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Fuentes-Granados Citlalli [a], Duran Pilar [a b], Carmona-Castro Agustín [a], Cárdenas-Vázquez René [a] & Miranda-Anaya Manuel [a]
[a] Departamento de Biología Celular, Facultad de Ciencias
[b] Instituto de Neurobiología, Universidad Nacional Autónoma de México, D.F., 04510, México
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# Obesity alters the daily sleep homeostasis and metabolism of the volcano mouse *Neotomodon alstoni*

Fuentes-Granados Citlalli[a], Duran Pilar[a,b], Carmona-Castro Agustín[a], Cárdenas-Vázquez René[a] and Miranda-Anaya Manuel[a]*

[a]*Departamento de Biología Celular, Facultad de Ciencias;* [b]*Instituto de Neurobiología, Universidad Nacional Autónoma de México, D.F. 04510, México*

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Some Mexican volcano mice (*Neotomodon alstoni*), when in captivity and fed regularly on a laboratory rodents' diet, develop obesity. The aims of the present work are to compare lean and obese mice with regard to the main characteristics of the sleep–wake cycle in 12:12 LD, and to investigate if there is a correlation with changes in metabolic-related blood parameters (leptin, insulin, triacylglycerides, corticosterone and the glucose tolerance test). The analysis of the sleep–wake cycle evaluated the temporal distribution of vigilance states and indicated a polyphasic architecture. Trends were observed in obese mice to have increased percentages of slow wave sleep and decreased wakefulness, but the percentage of rapid eye movement sleep was not different. Obese mice had higher concentrations of leptin and insulin, and showed a slower glucose removal from blood at noon than at midnight, indicating a possible resistance to these hormones. We propose that volcano mice are a good model for studying daily rhythms and metabolic disorders related to obesity.

**Keywords:** obesity; insulin; leptin; glucose; sleep homeostasis; *Neotomodon alstoni*



## Introduction

Epidemiological surveys indicate that obesity is becoming a major health problem in modern societies, and its frequency continues to increase in many industrialized countries (Kelishadi 2007). Increased dietary energy content and reduced physical activity are the main factors that have contributed to this. The impact of obesity on sleep physiology due to metabolic deregulation is being increasingly recognized (Laposky et al. 2008; Sharma and Kavuru 2010). In obese humans, sleep patterns and the total amount of sleep are affected (Kalsbeek et al. 2001), and studies in animal models of obesity and metabolic syndrome show similar alterations to those observed in humans. Obese leptin-deficient *ob*/*ob* mice present an increase in sleeping time and a more fragmented sleep–wake pattern, as well as a disrupted circadian distributions of the rapid eye movement (REM) sleep, non-REM (NREM) sleep and slow wave sleep (SWS) (Laposky et al. 2006). Similar findings have been reported in the obese/diabetic mouse with a mutation in the leptin receptor, which exhibits sleep

*Corresponding author. Email: miranmanuel@ciencias.unam.mx

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fragmentation, decreased compensatory response to sleep deprivation and decreased locomotor responses (Laposky et al. 2008). In mice with diet-induced obesity (DIO), wakefulness is reduced, whereas NREM sleep increases primarily during the dark period without sleep defragmentation (Guan et al. 1997; Jenkins et al. 2006).

The rodent models previously used to study obesity require genetic or physiologic intervention or the use of hypercaloric diets (for reviews, see Ghosh et al. 2010; Kennedy et al. 2010; Tschöp and Heiman 2001). However, the volcano mouse, *Neotomodon alstoni*, is an endemic species from the trans-volcano belt of Mexico. This species has been the subject of diverse biological and physiological studies including circadian rhythms (Fuentes-Granados et al. 2010) and the sleep–wake cycle (Ayala-Guerrero et al. 1998). Young animals captured in the field and kept under vivarium conditions, show body weight increases when fed a regular rodent chow, around 50% of them developing obesity. Mice that develop obesity may show some metabolic syndrome signs, such as hepatic steatosis, hypertriglyceridemia, glucose intolerance and hyperleptinemia (Carmona-Castro 2006; Fuentes-Granados 2011). Therefore, this species is a putative animal model to study the relationship between obesity and the sleep–wake cycle.

