and 22:00 hours (ANOVA); b) statistically significant with respect to the values obtained at 12:00, 14:00, 16:00, 18:00, and 22:00 hours (ANOVA); c) statistically significant with respect to the values obtained at 12:00, 16:00, and 22:00 hours (ANOVA).

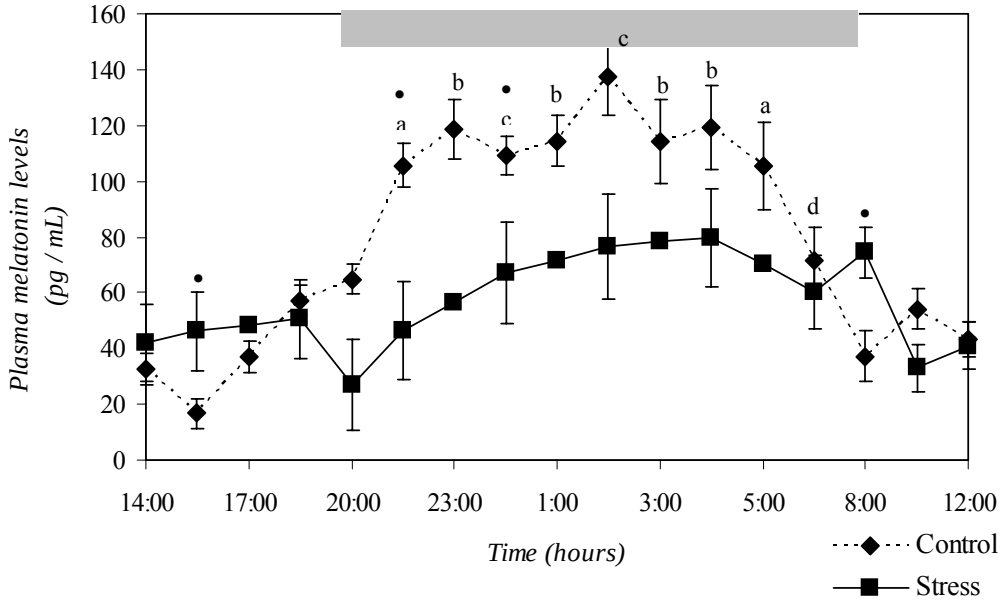

Fig. 4. **The daily variations of plasma melatonin levels in three-month-old male Wistar rats: control group and a group subjected to stress.** Each value represents the mean ± SD of ten determinations carried out in duplicate. The dark band represents the determinations carried out during the period of darkness. a) statistically significant with respect to the values obtained at 08:00, 10:00, 12:00, 14:00, 16:00, and 17:00 hours (ANOVA); b) statistically significant with respect to the values obtained at 08:00, 10:00, 12:00, 14:00, 16:00, 17:00, 18:00, and 20:00 hours (ANOVA); c) statistically significant with respect to the values obtained at 06:00, 08:00, 10:00, 12:00, 14:00, 16:00, 17:00, 18:00, and 20:00 hours (ANOVA); d) statistically significant with respect to the values obtained at 16:00 hours (ANOVA); ●) statistically significant with respect to the values obtained at the same time of day in the control group (Student's t).

RESULTS

The mean number of paddle movements made by each group of animals for each of the times of day studied is shown in Fig. 2 (first minute) and Fig. 3 (last five minutes). One observes that, in the case of the last five minutes (Fig. 3), the values were generally greater in the nocturnal than in the diurnal tests, with the differences being statistically significant at 00:00 (197±28 paddle movements), 02:00 (188±31 paddle movements), 04:00 (203±41 paddle movements), 06:00 (181±9 paddle movements), and 08:00 (166±2 paddle movements).

Figure 4 shows the plasma melatonin levels over a 24-h period at 1-h intervals in the periods from 22:00 to 06:00 and from 16:00 to 18:00, and at 2-h intervals during the remaining periods, in both the control and stressed groups. In the control animals, a maximum melatonin peak of 137±14 pg/mL occurred at 02:00. All the values in the dark period were statistically significantly different from those in the light period. In the stressed animals, the melatonin levels were more or less uniform over the 24-h study period, there being no significant differences between the values corresponding to the different times, so that one can state that the circadian rhythm observed in the control group is lost. There were statistically significantly differences with respect to the controls at 16:00 and at 08:00.

Figure 5 shows the plasma corticosterone levels in the two groups. In the control animals, a maximum corticosterone peak of 285±19 ng/mL, significantly different from the other hours of the day, occurred at 17:00. In the stressed animals, there was a peak in corticosterone secretion of 774.5±59 ng/mL at 14:00 which was significant only with respect to the value at 22:00 (414±62 ng/mL). At all the times of the day analysed, the corticosterone levels were significantly higher in the stressed animals than in the controls.

