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The Influence of Red Light Exposure at Night on Circadian Metabolism and Physiology in Sprague–Dawley Rats

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Early studies on rodents showed that short-term exposure to high-intensity light (> 70 lx) above 600 nm (red-appearing) influences circadian neuroendocrine and metabolic physiology. Here we addressed the hypothesis that long-term, low-intensity red light exposure at night (rLEN) from a 'safelight' emitting no light below approximately 620 nm disrupts the nocturnal circadian melatonin signal as well as circadian rhythms in circulating metabolites, related regulatory hormones, and physiologic parameters. Male Sprague–Dawley rats (n = 12 per group) were maintained on control 12:12-h light:dark (300 lx; lights on, 0600) or experimental 12:12 rLEN (8.1 lx) lighting regimens. After 1 wk, rats underwent 6 low-volume blood draws via cardiocentesis (0400, 0800, 1200, 1600, 2000, and 2400) over a 4-wk period to assess arterial plasma melatonin, total fatty acid, glucose, lactic acid, pO_2, pCO_2, insulin, leptin and corticosterone concentrations. Results revealed plasma melatonin levels (mean ± 1 SD) were high in the dark phase (197.5 ± 4.6 pg/mL) and low in the light phase (2.6 ± 1.2 pg/mL) of control conditions and significantly lower than controls under experimental conditions throughout the 24-h period ($P < 0.001$). Prominent circadian rhythms of plasma levels of total fatty acid, glucose, lactic acid, pO_2, pCO_2, insulin, leptin, and corticosterone were significantly ($P < 0.05$) disrupted under experimental conditions as compared with the corresponding entrained rhythms under control conditions. Therefore, chronic use of low-intensity rLEN from a common safelight disrupts the circadian organization of neuroendocrine, metabolic, and physiologic parameters indicative of animal health and wellbeing.

Abbreviations: rLEN, red light exposure at night; SCN, suprachiasmatic nuclei; TFA, total fatty acid.

Light is a powerful biologic force that entrains circadian rhythms of behavior, physiology and metabolism for all mammals.[1-3,4,7-12,26,27,29,56,63] Alterations in intensity, duration, and spectral transmittance (that is, wavelength; perceived as color) of light at an inappropriate time of the day can induce the disruption of many circadian rhythms, including those of locomotor activity,[2,3,21,44,54] the sleep–wake cycle,[2,3,21] dietary and water intake,[2,3,21,38] metabolism,[5,6,13-18,38,39,44,60] and neurologic functions,[43] among others. Circadian disruption is defined here as a significant change in the phasing, periodicity, amplitude, or duration of a circadian rhythm from its usual entrained pattern under 12:12-h light:dark conditions. Our early work provided the first in vivo experimental evidence demonstrating that the increased risk of breast cancer in humans, as occurs in the night-shift worker population, may be mediated by light exposure at night.[19,31,57] Our previous studies[4,5,13-18,64] also showed that adherence to appropriate lighting and lighting protocols, as outlined in *The Guide for the Care and Use of Laboratory Animals*,[37] is not only beneficial for but essential to the health and wellbeing of laboratory animals and experimental outcomes. For example, we found that a small light leak of as little as 0.2 lx (0.08 μW/cm^2) intensity during an otherwise normal dark phase (12 h) in our animal rooms elicited a disruption in circadian rhythms of plasma measures of endocrine physiology and metabolism in rats.[13-18,64] In a process termed phototransduction, photic information from the eyes is transmitted via the retinohypothalamic tract, which projects to the suprachiasmatic nuclei (SCN), or master biologic clock; in turn, signals from the SCN to the pineal gland via a polysynaptic pathway regulate the nocturnal production of the hormone melatonin (N-acetyl-5-methoxytryptamine).[36,48,53,54] More recent studies[17,18,64] revealed that the spectral transmittance of light (blue-, amber-, or red-appearing) through laboratory rodent cages dramatically influences the temporal coordination of circadian patterns of plasma melatonin, total fatty acid (TFA), glucose, lactic acid, corticosterone, pO_2 and pCO_2 levels in both pigmented and nonpigmented rats.[17,18,64]

For many years, it has been common practice to make use of red 'safe lamps' (wavelengths above 600 nm) during dark-phase laboratory animal exposure or to cover animal room rodent racks, lights, and observation windows with red-tinted film primarily for nighttime observation.[24,44,45,62,65] It was assumed light with wavelengths above 600 nm (red) had little or no effect on neurodendocrine or circadian systems, given that several nonpigmented species (felines, canines, ungulates, and most species of marine mammals) cannot visually perceive red light via the primary optic tract,[43,47] despite a few early studies on nonpigmented rodents showing otherwise.[43-45,60] Both polychromatic and analytic action spectra studies in humans subsequently showed that these responses to red-appearing light were weak.[8,28,29] At similar irradiances, the acute melatonin-suppressive effects of full-spectrum light were not observed with red light in most mammalian species.[7,38,53] This finding led to the use of red-tinted observation windows and red safety lights for nighttime laboratory animal observation.

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Recent studies in both rats and humans have shown, however, that high-intensity red light[8,30,51,59] over a short duration (that is, minutes) elicits marked suppression of the nocturnal melatonin signal. Long-wavelength light in the spectral region above 550 nm (perceived as yellow to red) of sufficiently high intensity and duration can acutely suppress melatonin and cause phase shifts or entrain circadian rhythms primarily by means of the nonvisual neural pathway of the retinohypothalamic tract.[30,34,45-47,50] Input to the retinohypothalamic tract differs from the classic visual rod-and-cone system of the primary optic tract, relying rather on a small subset of retinal ganglion cells called the intrinsically photosensitive retinal ganglion cells.[3,26,32,33,43,52,60] The neurophysiology of this newly detailed sensory pathway has important implications for consideration in the measurement, production, and application of light in all settings, including those of both human and laboratory animal environments.[43] Many investigators and animal care personnel, however, still assume that the use of light above 610 nm (orange- to red-appearing) for observation purposes, particularly at nighttime, results in little or no circadian disruptive effect on normal circadian physiology and behavior.[45,65] Arguably, sufficient scientific justification for the chronic use of red-tinted observation windows or red safelights, for examining potential neuroendocrine, neurobehavioral, metabolic, and physiologic parameters in laboratory animals is unavailable.

