Original Article
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Obesity

Predation Risk Modulates Diet-Induced Obesity in Male C57BL/6 Mice

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Objective: In this study, the behavioral and physiological changes induced by experimentally varying the risk of predation in male mice fed a high-fat diet were examined. In particular, the study aimed to assess whether the risk of being predated modulates the body weight gain, providing an ecological context for the obesity resistance observed in many species of small mammals.
Methods: Body weight, food intake, physical activity, and core body temperature of 35 male C57BL/6 mice were monitored for 20 days, while feeding a high-fat diet. A third of the animals were exposed to elevated risk of predation through exposure to the sounds of nocturnal predatory birds, and these were compared to animals exposed to a neutral noise or silence.
Results: Male mice exposed to predation risk had significantly lower weight gain than the neutral or silent groups. Reduced food intake and increased physical activity were the main proximal factors explaining this effect. The risk of predation also induced changes in boldness.
Conclusions: This study provides evidence supporting the role of predation risk on body weight gain of small mammals.

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Introduction

Obesity has been explained as an imbalance between energy intake and expenditure, influenced by a complex interplay of genetic, social, and environmental factors (1,2). Observations on many wild small mammal species have suggested that, contrary to humans, they have a strong regulation mechanism that allows them to avoid the risk of becoming obese (3,4). One explanation for this strong regulatory system is that body weight fluctuates according to a dual intervention point mechanism (5-7). The dual point intervention model suggests that there are upper and lower limit points of regulation (5,6,8). Between these points, mass may vary freely, but when the intervention point is reached, animals enable physiological mechanisms to avoid further weight gain or weight loss. This allows them to maintain the body weight within a limited range. The risk of predation has been suggested as a factor setting the upper limit point of intervention, given that an animal carrying large fat reserves may have an elevated risk of being predated (9). In contrast, the risk of starvation and the impact on reproduction (10), due to insufficient energy reserves, have been proposed as regulators of the lower limit point of intervention. One hypothesis for the diversity in human adiposity in modern societies is that the virtual absence of predation risk for 2 million years has led to genetic drift in the genes that control the upper intervention point (6).

Inbred C57BL/6 mice are sensitive to diet-induced obesity when fed a high-fat diet and have been a popular model for the study of obesity (11,12). In this study, we aimed to evaluate whether experimentally altering the risk of predation modulates weight gain of C57BL/6 mice, induced by feeding a high-fat diet. Second, we investigated the behavioral and physiological adjustments in response to the exposure to elevated risk of predation that might underpin this effect, through monitoring multiple parameters including the food intake, physical activity, body temperature, and assessing behavioral changes by observing their exploratory behavior and boldness.

Methods

Animals and predation treatment

Thirty-five male mice C57BL/6 were purchased from a commercial supplier (Vital River Ltd, Beijing, China), aged 10-12 weeks old and individually housed in standard mouse cages (30 cm × 15 cm × 20 cm); wood shavings and shredded paper were provided for bedding and environmental enrichment. Light conditions in the housing

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room were set as 12L:12D (lights on at 7 am). Animals had free access to food and water for the whole experimental period. First, mice were maintained on a 10% kcal/fat diet (D12450B—Research Diets, New Brunswick, USA), for transmitter implantation and recovery period (details below), lasting a total of 2 weeks. After this period mice were started on high-fat diet, 45% kcal/fat diet (D12451—Research Diets, New Brunswick, USA), and randomly assigned into one of three groups: control, predation, and neutral (respectively 13, 9, and 13 individuals per group). Body weight and food intake were monitored on a daily basis, for all the animals, between 6 and 7 pm. Each group was monitored for 20 days; the differential treatments started on the 8th day. Animals in the predation group were submitted to a treatment simulating the presence of predators near the cages and risk of being predated. The treatment consisted of a 2-min playback of owl calls (*Tyto alba* and *Bubo bubo)* accessed from commercially available compilations of bird songs (European bird calls; Jean C. Roché, Kosmos Verlag, Germany), broadcasted every 2 h, for a total of six events per night, starting 1 h after lights switched off. The neutral treatment was composed of playback of the recorded sound of a human voice reading a scientific paper (Neel, 1962—Diabetes Mellitus: A "Thrifty" Genotype Rendered Detrimental by "Progress"?) in English, monotonically during 2 min, played every 2 h, in a total of six events per night. This neutral treatment exposed animals to a sound that did not represent a threat of predation. All the sounds were played using a computer connected to audio speakers. Speakers were placed 2 m way from the cages, and sound volume was regulated by human ear to guarantee that it was heard by the animals. Animals in the control group were kept in a silent room for the entire period of the experiment.