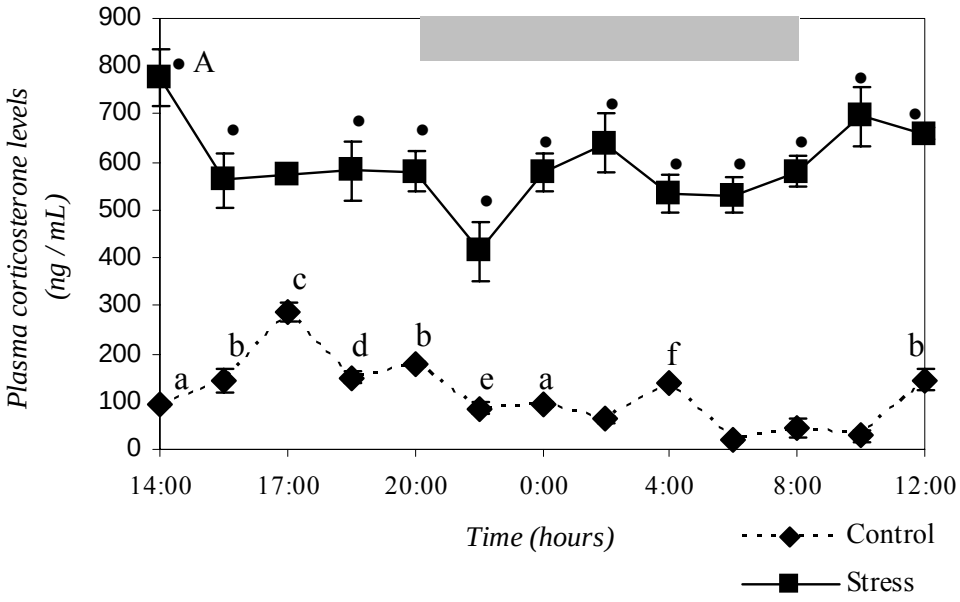

Fig. 5. **The daily variations of plasma corticosterone levels in three-month-old male Wistar rats: control group and a group subjected to stress**. Each value represents the mean ± SD of ten determinations carried out in duplicate. The dark band represents the determinations carried out during the period of darkness. a) statistically significant with respect to the values obtained at 06:00, 08:00, and 10:00 hours (ANOVA); b) statistically significant with respect to the values obtained at 02:00, 06:00, 08:00, 10:00, and 22:00 hours (ANOVA); c) statistically significant with respect to the values obtained at 00:00, 02:00, 06:00, 08:00, 10:00, 14:00, and 22:00 hours (ANOVA); d) statistically significant with respect to the values obtained at 00:00, 02:00, 04:00, 06:00, 08:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00, and 22:00 hours (ANOVA); e) statistically significant with respect to the values obtained at 00:00, 02:00, 04:00, 06:00, 08:00, 10:00, and 22:00 hours (ANOVA); f) statistically significant with respect to the values obtained at 22:00 hours (ANOVA); •) statistically significant with respect to the values obtained at the same time of day in the control group (Student's t).

DISCUSSION

Circadian rhythms are an evolutionary adaptation to day/night alternations, i.e., an adaptation to the environmental changes caused by the Earth's rotation (Berger 2003). They are defined operationally as biorhythms that persist (or free-run) with a period of approximately 24 hours in the absence of external time cues (or zeitgebers), are reset by changes in environmental conditions, most notably the daily dark-light and temperature cycles, and have an invariant period length over a wide range of physiologically relevant temperatures (temperature compensation) (Edery 2000). The plasma level of the pineal hormone melatonin characteristically presents rhythms that show a clear-cut circadian pattern, with secretion of the hormone occurring almost exclusively during the night (Vaughan et al. 1976, Luboshitzky 1998). This is because the activity of the limiting enzyme in its synthesis pathway – arylalkylamine-N-acetyltransferase – has a circadian rhythm with a maximum during the dark period (Falcón 1999). In the case of the laboratory rat (and of man), the pattern of melatonin secretion is said to be of type B, which is characterized by a gradual increase in melatonin synthesis from the time when lights are switched off with a peak occurring approximately in the middle of the dark phase (Urbanski 2000). The results of the present work were coherent with this pattern – there was the aforementioned gradual increase in melatonin levels from 20:00 (the time of lights out), and a maximum in secretion at 02:00, exactly half-way through the dark period.

The FST was originally proposed as an antidepressant screening test, and was based on the observation that mice forced to swim in a restricted space rapidly cease moving and become lethargic (Porsolt et al. 1978). Since the measurement of mobility may be biased by the subjectivity of each experimenter, the test was later modified by adding a Perspex (polymethyl methacrylate) water tank with a water wheel in its centre, in which the number of rotations of the wheel were counted by a photointerrupter attached to the shaft (Nomura et al. 1982). Many studies of the effects of forced-swimming-induced stress on the secretion of hormones (cortisol, vasopressin, glucagon, prolactin, thyroid stimulating hormone, and opioids) and neurotransmitters (catecholamines) have centred on the changes subsequent to the stress episode. Perhaps this was due to the lack of suitable recording equipment. Nevertheless, taking the physical structure of the FST as the basis, we designed an experiment that allows one to see how the animal's response varies over the course of an exercise session by counting the number of paddle movements performed by each rat corresponding to the different time-of-day groups. This parameter reflects the physical activity carried out by the animal. The experiment revealed a fundamental difference in the rats' behaviour under diurnal and nocturnal conditions. Thus, the number of paddle movements made during the final 10-minute swim by animals that performed the trial in the dark period was generally greater than those corresponding to the trials performed during the light period. In particular, this difference was observed in the last five minutes of each trial, but not during the first minute.