In this study we examined the hypothesis that chronic, low-intensity red light exposure throughout an otherwise normal dark phase (that is, red light exposure at night, rLEN) disrupts circadian melatonin production and the temporal coordination of normal metabolic and physiologic activities in the nonpigmented rat species commonly used for the preclinical study of human physiology or pathophysiology. Our approach was to expose experimental rats, for several weeks and under highly controlled conditions, to a low-intensity safelight (1A Red Safelight, Eastman Kodak, Rochester, NY) during the daily 12-h dark phase and compare their responses with those of control animals maintained under total dark-phase conditions. Use of the safelight eliminated potentially confounding factors in spectral transmittance variability of the many types of red-tinted observation windows and coverings in common use today by ensuring that experimental animals were exposed only to red light in the visible spectrum of wavelengths above 620 nm. Light intensity, duration, and wavelength during light phase were the same for all animals.

Materials and Methods

Reagents. HPLC-grade chloroform, ethyl ether, methanol, heptane, and hexane were purchased from Fisher Chemical (Pittsburgh, PA). Fatty acid and rapeseed oil methyl ester standards, as well as boron trifluoride–methanol, potassium chloride, sodium chloride, sodium hydroxide, and perchloric and trichloroacetic acids were purchased from Sigma Scientific (St Louis, MO). Ultrapure water (catalog no. 400000) was purchased from Cayman Chemical (Ann Arbor, MI).

Animals, housing conditions, and diet. Male adult (age, 3 to 4 wk), nonpigmented, outbred, Sprague–Dawley rats (Crl:SD; SAS SD) used in this study were purchased from Charles River (Kingston, NY) and were certified by the vendor to be SPF. Animals were maintained in an AAALAC-accredited facility in accordance with the *Guide*.[37] All procedures for animal use were approved by the Tulane University IACUC.

Rats were maintained in cages using hardwood maple bedding, which was changed twice weekly (catalog no. 7090, Sanichips, Harlan Teklad, Madison, WI). To safeguard that SPF animals remained infection-free from potential environmental pathogens, serum samples from sentinel animals housed only on the combined soiled bedding from other study cages in the same housing unit were tested quarterly and during the course of this study as described previously.[16-18,64] Rats were given free access to food (5053 Irradiated Laboratory Rodent Diet, Purina, Richmond, IN) and acidified water. Quadruplicate determinations of this diet contained 4.2 g TFA per 100 g of diet composed of 1.06% myristic (C14:0), 15.98% palmitic (C16:0), 1.45% palmitoleic (C16:1n7), 3.93% stearic (C18:0), 22.26% oleic (C18:1n9), 55.05% linoleic (C18:2n6), and 0.22% arachidonic (C20:4n6) acids. Minor amounts of other FA comprised 0.50%. Conjugated linoleic acids and trans fatty acids were not detectable. More than 90% of the TFA appeared in the form of triglycerides; more than 5% was in the form of free FA.

Parameters for rLEN. To better understand the red light exposure at night lighting parameters of illuminance and irradiance associated with a typical red-tinted observation window, measurements first were taken inside the laboratory animal cages located on an IVC isolator unit (Allentown, PA) located within a room with a red-tinted observation window. Polychromatic white light (model F32T8TL741, Philips, Somerset, NJ) from overhead luminaires (4 lamps per ballast) in the 24/7-lighted corridor outside the room (162.18 ± 0.23 lx; 395.50 ± 0.55μW/cm^2) passed through the red-tinted observation window (9 in. × 12 in.; Rtint_S-Red_12_11, RVinyl Tint and Auto Accessories, Charlotte, NC) into the animal room during the dark phase. The red-tinted light that entered the cages of rats maintained on an IVC isolator unit 8 ft (2.43 m) across the room from the observation window measured 7.56 ± 0.69 lx (3.10 ± 0.28 μW/cm^2) within the cages and at rodent eye level (n = 36 measurements). Measurements were taken at 6 different positions within each of the cages (front, middle, rear), and the means were determined.

Caging, lighting regimens, and spectral transmittance measurements. On arrival, rats were randomized into 2 designated groups of 6 animals each (3 per cage) and placed in translucent, clear, laboratory rodent cages (10.5 in. × 19 in. × 8 in.; wall thickness, 0.1 in.) purchased from Ancare (polycarbonate translucent clear, catalog no. R20PC; Bellmore, NY). Radiometric, photometric, and spectral transmittance characteristics of the cages used in this study were reported previously.[17,18,64] The rodents were maintained in environmentally controlled rooms (25 °C; 50% to 55% humidity) with diurnal lighting, 12:12-h light (300 lx; 123 μW/cm^2):dark (lights on, 0600). Animal rooms were lighted with a series of 3 overhead luminaires containing 4 cool-white fluorescent lamps each (model F32T8TL741, Philips, Somerset, NJ); animal rooms were normally completely devoid of light contamination during the dark phase.[4,5,17,18] Daily, during the course of this experiment, the animal room was monitored for lighting intensity during the light phase at 1 m above the floor in the center of the room (at rodent eye level) and outside and from within and at the front of the animal cages. Irradiance measures used a radiometer–photometer (model no. IL1400A, International Light Technologies, Peabody, MA) that included a silicon diode detector head (model no. SEL033) with a wide-angle input optic (W6849) and a filter (F23104) to provide a flat response across the visible spectrum. Illuminance measures used a silicon diode detector head (model no. SEL033) with a wide-angle input optic (W10069) and a filter (Y23104) to provide a photopic illuminance response. The meter and associated optics were calibrated annually, as described previously.[4,5,17,18,64] After a 1-wk acclimation period, the randomized rats were divided further into 2 groups (control and experimental) of 2 cages each (n = 3 rats per cage). Both groups continued to be maintained in the light phase lighting protocol as described earlier. Each day and

at the same time (0800), prior to light intensity measurements for that day, all cages on the rack shelves were rotated one position to the right (placed at an identical, premeasured distance apart) in the same horizontal plane; the cage at position 4 (last position at right on the shelf) was moved to position 1 (first position at left on the shelf). Although there were no significant differences in light intensity as measured from within the front of each cage at each of the 4 positions, the daily cage shift further ensured uniformity of intensity of ocular light exposure and accounted for the effects of any unforeseen subtle differences due to position on the rack shelf during light phase.