The aim of the present work was to study the effect of obesity, produced by vivarium conditions where there was some degree of space restriction and the animals consumed *ad libitum* a regular rodent laboratory diet, upon the sleep–wake cycle in adult male volcano mice, *N. alstoni*.



## Methods

### *Animals and housing*

Young adult males of the species *N. alstoni* ($n = 24$) were captured in the field and chosen according to the following characteristics (indicated in other studies: Nowak 1999; Ceballos and Oliva 2005): body weight below 40 g, body length from 100 to 130 mm, ears almost hairless, a whitish under-belly, and the upper parts of the body covered with a dense clear gray fur. All animals were kept in quarantine, housed individually at the vivarium facilities at the Facultad de Ciencias, UNAM. The mice were fed with a commercial diet for laboratory rodents (Rodent Lab Chow 5001, Purina Inc.) and tap water was provided *ad libitum*. Room temperature was between 18°C and 23°C, the light–dark cycle (LD) was set at 12:12 (photophase: 0600-1800 h, 200-250 lx; Quanto-radio-photometer, Li-Cor model Li180). After seven months in captivity, mice were divided according to their bodyweight (BW) in two groups: lean controls (CTL, BW 47 $\pm$ 4g) and obese mice (Ob, 72 $\pm$ 2g). All procedures described in this work were carried out in accordance with the official Mexican Regulation for Experimentation in Animals (NOM-062-Z00-1999, 2001).

### *Electrode implantation and sleep recordings*

Electrode implantation was performed in 10 adult males (5 obese and 5 lean). All animals were anesthetized via intramuscular injection of Ketamine (30 mg/kg) and Xilacin (10 mg/kg). Stainless steel bipolar electrodes were implanted stereotaxically in the left occipital cortex (3.0–3.2 mm lateral from the mid-line of sagittal suture and 2.0–2.2 mm anterior to the lambda). Bipolar stainless steel electrodes were also implanted in the dorsal neck muscles, and one reference screw electrode was fixed in the bone located above the cerebellar region. All electrodes were connected to an

Amphenol cap mounted on the animal's skull, fixed with dental acrylic cement. Recordings of vigilance states started seven days after surgery. At least 24 h before the start of electrocorticographic (ECoG) recordings, each mouse was placed individually in a clear glass box (60 × 47 × 50 cm) located in an electrically shielded, sound-attenuated chamber (120 × 75 × 120 cm), illuminated by a fluorescent white lamp maintaining the same LD schedule as when in the housing (see above). All animals remained undisturbed and with the electrode cables attached. Electrode cables were connected to a counter-weighted Airflyte 9-lead slip ring. Food and water were available *ad libitum*.

The recording chambers were maintained at room temperature (24–25°C) and 40–50% humidity. ECoG and electromyograms (EMG) were obtained with the use of AC voltage amplifiers ISODAM8A (WPI). Signals were digitized and captured into a PC every 10 min using a sample rate of 200 Hz via an acquisition data system (MP100) and the correspondent Acqknowledge 3.8.1, (BIOPAC®) software.



### *Blood analysis*

Blood samples from the retro-orbital sinus were taken to assay plasma concentrations of leptin, insulin, triacylglycerides, and corticosterone. Animals were entrained to LD 12:12, with lights on from 06:00 h to 18:00 h, and blood samples taken after anesthetization with volatilized isofluorane (Dorin, Halocarbon Labs., USA). Samples were taken from the same animal at mid-day and midnight, leaving at least three days between the two sampling times, samples being taken from animals that had been food-restricted for 4 h.