Physical activity and core body temperature

Two weeks before the experiment, a sub-set of animals were implanted with telemetry transmitters to monitor body temperature and physical activity (Model PDT-4000 E-Mitter, Mini-Mitter, Bend, OR). The transmitter was inserted intraperitoneally. Animals were anesthetized with a mixed flow of isoflurane and oxygen, allowing us to make a small incision of ∼1 cm in both the ventral skin and peritoneal wall. After the insertion of the transmitter, the two layers were sutured independently. The total surgical procedure took 15-20 min, per mouse. After the surgical procedure, mice were given a recovery period of 1 week. Receiver pads (ER-4000 Receiver, Mini-Mitter, Bend, OR) were installed below the mouse cages, receiving the information from the transmitters; data were then collected by a Windows-based software (VitalView^{TM}: Mini-Mitter, Bend, OR), at 15-s intervals. Due to the limited number of receiver pads available, the number of monitored mice for these parameters was seven per group.

Corticosterone levels

Corticosterone levels have been previously implicated as a mechanism linking elevated predation risk to food intake and weight regulation in rodents (13). Corticosterone was measured on the last day of the experiment, after 12 days exposed to the predation risk treatment. Given that fecal concentration of corticosterone peaks 6-12 h after a stressful event (14), each animal was placed in a clean cage, between 10 am and 3 pm, and fresh feces were collected and stored in 100% ethanol at −30°C, until being processed. Hormone extraction was performed following a modified method by Goymann et al. (15) . Briefly, ∼0.05 g of feces (Sartorius) were added to 1 ml of 80% methanol and pulverized using a small pallet knife. The blend was then vortexed for 1 h at 500 rpm, followed by 20 min of centrifugation at 2,500*g*. The supernatant was then transferred to another tube and diluted with a buffer solution. Corticosterone levels were measured using Enzyme Immunoassay (EIA) commercial kits (Cayman Chemical Item no. 500655).

Behavior analysis

Dominant behaviors. Animals were randomly video recorded over the 12-day experimental period. Each mouse was monitored for a total period of 10 h, starting 2 h before dark phase; cameras were set at 5 pm and left for 10 h. Dark phase recording were made using infrared night vision mode from the video cameras (JVC, GZ-MG20). Within each recording, the animals were observed for five random periods of 10 min, and their dominant behaviors were listed.

Dominant behaviors were classified into four categories, grooming, feeding, resting, and general activity, according with the ethogram previously described for this species (16). General activity included walking, climbing the cage, and all general movements; feeding included eating the pellets provided, occasional coprophagy, and drinking. Resting behavior was considered when the animal was sleeping or sitting not moving in the cage or grooming. Grooming was registered when the animals were licking the fur or tail, scratching with any limb, and not moving in the cage. Time spent on each behavior was registered with ETHOWATCHER® software using real-time ethography mode (17).