In our study, control rats continued to be maintained during dark phase (1800 to 0600) as described, whereas experimental animals (that is, those under rLEN conditions) were maintained during dark phase under exposure to the low-intensity (< 10 lx) red light provided by a 15-W incandescent white light (catalog no. 90001, Soft White Light, 120 V, GE, Cleveland, OH) covered with a Kodak 1A Red Safelight Filter (diameter, 13.9 cm; catalog no. 1521517, Eastman Kodak, Rochester, NY). The safelight was positioned at a distance of 9.83 ft (3.00 m) in front of the 2 translucent clear cages and at a slight downward angle of 25°. Only polychromatic red light of spectral transmittance of wavelengths greater than 620 nm pass through the filter,[23,59] thus controlling for anomalies in light spectral transmittance associated with the various red-tinted materials used for observation windows and coverings in the laboratory animal science industry today. Measurements of light illuminance and irradiance were taken within the cage at rodent eye level on both left and right sides facing forward toward the red light and at the front, middle, and rear of each cage, and the values were averaged for comparison between the 2 cages. Each day, 1 min prior to the onset of the dark phase, the 2 cages of experimental rats were gently removed from the shelving unit in the primary light-phase holding room and placed on a corresponding shelving unit in a separate, adjacent animal room (for red light exposure at night) less than 6 feet away; the cages were returned the following morning 1 min prior to onset of the light phase. Each day, the 2 cages of experimental rats were alternated in position, to ensure uniformity of intensity of ocular light exposure and to account for the effects of any unforeseen subtle differences due to position in front of the safelight.

The light source was characterized by using a handheld spectroradiometer (FieldSpec, ASD, Boulder, CO; Figure 1). For this characterization, the cage was placed at a distance of 3 m from the light, with the surface of the light unit tilted slightly downward at the cage at an angle of 25°. The optical sensor of the spectroradiometer was situated inside the cage, at the center and facing the red light, on standard woodchip bedding, at an approximation of rat eye-level, 2 in. above the bottom of the cage. A lid was placed on the cage to further simulate the animals' light exposures.

To minimize the potentially confounding effects of cage clouding or aging due to cage cleaning during the course of this study, only new cages were used. Cages were cleaned and sanitized as described previously.[17,18] Cage light intensity measurements made daily over the 6-wk course of this study showed no variation as a result of the cleaning procedure. Measures of spectral transmittance through the clear polycarbonate standard rodent cages used in this study were reported previously.[17,18] Under current convention, when discussing human and laboratory animal environments, the term 'luminous flux' (lx) is employed, which indicates the amount of light falling on a surface that stimulates the mammalian eye during daytime or the perceived brightness to the human eye (photometric values). Measures of photometric values in lx are appropriate for human daytime vision but are not appropriate for quantifying light stimuli that regulates circadian, neuroendocrine, or neurobehavioral physiology in animals or humans.[7,8,29,30] Consequently, radiometric values of irradiance ($\mu W/cm^2$) were measured in the cages by using the same equipment and system. Given these standards, the light stimuli in the investigation reported here are presented in terms of both lx and $\mu W/cm^2$ for ease of understanding.

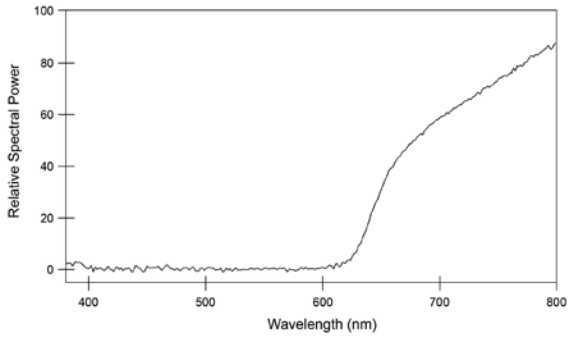

Figure 1. Spectral power distribution of the red light source at animal's eye level as quantified by a handheld spectroradiometer (FieldSpec, ASD, Boulder, CO) within a standard clear, polycarbonate cage. The resulting tracing shows that the filter eliminates visible light energy below 620 nm.

Arterial blood collection. After 2 wk of the lighting regimens described in the previous section, rats underwent a series of 6 low-volume blood draws via cardiocentesis to collect left ventricular arterial blood, as described previously,[4,5,13-18,64] over a period of 30 d. Briefly, blood collections on all rats in each group (control and experimental; $n = 6$ per group) were designated at 4-h intervals to include the 24-h feeding period (that is, 0400, 0800, 1200, 1600, 2000, and 2400). For instance, all rats in both groups were tested at the same time point, that is, 0400; the next time point for sampling (that is, 0800), was 5 d later; each animal was tested only once every 5 d to eliminate effects on feeding, stress, and potential mortality, for a combined total of 72 whole-blood samples (6 rats per group × 2 groups × 6 time points per rat). Each rat was lightly anesthetized by CO_2 inhalation by placement for 10 to 15 s into a 10 in. × 8 in. × 8 in. acrylic gas anesthetizing chamber (catalog no. AB2, Braintree Scientific, Braintree, MA) through which CO_2 and air were passed to approximate a 70% CO_2, 30% air environment. On first sign of unconsciousness (loss of righting reflex) and while still spontaneously breathing, the rat was removed from the chamber and placed in supine position and breathing room air unassisted in preparation for cardiocentesis; 0.5-mL samples were taken from the left ventricle (less than 4.2% total blood volume) via tuberculin syringe (25 gauge, 3/8 in.; Becton-Dickinson, Franklin Lakes, NJ) moistened with sodium heparin (1000 U/mL; Elkin-Sinn, Cherry Hill, NJ), as described previously.[4,5,13-18,64] For the brief 45-s cardiocentesis procedure, blood sampling during the dark phase (that is, 2000, 2400, 0400) was accomplished under the red safelight lamp conditions used for the experimental group. The investigators have nearly 4 decades of experience in using this IACUC-approved cardiocentesis technique developed in the laboratory; there were no complications, such as moribundity or morbidity, due to anesthesia or cardiocentesis during the course of the investigation; and rats were immediately active after the procedure. Plasma samples were stored at −20 °C until assayed for melatonin, corticosterone, insulin, leptin, and TFA. The timeline for this investigation was 7 to 8 wk, comprising a 1-wk acclimation period; 2-wk acclimation exposure to dim red light at night (experimental group) and continuing during

the study; and a 30-d (approximately 4 wk) period for blood collection from both control and experimental rats.