All samples were centrifuged at 3500 rpm, and the plasma was separated and kept at −70°C until assayed. Twelve lean and 12 obese mice were used to get blood samples to determine triacylglycerides and insulin, while samples from 6 lean and 6 obese were used to quantify leptin and corticosterone. Plasma triacylglycerides were quantified using Triacylglycerides-L kits (Spinreact); insulin, leptin and corticosterone were quantified using an Ultra Sensitive Mouse Insulin ELISA kit, Mouse Leptin ELISA kit (both from Cristal Chem. Inc, USA), and Corticosterone ELISA kit (from Assay Designs Inc, USA). All ELISA reactions were measured with a micro plate reader (Spectramax 190, Molecular Devices, USA) in accordance with the manufacturer's instructions.

### *Glucose tolerance tests*

Glucose tolerance tests (GTT) were performed in five obese and five lean mice, both at noon and midnight leaving at least three days between the two sampling times. Samples were taken from animals that had been food-deprived for 4 h. A solution of 50% glucose at a dose of 2.5 g glucose/kg per body weight (BW) was administrated i.p. Blood samples were taken before (time 0) and 30, 60, 90 and 120 min after glucose administration. Glucose was quantified using reactive bands and a glucometer (Accutrend GCT, Roche Diagnostics).

### *Data analysis*

Data obtained from metabolic parameters were compared with unpaired Student's *t*-tests. For GTTs comparisons in Relative Units (RU), area under the curve was

calculated using the Origin-Pro 7.5 software, and also compared by Student's *t*-tests. The sleep recordings were obtained using epochs of 10 s with the program Acqknowledge 3.8.1 (BIOPAC®), and three vigilance states were visually distinguished and scored: Wakefulness (W), slow wave sleep (SWS) and rapid eye movement sleep (REMS). The duration in minutes and the temporal distribution of each state were calculated by means of the software Estadsue (designed by M.A. Guevara at UNAM, 1997). Results were compared with a one-way ANOVA, using Tukey post-hoc tests to assess significant differences (Stat Graphics Centurion, 16.1.05). Statistical significance was set at $p < 0.05$.

## Results

### *Differences in sleep pattern*

Figure 1A shows the average ($\pm$SE) of the percentage for each vigilance state (W, SWS and REMS) quantified by ECoG every 2 h. Lean animals (left panel) presented an irregular distribution of these states, with a clear dominance of W over SWS and REMS; no clear difference between arousals and waking in the dark or light phases of the LD cycle was noted. In obese mice, even though this irregular pattern persisted, there was a clear decrease in percentage of the W state and increase of

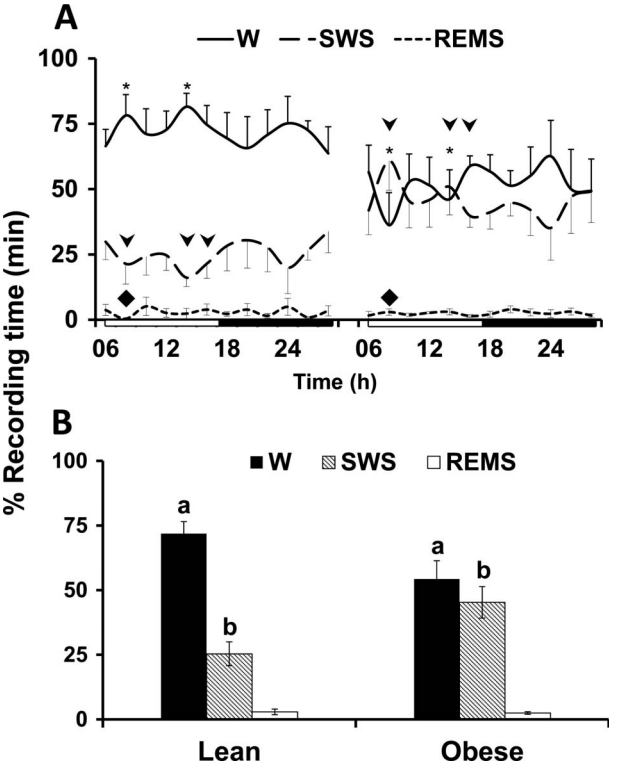


Figure 1. (A) Two-hourly distribution and (B) 24-h averages for each vigilance state: Wakefulness (W), slow wave sleep (SWS) and rapid eye movement sleep (REMS). Left panel, lean animals; right panel, obese animals. Values are mean $\pm$ SEM. Significant differences in a sleep stage between lean and obese mice are denoted with the same letter or symbol ($p < 0.05$).