Exploration and boldness. Individual reaction toward a novel situation, e.g., new and unknown environment, is commonly used as an index of animal's general behavior and to unravel fitness-related traits (18,19). The open-field test was used to assess these behavioral components. Trials were conducted after 12 days of the treatment. The tests were performed after "dusk" during the normal circadian active period, between 8 pm and 12 pm. The dimensions of the open-field arena were 50 cm × 50 cm × 40 cm, constructed with plastic-covered plywood. A video camera was positioned above the apparatus; to record the behavior of the animals using infrared sensitive night mode, the testing room was illuminated by a red bulb. For the test, each animal was placed in the same corner of the arena, using a plastic jar to transport each mouse to the home cage to the arena to minimize handling. Each mouse was allowed to explore the apparatus freely for a 10-min trial period. During the tests the experimenter was not in the room. Between trials, the apparatus was cleaned using 70% ethanol. Each animal was tested once.

The videos were analyzed using ETHOWATCHER® in activity analysis mode (17), and the following aspects were extracted: total distance traveled; % of time resting; % of time in the central area of arena (625 cm^2, 12.5 cm away from the walls).

Statistics

All the data were expressed as means ± S.E. General linear modeling (GLM) with repeated measures was used to test the body weight variation and food intake across the days of the study, including treatment as a fixed factor, and body weight as covariate when testing food intake variation. The effect of treatment on concentrations of fecal corticosterone were analyzed using one-way analysis of variance (ANOVA), followed by *post hoc* Tukey test, setting treatment

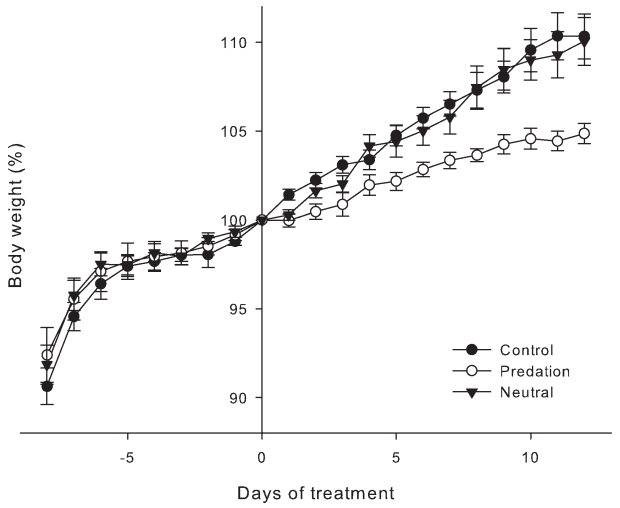

Figure 1 Body weight variation (%) (mean ± S.E) in male C57BL/6 mice throughout the baseline and predation treatment days.

group as a fixed factor. Physical activity and core body temperature data were averaged for each hour, and analyzed during the baseline and treatment periods, for variation over the 24-h period and daily across the study. GLM with repeated measures procedure was used, considering repeated measures accordingly along 24 h or days of study, setting treatment group as fixed factor, followed by *post hoc* Tukey test.

Dominant behaviors were analyzed through multivariate GLM followed by Tukey *post hoc* tests. Open-field data were analyzed through GLMl, including total distance traveled, % of time resting, and % of time in the central area of the area as variables and treatment group as factor. This procedure was followed by *post hoc* Tukey tests to examine the differences between treatment groups. All data were analyzed using SPSS 22.0 for Windows. Statistical significance was set at $P = 0.05$.

Results

To analyze the effects of predation risk on the body weight, mice were first fed a high-fat diet for 8 days. During this baseline period, body weight increased in all three groups (day effect: $F_{7,224}$= 72.656; $P < 0.001$) but did not differ significantly between the groups ($F_{2,32} = 0.423$; $P < 0.653$). After starting the predation risk treatments, the variation in body weight was also mostly affected by the day of measurement ($F_{12,384} = 100.403$; $P < 0.001$), but there was also a significant effect of the treatment ($F_{1,32} = 5.194$; $P = 0.011$) and the interaction between treatment and day of measurement ($F_{24,384} = 3.800$; $P < 0.001$). Over the 12 days of sound treatment, animals in both the control group and neutral group increased their body weight by 10% (Tukey: $P = 0.902$), consistent with the baseline rate of increase. However, animals under the predation treatment increased their body weight by only 4% (Tukey: predation vs. control $P = 0.013$; predation vs. neutral $P = 0.032$) (Figure 1).