Arterial glucose, lactate and acid–gas measurements. During the course of this study, arterial whole-blood samples for measurement of pH, pO_2, pCO_2, glucose, and lactate levels and Hct were obtained by using a handheld analyzer (iSTAT1 with CG4+ and CG8+ Cartridges, Abbott Laboratories, East Windsor, NJ).

Time-course study of rLEN and acute melatonin suppression. During the initial long-term investigation, the importance of defining the acute melatonin-suppression response to rLEN conditions became important. Therefore, in a subsequent kinetic study, plasma samples were taken at 2400 from a second set of control rats (n = 6) maintained on a normal 12:12-h light:dark lighting cycle to ascertain nighttime melatonin levels. After a 5-d rest period between cardiocentesis events as previously, the rats then were placed in the same rLEN conditions at 2400 (arterial plasma melatonin at peak levels) for exposure to rLEN (8.07 ± 0.95 lx [3.31 ± 0.38 μW/cm^2]) for 30, 60, 90, and 120 min, followed by arterial blood collection to examine the effects of short-term rLEN on suppression of the normal nighttime melatonin surge. The experiment was repeated once, and the results were combined for data analysis.

Melatonin analysis. Arterial plasma melatonin levels were measured by radioimmunoassay (catalog no. 01-RK-MEL2, Melatonin Rat ^{125}I Radioimmunoassay Kit, Alpco, Salem, NH; lot no. 1429.18, prepared by Bühlmann Laboratories AG, Schönenbuch, Switzerland) and analyzed (Cobra 5005 Automated Gamma Counter, Packard, Palo Alto, CA), as previously described.[17,18,64] The minimal detection level for the assay was 1 to 2 pg/mL.

FA extraction and analysis. Arterial plasma TFA, triglycerides, phospholipids, and cholesterol esters were extracted from 0.1-mL samples, as previously described.[4,5,13-18,64] Prior to extraction, heptadecanoic acid (100 μg), which had been dissolved in chloroform (Fisher Scientific, Fair Lawn, NJ), was used as an internal standard. Methyl esters of FA were analyzed on a gas chromatograph (model 5890A, Hewlett Packard, Palo Alto, CA) fitted with a flame ionization detector (model 7673A), autoinjector (model 7673S), and integrator (model 3396A). All separations used a capillary column (0.25 mm × 30 m; model 2380, Supelco, Bellefonte, PA) at 190 °C, with helium as the carrier gas (linear rate, 20 cm/s; split, 100:1). Injection port and detector were adjusted to 220 °C. All methyl esters were identified on the basis of their retention time compared with those of known standards. Minimum detectable limit for the assay was 0.05 μg/mL.

ELISA of corticosterone, insulin, and leptin. Arterial plasma samples were prepared in duplicate for measurement of corticosterone, insulin, and leptin levels by using chemiluminescent ELISA diagnostic kits (corticosterone: catalog no. 55-CORMS-E01, mouse–rat, protocol version 9 April to 13 September 2011; insulin: catalog no. 80-INSRTH-E01; rat, high range; protocol version 2.0 to 2 December 2011; and leptin: catalog no. 22-LEPMS-E01; mouse–rat; protocol version 030112 to 29 February 2012; all from Alpco). Samples were measured by using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA) at 450 nM. Detection sensitivity for corticosterone, insulin, and leptin plasma analyses were 4.5, 0.124, and 10 pg/mL, respectively; lower limits of the assays were 15, 0.15, and 10 pg/mL, respectively; and the CV of all assays were less than 4.0%.

Statistical analysis. All data are presented as mean ± 1 SD unless otherwise noted (n = 6 per group). The experiment was repeated once for measurements of corticosterone, insulin, leptin, linoleic acid, melatonin, and TFA, and all data for both replicate experiments are combined for a total of 12 values per group. The nonparametric JTK_CYCLE algorithm,[35] as implemented in scripts for the R software package (R version 3.1.0, http://openwetware.org/wiki/HughesLab:JTK_Cycle), was used to determine the statistical significance of 24-h cycling for each analyte, with adjustments for multiple comparisons. This algorithm also was used to estimate phase (time of peak levels) and amplitude of cycling. Statistical differences between mean values in the rLEN group compared with the control group at each circadian time point were assessed by using an unpaired Student t test. Differences between the group means were considered statistically different at a P value of less than 0.05.

Results

Animal room illumination and caging spectral transmittance. The mean daytime animal room illumination at the center of the rooms and at 1 m above the floor was reported previously.[17,18,64] Measurements of photometric illuminance (lx) and radiometric irradiance (μW/cm^2) from outside and inside the front of each cage made daily at all 4 positions, as cages were shifted left to right at the same level on the caging racks, showed little to no intercage variability. Mean values for clear cages were 132.10 ± 3.26 lx (54.16 ± 1.34 μW/cm^2) at the outside and front of the cage and 98.71 ± 3.61 lx (40.47 ± 1.48 μW/cm^2) inside at the front of cage (n = 100 measurements). Spectral power distributions of light measured through the wall of these cages, including measures of correlated color temperature and photon flux, as well as interior radiometric and photometric measures, were reported previously.[17,18,64] Measurements made within the cages during the dark phase revealed 0 lx (0 μW/cm^2, no light exposure at night) for the control group and 8.07 ± 0.95 lx (3.31 ± 0.38 μW/cm^2) for the rLEN group.

Dietary, water intake, and body growth rates. Dietary and water intake and body growth rates between the 2 groups did not differ significantly and are reported here as the combined means. Mean daily dietary intake was 20.02 ± 0.71 g/d, and water intake was 31.06 ± 1.46 mL/d (n = 63 measurements). The percentage of total daily dietary and water intake during the course of the 24-h day measured, respectively, 96.23% ± 1.47% and 97.98% ± 1.36% (1800 to 0600), 1.24% ± 0.40% and 0.66% ± 0.29% (0600 to 1200), and 2.53% ± 1.45% and 1.36% ± 0.75% (1200 to 1800 h). The mean daily body growth rate was 2.82 ± 0.36 g/d (n = 32 measurements/group).