SWS. Averages over the 24 h are shown in Figure 1B. Significant differences ($p < 0.05$) were observed between lean and obese, respectively in W (72 ± 4.7% and 54.3 ± 7.1%), SWS (25.4 ± 4.6% and 45.3 ± 6.1%), but no differences in REMS (2.9 ± 1.1 and 2.45 ± 0.5%) were found between the two groups.

### *Differences on serum metabolites*

Figure 2A shows the differences between noon and midnight in plasma leptin, insulin, triacylglycerides and corticosterone. Average concentrations (±SE) are shown, as are comparisons between lean (white bars) and obese (gray bars) mice. Significant differences were found in the content of leptin at midnight (0.171 ± 0.037 vs. 1.059 ± 0.33 ng/ml, lean and obese mice, respectively); similar but not significant differences were found at noon (0.201 ± 0.038 vs. 0.947 ± 0.35 ng/ml). Insulin levels were lower in lean than in obese mice, although no significant differences were found either at noon (0.99 ± 0.26 vs. 1.35 ± 0.20 ng/ml) or at midnight (0.58 ± 0.24 vs. 0.82 ± 0.19 ng/ml). Triacylglyceride concentrations were not different at noon (117.23 ± 19.80 vs. 118.33 ± 17.12 mg/dl) but were different at midnight, being higher in obese animals (26.53 ± 4.31 vs. 67.84 ± 13.45 mg/dl). Corticosterone levels tended to be lower in obese animals, though the differences with lean were not significant either at noon (30.7 ± 4.1 vs. 30.0 ± 3.2 ng/ml) or at midnight (46.0 ± 4.6 vs. 53.5 ± 7.1 ng/ml). Significant day/night differences in concentrations (indicated by "a") were found in lean mice only for corticosterone and triacylglyceride levels.

### *Glucose Tolerance Test*

Figure 2B shows the GTT curves for lean (left) and obese mice (right). Comparing the areas under the curves in relative units (RU), no significant differences were found between noon (9096 ± 2642.7 RU, open circles) and midnight (8106 ± 2524 RU, filled circles) in lean mice, whereas glucose removal in obese mice was faster at midnight than at noon (midnight = 7302 ± 894.2; noon = 17507 ± 4440, RU; $p < 0.05$, Student *t*-test). A star on the plot denotes a significant difference between the two curves at this time-point.

## Discussion

Obese mice of the species *N. alstoni* showed a clear increase in SWS percentage and this may be related to the decrease in W percentage according with ECoG. Plasma analyses of several metabolites indicated differences in leptin and triacylglycerides concentrations between obese and lean mice, also with a tendency for higher insulin levels in obese animals. GTT indicated slower glucose removal from the blood, even though obese animals exhibited higher levels of insulin and leptin, and this indicates resistance to these hormones in obese mice. These results are consistent with those from other mouse species, where obesity seems to affect particularly those mechanisms involved in the homeostasis of the sleep process. Examples of this altered sleep homeostasis are seen in leptin-deficient *ob*/*ob* mice, leptin-resistant *db*/*db* mice (Laposky et al. 2006, 2008) and, particularly, mice with diet-induced obesity (DIO), where W is reduced but NREM sleep is increased (Guan et al. 1997; Jenkins et al. 2006).