The analysis of food intake (Figure 2) revealed that during the baseline period, the variation in food intake was mostly correlated with variations in the body weight ($F_{1,30} = 15.479$; $P < 0.001$). Day of measurement ($F_{6,180} = 0.778$; $P < 0.588$) and treatment group ($F_{2,30} = 1.592$; $P = 0.220$) had no significant effects. Throughout the treatment period, the food intake in the predation group was reduced (2.72 ± 0.11 g), when compared with animals in the control group (3.36 ± 0.08 g) and neutral group (3.54 ± 0.09 g) ($F_{2,30} = 16.034$; $P < 0.001$). During this period, body weight ($F_{1,30} = 2.850$; $P = 0.102$) and day of measurement ($F_{12,360} = 0.709$; $P = 0.743$) were not significantly related to food intake.

Analysis of corticosterone levels showed a significant difference between the treatment groups ($F_{3,18} = 20.408$; $P < 0.001$). *Post hoc* tests revealed ($P = 0.005$) that corticosterone levels were elevated in both the neutral (544.1 ± 51.1 ng/ml) and predation groups (323.4 ± 136.2 ng/ml) relative to the control animals (95.5 ± 55.9 ng/ml).

Physical activity and core body temperature

Animals exhibited similar patterns of circadian physical activity. Briefly, mice were active during the dark phase and inactive during the light phase. Two peaks in physical activity occurred during the light changes, at 7 pm "dusk" and 7 am "dawn" ($F_{23,391} = 39.193$; $P < 0.001$). Throughout the baseline period (Figure 3A) animals showed inconsistent activity variation during the dark period which resulted in a small but statistically significant group effect, specifically between the control group and neutral treatment group ($F_{2,17} = 6.185$, $P = 0.01$; Tukey $P = 0.007$). During the treatment period (Figure 3B), the physical activity pattern slightly changed, resulting in significant effects due to the treatment ($F_{2,17} = 13.886$, $P < 0.01$); animals in the predation treatment group increased their activity during the dark period compared with controls (Tukey $P = 0.001$) and the neutral group (Tukey $P = 0.001$).

Daily activity (Figure 4) was primarily affected by the day of measurement during the treatment period ($F_{12,204} = 8.349$; $P < 0.01$) but not during the baseline ($F_{6,102} = 1.047$; $P < 0.4$). During the

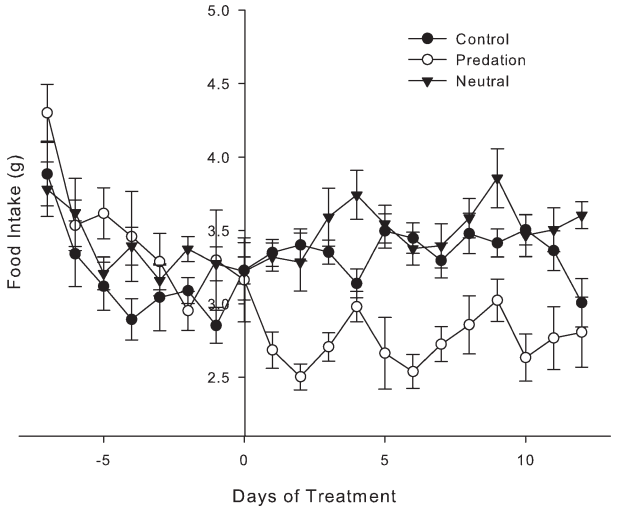

Figure 2 Food intake (g) (mean ± S.E) variation in male C57BL/6 mice throughout baseline and treatment periods.