Plasma melatonin values. Circadian rhythms in concentrations of plasma melatonin are shown in Figure 2. The overall pattern of daily plasma melatonin level rhythms was similar for both groups: low (< 5 pg/mL) during daytime and significantly (P < 0.001) higher during the dark phase, with peak levels occurring between 2400 and 0400, and decreasing to a nadir between 1200 and 1600. There were no differences in either the phase (for example, timing) or duration of the nocturnal melatonin signal between the 2 groups of rats. Peak dark-phase melatonin levels for rLEN rats, however, were nearly 95% lower (P < 0.001) than those of control rats. In addition, the integrated mean level of melatonin over the 24-h period of rLEN rats was less than 90% that of control rats.

Arterial plasma TFA. Circadian rhythms in concentrations of arterial blood plasma TFA with free access to the food were measured in male rats (Figure 3). The plasma lipid levels in control rats followed the levels reflecting the usual circadian feeding pattern observed in 12:12-h entrained rats, as reported earlier,[4,5,13-18,64] but were elevated throughout the 24-h day in rLEN rats, such that the total TFA areas assessed over the 24-h day did not differ significantly between groups (control, 46.8 mg/mL; rLEN, 45.3 mg/mL). Circadian cycling was evident for both groups but showed a severely dampened amplitude in rLEN rats (Table 1).

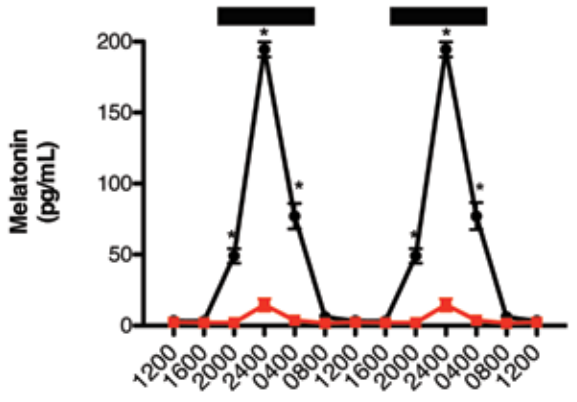

Figure 2. Diurnal plasma melatonin levels (pg/mL; mean ± 1 SD) of male Sprague–Dawley rats (n = 12 per group) maintained for 6 wk in standard polycarbonate, translucent, clear cages under 12:12-h photoperiods. Both groups were exposed similarly during the light phase (300 lx; 123 μW/cm^2); during the 12-h dark phase, control rats (solid black circles) had no LEN, and experimental animals (solid red squares) were maintained on rLEN (3.31 ± 0.38 μW/cm^2; 8.07 ± 0.95 lx). Data are plotted twice to better demonstrate rhythmicity. Rats were exposed to dark-phase lighting conditions from 1800 to 0600 (dark bars). Rhythmicity analysis (Table 1) revealed robust and highly significant ($P = 5.85 \times 10^{-32}$) rhythmic patterns under control lighting conditions, with a 40-fold decrease in amplitude observed under dim rLEN conditions. *, $P < 0.001$ rLEN compared with control conditions (Student t test). Values with asterisks are different ($P < 0.05$) than those without asterisks.

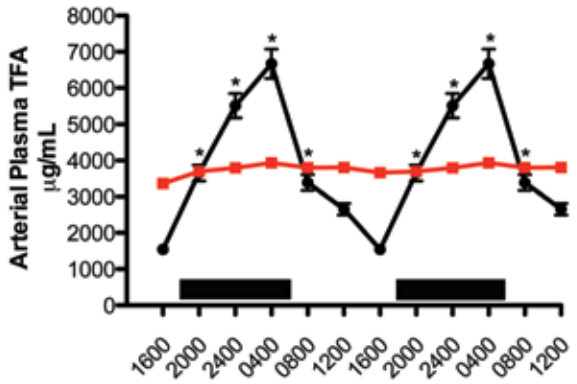

Figure 3. Diurnal changes in the blood plasma lipid concentrations in the arterial blood of male Sprague–Dawley rats fed normal chow ad libitum and maintained on either control (solid black circles) or experimental (solid red squares) lighting conditions. Rats were exposed to dark-phase lighting conditions (see Methods) from 1800 to 0600 (dark bars). TFA values (mean ± 1 SD; n = 12 per group) are the sums of myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic acid concentrations collected at the various time points. Data are plotted twice to better demonstrate rhythmicity. Rhythmicity analysis (Table 1) revealed robust and highly significant rhythmic patterns in arterial plasma TFA in control rats; these rhythms were severely abrogated under dim rLEN. *, $P < 0.01$ LEN compared with control (Student t test). Values with asterisks are different ($P < 0.05$) from those without asterisks.

Arterial blood glucose, lactate and acid/gas levels. Figure 4 depicts daily rhythms in levels of arterial blood glucose, lactate, pO_2, and pCO_2 in the male rats comprising the control and rLEN groups. Phase shifts were determined by comparing peak values (acrophases) of rLEN rats with those of controls. A phase advance was defined as a shift in the group peak level of rLEN rats to an earlier time (that is, from 1200 to 0800), whereas a phase delay was defined as a shift to a later time (that is, from 0400 to 0800), as compared with values for control rats. Daily rhythms for arterial glucose and lactate concentrations (Figure 4 A and B) followed near-identical curves in both groups and peaked near the middle of the dark phase (2400). However, concentrations were significantly ($P < 0.05$) higher over the 24-h day in rLEN compared with control rats. Total calculated areas under the glucose curves over the 24-h day were higher ($P < 0.05$) for rLEN rats than in control rats. Mean blood glucose concentrations calculated over the 24-h day were 133.9 ± 3.5 mg/dL for control rats and 155.2 ± 5.0 mg/dL for rLEN rats. Calculated mean daily arterial lactate assessed over the 24-h day was higher ($P < 0.05$) rLEN rats than control animals. Mean blood lactate concentrations calculated over the 24-h day were 1.36 ± 0.08 mmol/L for control animals and 1.66 ± 0.11 mmol/L for rLEN rats.

Daily rhythms in arterial pO_2 and pCO_2 (Figure 4 C and D) were nearly identical between control and rLEN groups, with peak values occurring at late dark phase (2400) and lowest values at the midlight phase (1600). However, once again, values over the 24-h day were significantly ($P < 0.05$) higher in rLEN compared with control rats. Calculated mean daily arterial pO_2 assessed over the 24-h day (Figure 4 C) were significantly different between groups (controls, 134.6 ± 3.4; rLEN, 152.3 ± 3.9 mm Hg; n = 72 measurements). Total daily mean arterial pCO_2 values were significantly ($P < 0.05$) lower in the control group than in the rLEN group. Calculated mean daily arterial pCO_2 assessed over the 24-h day for the curves shown in Figure 4 D were significantly ($P < 0.05$) different from one another (control, 28.1 ± 0.6 mm Hg; rLEN, 30.0 ± 0.7 mm Hg; n = 72 measurements).