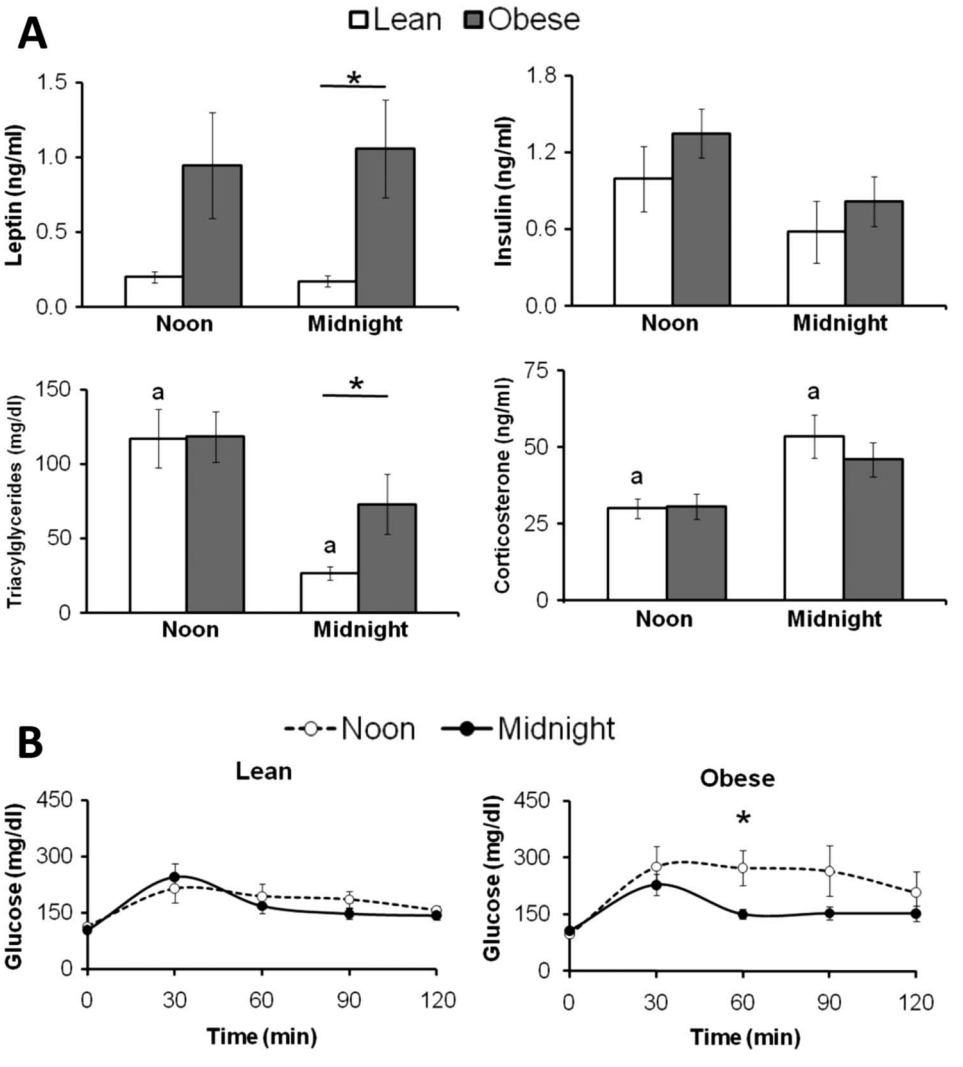


Figure 2. (A) Comparisons (mean ± SEM) between noon and midnight of average concentrations of leptin and insulin (top), and of triacylglycerides and corticosterone (bottom) in lean (open bars) and obese (closed bars) mice. A star indicates a significant difference between the two groups. Significant differences between noon and midnight are denoted by "a". (B) plasma glucose concentrations during the GTT for lean (left panel) and obese (right panel) mice at noon (open circles) and midnight (closed circles).