Since noradrenaline is involved in the control of melatonin synthesis and catecholamine secretions increase markedly during physical exercise, the effect of exercise on different aspects of the circadian rhythmicity of melatonin has been extensively analysed (Kjaer and Dela 1996). The findings, however, have been quite contradictory. Considering, for example, only the type of stressor used in the present study – swimming-induced stress – in one set of experiments, a decline in the nocturnal levels of melatonin but with the pineal NAT activity remaining unsuppressed has been observed in animals forced to swim during the night (Troiani et al. 1988a, Troiani et al. 1988b), in another, the serum melatonin levels in rats subjected to forced swimming at night remained high 15 min after the swimming was initiated, but had dropped slightly by 30 min. Yaga et al. (1993), and Monteleone et al. (1990, 1992) observed a decrease in melatonin synthesis caused by physical exercise. Such conflicting findings may be due to differences in lighting conditions and the time of day at which the study subjects exercised. In the present study, the melatonin concentrations declined with respect to the control group, although the nocturnal levels were still higher than the diurnal. This indicates that the plasma catecholamine concentration does not affect NAT activity (Joshi et al. 1986), and is coherent with previous results obtained by our laboratory (Barriga et al. 2001). Little is known, although much has been speculated, about the mechanisms which account for the large reduction in plasma melatonin levels in rats that are forced to swim at night. Early studies have suggested that it could be due to the inhibition of melatonin synthesis by corticosterone (Lowestein et al. 1984, Roberts et al. 1985), the principal glucocorticoid released in response to a stressing exercise in rodents (Ferry et al. 1991, Simon 1991, Borer et al. 1992). In a basal state, contrary to the case of melatonin, which reaches its maximum secretion during the period of darkness regardless of whether the animal is diurnal (such as man, with mental and locomotor activities as a rule enhanced during the light period of the day, while sleep or resting coincide with the period of darkness) or nocturnal (active during the dark period, such as mice and rats – the commonest laboratory animals), plasma glucocorticoid concentrations rise in anticipation of the beginning

of the period of locomotor activity, i.e., during the afternoon in nocturnally active species and during the day in diurnally active species (Płytycz and Seljelid 1997). In the present work, we observed a circadian rhythm of plasma corticosterone with a maximum at 17:00, in agreement with the pattern described for the basal secretion of this hormone in nocturnally active species. Also, the secondary peaks of secretion found during the cycle reflect the pulsatile secretion described by other workers (Windle et al. 1998, López-Calderón 1999). In the stressed animals, there was a loss of this corticosterone rhythm. Instead, there was a generalized increase in the release of this hormone at all the times evaluated with respect to the control group. Other work suggests that a decline in the circulating levels of L-tryptophan occurs because the glucocorticoids activate the enzyme tryptophan 2,3-dioxygenase (Feigelson and Greengard 1962) which catalyses the opening of tryptophan's five-atom ring to form N-formyl-kynurenine, and reduces the readiness of the pinealocytes to produce serotonin, the precursor of melatonin (Bartsch et al. 1999). Because of its low plasma levels found in stressed animals, there have been proposals to administer the hormone as a buffer to neutralize the adverse effects of glucocorticoids (Aoyama et al. 1987, Maestroni 1993). There is direct evidence that exercise increases the production of potentially tissue-damaging free radicals (Freeman and Crapo 1982). By interacting destructively with practically every cell component (Davies et al. 1982, Jenkins 1988), these increase lipid peroxidation in various tissues including skeletal and cardiac muscle, liver, brain, and erythrocytes (Venditti and Di Meo 1997). In this sense, it has been observed that melatonin at least partly prevents these adverse effects in rats subjected to swimming stress (Hara et al. 1996, Hara et al. 1997). It is evident that, if free radicals are involved in promoting muscle fatigue, manipulations changing the cellular antioxidant system will lead to alterations in muscle endurance, as could be the case of the increased melatonin levels typical of the dark period. There has been evidence of a relationship between endurance and the generation of free radicals during exercise, in particular that vitamin E and spin-trappers prolong endurance to physical exercise (Novelli et al. 1990), thus supporting the idea that free radicals have a physiological role during exercise. Also, melatonin has been found to affect other physiological functions thought to be limiting factors in endurance, e.g., water and electrolyte balance, and glycogen metabolism (Mazepa et al. 2000, Skotnicka and Hynczak 2001).

In sum, our results show, first, that swimming stress leads to a flattening of melatonin and corticosterone circadian rhythms, and second, that the capacity to endure a bout of extenuating exercise depends on the time of day at which the animal is subjected to the stressor, with the greater endurance during the night of a nocturnal animal such as the rat pointing to the capacity of melatonin to revert the effects of the stress. It would be interesting to observe the behavioural response of a diurnal animal to a similar stressor.

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