Arterial blood pH, O_2 saturation, and Hct remained relatively constant for both groups over the 24-h day (pH, 7.44 ± 0.074; O_2, 99.1% ± 0.01%; Hct, 45.3 ± 0.05; n = 72). These values are consistent with carotid arterial blood acid–gases values determined in previous cardiocentesis investigations at this time of day.[4,5,13-18,64]

Arterial plasma corticosterone, insulin, and leptin concentrations. Figure 5 depicts daily rhythms in concentrations of arterial blood plasma corticosterone, insulin, and leptin. Plasma corticosterone levels revealed clear differences between groups regarding integrative concentrations and diurnal rhythms. Values for arterial plasma corticosterone in rats of both groups began to increase after 1200 ($P < 0.05$), with peak levels occurring at 2400 in the control group ($P < 0.05$) that eventually decreased to a nadir at 1200 ($P < 0.05$). Peak corticosterone levels for the rLEN group were phase-advanced 8 h in comparison to those in control rats, peaking at 1600 ($P<0.05$) and decreasing to a nadir after the dark phase, at 0800. Integrated plasma corticosterone concentrations calculated over the 24-h day were significantly ($P < 0.001$) different from one another at 701.5 ± 8.3 ng/mL (2034.5 ± 24.1 nmol/L) for control rats and 377.7 ± 3.6 ng/mL (1095.4 ± 10.4 nmol/L) for rLEN animals.

Plasma concentrations of insulin for animals in both groups (Figure 5 B) showed clear differences in daily rhythms and integrative levels. Values for arterial plasma insulin in control animals were highest at 6 h after the onset of the dark phase (2400), with a secondary minor peak after the onset of light phase (0800); lowest levels occurred during the light phase (1200 to 1600). Rats in the rLEN group showed peak insulin levels at 2 h after the onset of the dark phase (2000), with a second minor peak at 0800, and that were phase-advanced 4 h compared with those of control rats. However, peak levels in rLEN rats were less than half of ($P < 0.05$) those in control animals. In addition, control rats experienced a rapid increase to peak insulin levels at 2400, followed by a rapid decline. In the rLEN group, this process was more protracted, with insulin levels gradually declining over 16 h from their peak at 2000 to their lowest levels at

Table 1. Summary of rhythmicity analysis for control and experimental (rLEN) groups

	Estimated phase			Amplitude			*Q* value for circadian cycling	
	Control	rLEN	Phase shift	Control	rLEN	Fold change	Control	rLEN
Corticosterone	0200	2200	−4 h	30.80	14.79	−2.08	1.05×10^{-36}	5.17×10^{-08}
Glucose	2400	0200	+2 h	10.10	7.77	−1.30	4.64×10^{-05}	4.50×10^{-02}
Insulin	0200	2400	−2 h	0.61	0.35	−1.72	1.35×10^{-10}	8.71×10^{-04}
Lactate	2400	0200	+2 h	0.30	0.43	1.45	1.46×10^{-07}	5.09×10^{-13}
Leptin	0200	NS	—[a]	0.28	0.02	−15.97	5.49×10^{-18}	2.03×10^{-01}
Linoleic acid	0400	0600	+2 h	563.56	37.48	−15.04	6.12×10^{-29}	1.15×10^{-09}
Melatonin	0200	0200	0	53.00	1.32	−40.24	5.85×10^{-32}	2.02×10^{-06}
pCO_2	0400	0200	−2 h	2.83	2.76	−1.03	9.10×10^{-08}	5.09×10^{-11}
pO_2	2400	2400	0	12.02	10.15	−1.18	4.55×10^{-10}	5.09×10^{-11}
TFA	0400	0600	+2 h	1797.86	85.56	−21.01	3.60×10^{-29}	1.63×10^{-07}

NS, not significant (not cycling)
Phase, amplitude, and multiple testing-adjusted *P* value (Q) estimated by rhythmicity analysis with a fixed 24-h period using original units as described in the text and shown in Figures 2 through 5.
[a]Not applicable; if there is not significant cycling, then the algorithm will not return an amplitude.