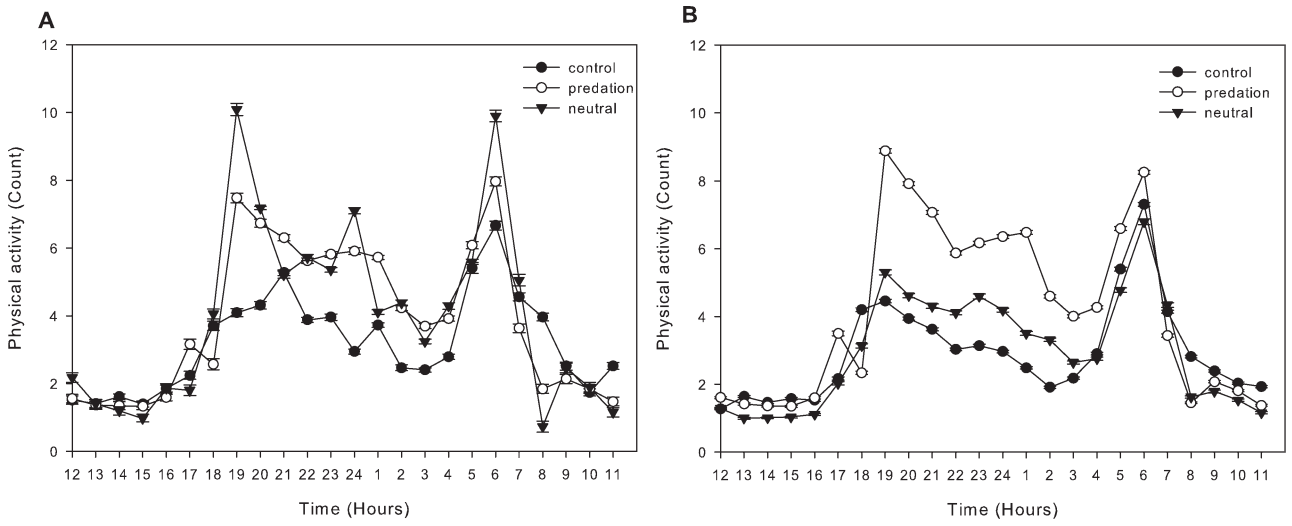

Figure 3 Physical activity of male C57BL/6 mice for 24-h periods (mean ± S.E). Light period: 7 h to 19 h; dark period: 19 h to 7 h. (**A**) Physical activity during the baseline period. (**B**) Physical activity during the predation risk treatment period.

treatment, daily activity was 42% higher in the group submitted to the predation treatment (4.19 ± 0.18 counts/h) compared to controls (2.94 ± 0.19 counts/h) and 22% higher than the neutral group (3.46 ± 0.2 counts/h). Between the baseline period and treatment, the animals in the predation group increased their activity by 23%; the control and neutral groups reduced activity by 10% and 20%, respectively.

Variation in core body temperature of all the animals followed a circadian pattern that peaked between 6 pm and 7 pm and between 5 am and 6 am, about 1 h before dusk and dawn, respectively (Figure 5). The core body temperature was generally about 1°C higher during the dark phase. The time of measurement was the main factor associated with the variation of core body temperature during the baseline period ($F_{23,391} = 72.685$; $P < 0.001$) and the treatment period ($F_{23,391} = 157.731$; $P < 0.001$). The treatment did not affect the daily core body temperature variation ($F_{2,17} = 0.085$; $P = 0.919$; Control: 37.0 ± 0.11°C; Neutral: 37.1 ± 0.12°C; Predation: 37.1 ± 0.11°C). During the predatory risk treatment, the core body temperature was not affected by the treatment effect ($F_{2,17} = 0.060$; $P = 0.942$; Control: 36.8 ± 0.08°C; Neutral: 36.8 ± 0.08°C; Predation: 36.6 ± 0.08°C). Rather, the variation was mostly correlated with the hour of measurement ($F_{23,391} = 157.731$; $P < 0.001$) (Figure 6).