Even though there is an association between sleep regulation and obesity, and sleep is important in maintaining energy balance, there are also several examples that indicate that metabolic pathways reciprocally interact to influence sleep–wake regulation (Laposky et al. 2008). Orexins have been found to be influenced by peripheral metabolic cues such as leptin, ghrelin and glucose; these findings suggest that orexin may provide an important link between sleep and metabolism, as it may play a role in co-ordinating periods of sleep, food consumption and energy storage (Sharma and Kavuru 2010). Orexinergic neurons localized in the hypothalamus

project to several brain areas, including the paraventricular nucleus of the thalamus, the arcuate nucleus and, most notably, the locus coeruleus and tubero-mammillary nucleus (areas involved in the regulation of wakefulness). An internal imbalance may occur through many pathways involving, for example, sympathetic overstimulation, hormonal imbalance and subclinical inflammation (Sharma and Kavuru 2010). Therefore, animal models that can help achieve a better understanding on how these imbalances occur are desirable.

The results in the present work were obtained from wild-type laboratory-raised animals, and so a higher natural variability occur in such a population is to be expected. All animals chosen for the present study were young males when captured, but details of age variability are not known. By the time the sleep recordings were performed and blood samples were taken, the animals had been kept under vivarium conditions for seven months, reducing the possibility that the findings were a consequence of incomplete acclimation to laboratory conditions. Obesity in this species is particularly easy to develop in captured wild-type animals when kept in the laboratory – by the seventh month of captivity, differences in body weight are apparent.

The 24-h temporal distribution of the sleep–wake cycle showed a poly-phasic pattern, as found in humans. SWS increased in obese mice, both in the photophase and scotophase, whereas no differences in REMS were observed. The distribution of activity during the course of the LD cycle indicates that these animals present frequent arousals and quiet waking periods, a behavioral pattern which is sometimes observed in non-wheel running environments (Fuentes-Granados et al. 2010) and is also consistent with previous studies of sleep in this species (Ayala-Guerrero et al. 1998). The difference in W evaluated by ECoG, is however not detected when activity is tested by infrared crossings (data not shown).

Day/night differences in control, lean mice were found in basal concentrations of triacylglycerides and corticosterone, and a tendency existed for insulin levels to be higher at mid-day. However, no differences were detected in basal concentrations of glucose or leptin, possibly because of the time-points studied. Basal concentrations of blood parameters were tested after four hours of fasting; had we taken blood samples in animals that had been fed *ad libitum*, higher differences might have been found, particularly if the mice were active and feeding. Leptin showed higher levels (around four to five times) in obese than in lean mice at both times of sampling. Since this hormone is involved in regulation of feeding behavior (Anubhuti 2008), the high levels observed in the obese mice suggest that leptin-resistance was present.

Glucose removal from the blood, evaluated thorough GTT, showed a return to basal values within 2 h. There were no differences in this process between noon and midnight in lean mice, but glucose removal was less efficient at noon in obese mice, despite a tendency for higher insulin levels than in lean mice (Figure 2B), a result which may indicate insulin resistance. This resistance needs to be confirmed in further studies; however, in preliminary analyzes in our laboratory, we have observed that obese mice respond with higher plasma insulin concentrations when glucose level rise during the GTT (unpublished observations).

The use of non-traditional animal models allows investigations of variation in physiological responses, typical within natural populations (Heideman 2004), and may lead to the discovery of useful animal models of human disease. Such a discovery occurred with the mouse *Peromiscus californicus*, which was found to develop diabetes mellitus type 2 when fed commercial diets (Krugner-Higby et al.

2000, 2006). The results obtained in the present work lead us to conclude that a high percentage of wild-cached Mexican volcano mice, *N. alstoni*, develop obesity, with some of the parameters typically present in metabolic syndrome in humans (for example, hyper-triacylglyceridemia, hyper-insulinemia and hyper-leptinemia). In this species, obesity was reached in around 50% of the population by using a regular rodent chow; it did not require any special treatment and, importantly, the obese phenotype presents some metabolic signs that resemble metabolic syndrome. These attributes make *N. alstoni* an important candidate for an animal model to study, within a natural population, obesity and some characteristics of metabolic syndrome, both high-risk factors for the development of diabetes and cardiovascular disease in humans.

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