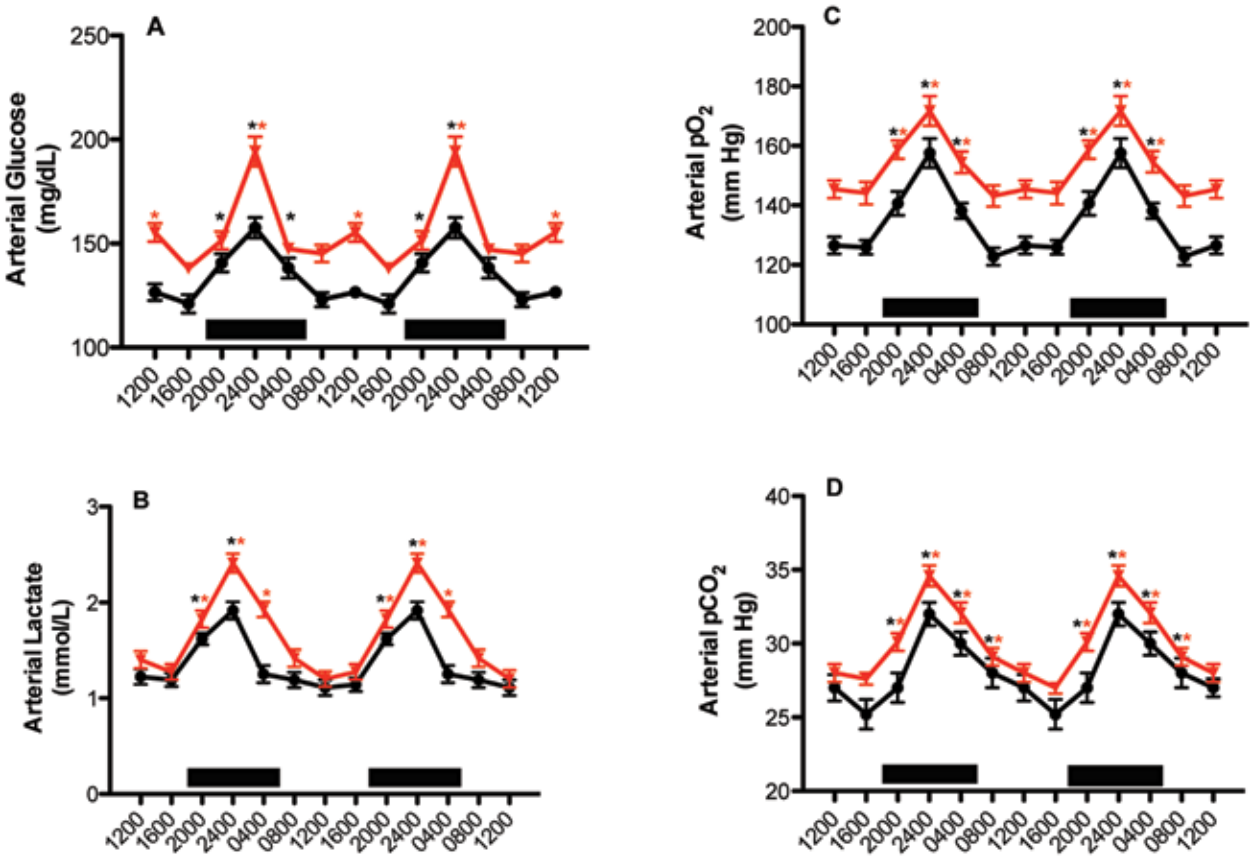

Figure 4. Diurnal changes in arterial blood (A) glucose, (B) lactate, (C) pO_2, and (D) pCO_2 levels (mean ± 1 SD; $n = 12$ per group) of male Sprague–Dawley rats maintained on either control (solid black circles), or experimental (solid red squares) lighting conditions. Rats were exposed to dark-phase lighting conditions from 1800 to 0600 (dark bars). Data are plotted twice to better visualize rhythmicity. Rhythmicity analysis (Table 1) revealed robust and highly significant ($P < 0.0001$) rhythmic patterns under control conditions and significantly different ($P < 0.05$) rhythmic patterns under dim rLEN conditions. *, $P < 0.001$ rLEN compared with control conditions (Student *t* test). Values with asterisks are different ($P < 0.05$) than those without asterisks.

1600. Integrated mean plasma insulin concentrations calculated over the 24-h day differed ($P < 0.001$) between groups (control, 17.4 ± 0.06 ng/mL; rLEN, 15.5 ± 0.10 ng/mL).

Plasma concentrations of leptin (Figure 5 C) also revealed clear differences between control and rLEN groups with regard to diurnal rhythms and integrated levels. Values for arterial plasma leptin in control animals began to increase 2 h after onset of the dark phase ($P < 0.05$), with peak levels occurring at 2400 and gradually decreasing to a nadir at 1600 ($P < 0.05$). rLEN rats showed a sharp peak in arterial plasma leptin levels at 0400, which was more than 50% higher ($P < 0.05$) and phase-delayed 4 h compared with that in control rats. Blood leptin concentrations reached a nadir at 0800 in rLEN rats. A second similar, albeit broader, peak amplitude in leptin levels occurred

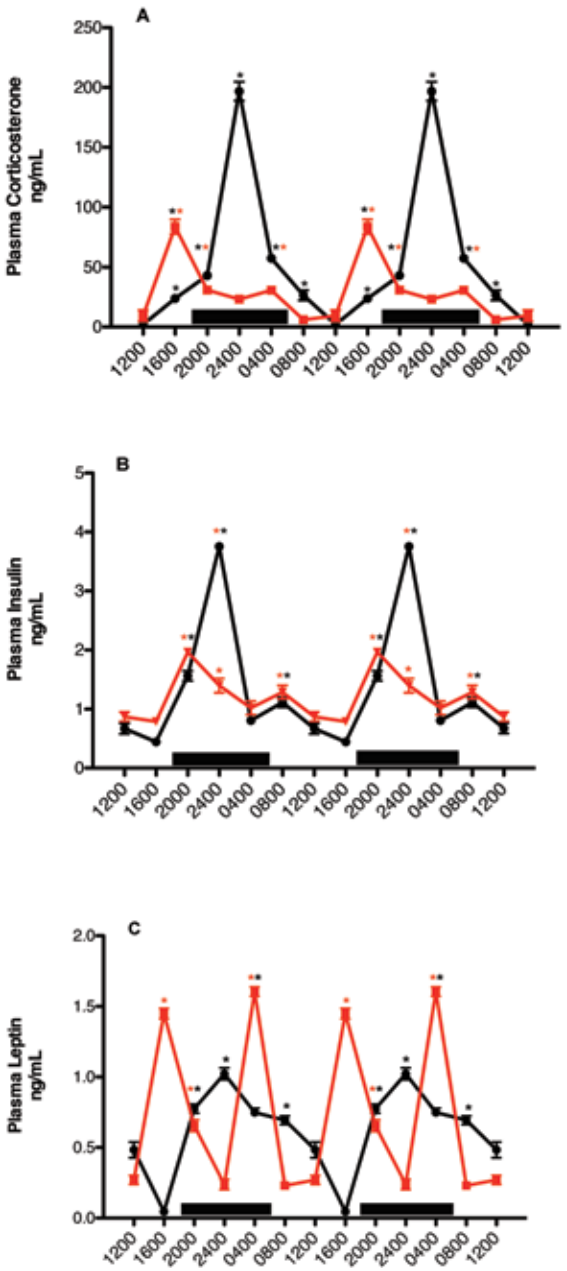

Figure 5. Diurnal changes in plasma (A) corticosterone, (B) insulin, and (C) leptin concentrations (mean ± 1 SD; $n = 12$ per group) in the arterial blood of rats maintained on either control (solid black circles) or experimental (solid red squares) lighting conditions. Data are plotted twice to better demonstrate rhythmicity. Rats were exposed to dark-phase lighting conditions from 1800 to 0600 (dark bars). Rhythmicity analysis (Table 1) revealed robust and highly significant ($P < 0.0001$) rhythmic patterns under control conditions, significant ($P < 0.05$) but disrupted rhythmic patterns under dim rLEN conditions for corticosterone and leptin, and an absence of significant circadian cycling for leptin under dim rLEN. *, $P < 0.001$ rLEN compared with control conditions (Student t test). Values with asterisks are different ($P < 0.05$) than those without asterisks.