Behavior observations

The time spent on each of the main behavior categories (Figure 7) was not different between treatment groups ($F_{6,40} = 0.716$; $P = 0.639$). Animals spent 39.4 ± 3.1% of time on general activity, 33.7 ± 4.0% of time resting, 10.3 ± 1.8% of time feeding, and 16.6 ± 1.5% of time grooming.

The analysis of boldness and exploration variables revealed that the treatment had a significant association with the % time resting ($F_{2,32} = 3.501$, $P < 0.042$) and the % of time in the center of the arena ($F_{2,32} = 14.866$, $P < 0.001$), but not with the total distance traveled ($F_{2,32} = 1.627$, $P = 0.212$) (Figure 8). However, the apparent tendency was not validated by the *post hoc* Tukey tests, when analyzing the % of time resting (Tukey: Control vs. Predation $P = 0.076$; Control vs. Neutral $P = 0.085$; Neutral vs. Predation $P = 0.973$). Animals in the group submitted to elevated predation risk spent significantly more time at the central area of the arena (11.7 ± 1.7%) than both the control and neutral groups (Tukey: $P < 0.001$ and $P = 0.001$; Control: 3.7 ± 0.6%; Neutral: 5.3 ± 0.9%).

Discussion

In this study, we analyzed the effect of the perceived risk of predation on energy balance of male C57BL/6 mice, by inducing weight

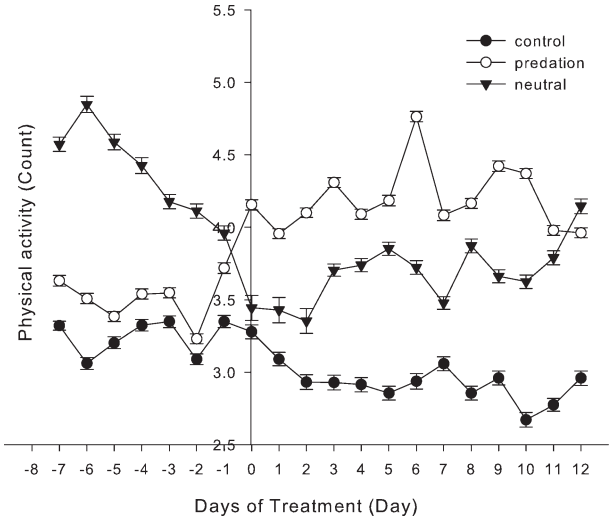

Figure 4 Daily activity (mean ± S.E. for 24 h) of male C57BL/6 mice across baseline and treatment periods.

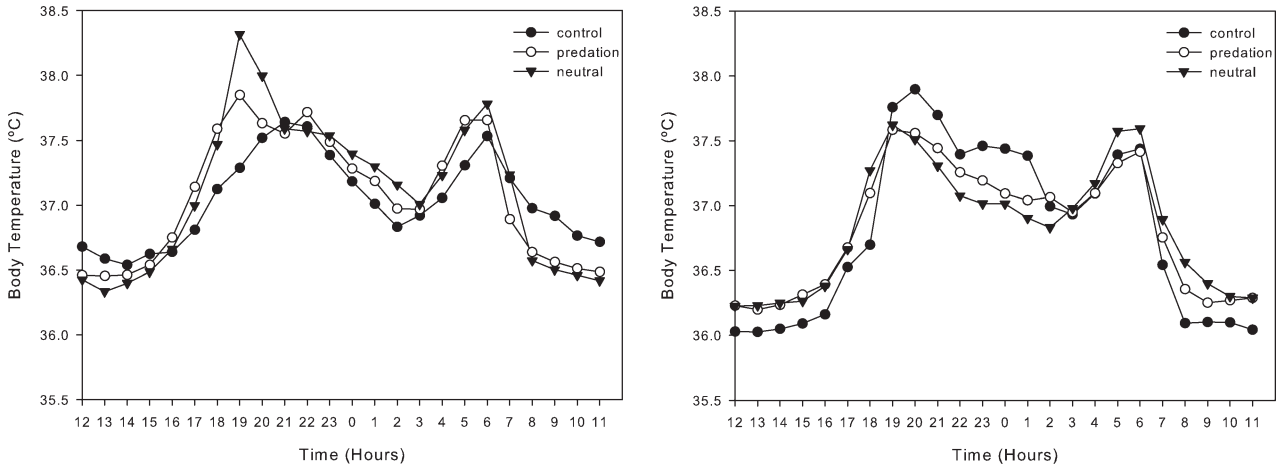

Figure 5 Core body temperature of male C57BL/6 mice over 24-h period. (**A**) Core body temperature during the baseline period. (**B**) Core body temperature during the predation risk treatment period.

gain through feeding a high-fat diet. These animals are sensitive to diet-induced obesity but the propensity to gain weight is variable between individuals (12). Our data showed that the rate of body weight gain was consistent between animals that were exposed to a neutral noise (human speech) or to no noise at all. However, body weight gain was significantly reduced in animals submitted to the predation risk treatment, strongly supporting the original hypothesis that predation risk is a modulating factor over weight control. Analyzing the parameters that influenced energy balance, the reduced rate of gain in body weight of the predation treatment group was explained by a combination of lower food intake and increased physical activity, particularly during the dark phase.

Since we only studied young males, we do not know whether this response would also be observed in females or in older individuals. In fact, loss of hearing in older C57BL/6 mice (20) may render them unresponsive to sound cues, suggesting the response may be specific to younger animals.

Previous studies have also found impacts of perceived predation risk on foraging behavior and food intake in several species (21-23). Our study showed a clear reduction of food intake when mice were exposed to the predator calls. Noise and other stress sources have been demonstrated to cause reduction of body weight gained in rats (24) and mice (25). Because we used a neutral noise treatment, which did not cause a reduction in weight gain relative to mice kept in silence, we can rule out the possibility that the retarded weight gain was a generalized stress effect due to noise. Previous work has suggested that a mediating mechanism by which predator risk may influence energy balance and hence body weight is via an increase in stress hormones. Indeed we found that corticosterone levels were elevated in the mice exposed to predator calls relative to the mice kept in silence. However, the levels of corticosterone were also

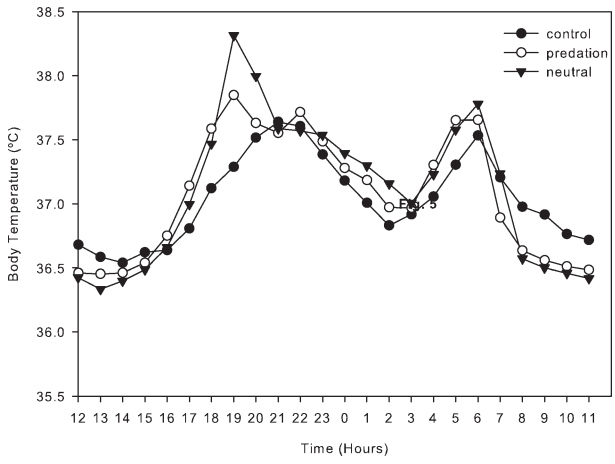

Figure 6 Daily variation of core body temperature of male C57BL/6 mice measured for 24-h periods across the baseline period and 12 treatment days.

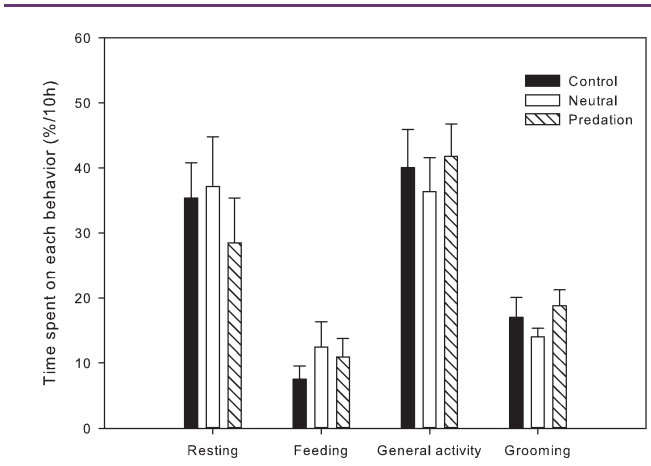

Figure 7 Effects of experimental treatment over the time spent on each category of dominant behaviors (mean ± S.E.%time/10 h).

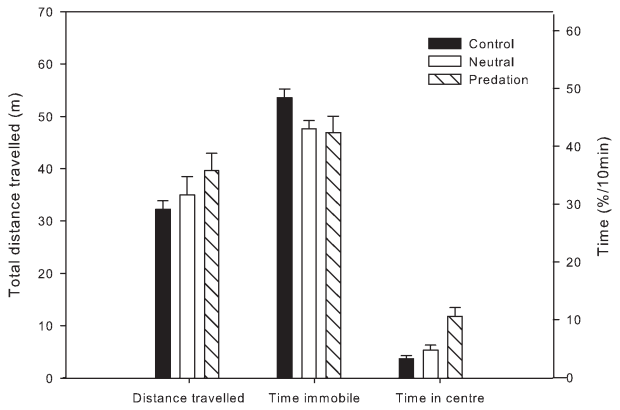

Figure 8 Boldness and exploration behavior parameters (mean ± S.E.).

increased in mice exposed to the neutral sounds, which did not have retarded weight gain. Hence it seems unlikely that these increased levels of corticosterone mediated the weight reduction effect. Rather, it suggests that mice may be able to distinguish between sounds and discriminate whether they represent a predation threat necessitating modulation of energy balance and body weight.

These observations contradict the suggestion of a generalization process made by previous studies (26), which suggests that animals may have a generalized response toward auditory cues, not distinguishing whether the source represents a threat. Nevertheless, recognition of predation cues has been discussed to comprise both innate and learned components, being often dependent upon experience (27).

The imbalance between energy intake and expenditure was reinforced by the variation in activity patterns. The predation risk caused a general increase of the physical activity in the animals, supported by the behavior observations. This effect was unexpected because freezing and reduced physical activity is a commonly used strategy by prey species to avoid detection by predators, as reported for fish (28), larval frogs (29), and voles (30). In contrast, increased activity or "fleeing" is used mostly after being spotted by the predator, to reduce the distance between predator and prey (31). Moreover, this variation occurred during the naturally active period, meaning that the sound disturbance did not disrupt the circadian cycle, but involved modification of the level of activity within the active phase of the cycle. We should also take into consideration the diet component given that fat-enriched diets have been described to cause alterations on the circadian clock (32,33) and induce depressive and anxiety-like behaviors (34). The data obtained from the open-field test also resulted in unexpected observations. According to Eilam (35), animals avoid the open area in the center of the arena and seek the area close to the walls, which provides a more secure environment, which reflects observations made in natural patches (36). Our data suggest an increased boldness in the animals under elevated risk of predation. This may appear counterintuitive as predation avoidance strategy, but boldness has been associated with the capacity to make quick decisions (37); therefore it may have an adaptive role, being beneficial in a high-risk environment (38).

Another feature of energy balance that should not be neglected is the body temperature. Lowering body temperature is among the mechanisms used by animals to save energy, as exemplified in hibernation, torpor (39), and caloric restriction studies (40). However in our study, we did not find significant variations in body temperature associated with the experimental treatment and weight gain.

In summary, the current data support the role of the predation risk in the regulation of body weight, modulating obesity levels by reducing food intake and promoting energy expenditure.O

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