12 h later, near the end of the light phase (1600), in rLEN rats. Integrated plasma leptin concentrations calculated over the 24-h day differed significantly ($P < 0.05$) between groups (control, 8.02 ± 0.05 ng/mL; rLEN, 9.16 ± 0.06 ng/mL).

Time-course study of rLEN and acute melatonin suppression. Normal nighttime (2400) arterial plasma concentrations of melatonin after time-course exposure to rLEN are presented in Figure 6. Results show that plasma melatonin levels began

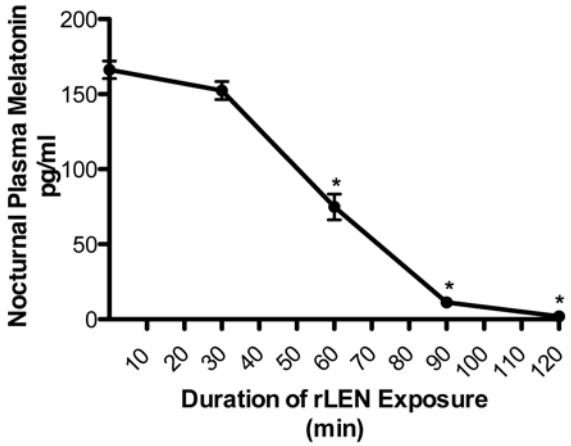

Figure 6. Effect of red safelight exposure (8.07 ± 0.95 lx; 3.31 ± 0.38 μW/cm^2) during the dark phase (2400) on arterial plasma melatonin concentration in control rats at 0 min (no rLEN exposure) and after 30, 60, 90, and 120 min of exposure to rLEN. *, Concentration significantly ($P < 0.05$, Student t test) different from that at time 0.

to decrease as soon as 30 min after animals' exposure to rLEN, with 45% suppression ($P < 0.05$) after 60 min of exposure. Arterial plasma melatonin levels after 90 to 120 min rLEN were suppressed as low as normal daytime values.

Discussion

Exposure to white, polychromatic light at night disrupts the circadian system in all mammals, including humans and laboratory animals.[4,5,10-12,15,16,19,21,28,30,39,43,53,55,57,59] Nocturnal rodents, such as rats, mice, and hamsters, however, are more than 100-fold more sensitive to light for suppression of the nighttime pineal melatonin signal than are humans.[7,8] We tested the hypothesis that the long-term exposure of Sprague–Dawley rats, a strain frequently used in preclinical biomedical research investigations of human health and disease, to dim red light (above 620 nm) from a safelight during the entire 12-h dark phase of a 12:12-h light:dark period over several weeks would disrupt circadian rhythms of physiology and metabolism. Both control and experimental groups were maintained in an environment where both the intensity and duration of lighting remained constant during the 12-h light phase. We confirmed previous work[17,18,64] that these photic environments did not differ in spectral transmittance of light (color) passing into the cage during the normal light phase. Irradiance, photon flux, illuminance, and correlated color temperature values for the translucent, clear, polycarbonate cages were normalized via the location of the cages on the laboratory rodent holding racks under the light source. To comprehensively describe the rLEN experienced by the circadian, neuroendocrine, and neurobehavioral systems of the rats, we included measurements of corneal spectral power distribution in this study. Nonvisual responses in most mammals are initiated by as many as 5 biologic and meaningful representations of irradiance that include the rod, cone, and intrinsically photosensitive retinal ganglion cell melanopsin photoreceptor systems: rhodopic, melanopic, cyanopic, and erythropic illuminance.[42] In this manner, we obtained a consistent and appropriate method for quantification of the light provided, thus facilitating the comparison of polychromatic light of different spectral qualities across studies, including the red-appearing light used in this study.

Although the timing of the nocturnal circadian plasma melatonin peak was not different between rats housed in the 2 different nighttime lighting environments, there were very marked differences in both melatonin duration and amplitude. The amplitude of nocturnal plasma melatonin in the rLEN animals was nearly 95% lower, as compared with that in rats maintained under a normal 12-h dark phase that was devoid of all light exposure at night. The acute melatonin-suppressive effects of rLEN began to occur after 30 min of exposure during the dark phase. Within 60 to 90 min of exposure, melatonin levels reached low values similar to those previously observed after exposure to dim white light at night or during the regular light phase.[5,13-16] This rate of acute melatonin suppression is markedly slower than that observed in response to bright polychromatic light, which occurs within a few minutes.[4,5,8,29,30, 44,50,59] This difference suggests that red light may be appropriate for use in experiments that require a very brief manipulation during the nighttime in this laboratory rat strain. Animals maintained in rooms with red-tinted observation windows that enable chronic ocular exposure to a similar spectral transmittance and illuminance of rLEN, however, may undergo similar responses to those we observed here. To our knowledge, this study represents the first time that the exposure of a rodent species to a spectral transmittance of light above the 620 nm range (red) of similar intensity and duration has been shown to induce an acute and marked decrease in the melatonin amplitude during the dark phase. Others[59] have shown in Sprague–Dawley rats that a 1-h exposure at 2300 to a red safelight at an intensity of 414.6 lx (170 $\mu W/cm^2$), 1024.4 lx (420 $\mu W/cm^2$), or 2536.6 lx (1040 $\mu W/cm^2$), all of which are much higher than the intensity that we used in the current study, resulted in a corresponding nighttime melatonin suppression of 40%, 60%, and 90%, respectively. Indeed, in our study, the highest intensity of red light during the dark phase was as effective as bright polychromatic white light (1634.1 lx; 670 $\mu W/cm^2$) in suppressing nighttime serum melatonin levels. We conclude that red light use during the dark phase, at least at these intensities, cannot be regarded as 'safe' with respect to nocturnal circadian melatonin production.

Daily oscillations in arterial TFA concentrations (Figure 3) were almost completely abrogated in rLEN rats, in contrast to the robust rhythms in animals maintained in a normal 12-h dark phase (controls). This result is in marked contrast to rats maintained on dim white light (0.2 lx), in which the entrained TFA rhythm typically present in control rats persisted in experimental animals.[4,5] Arterial TFA levels for rLEN animals were constantly increased to a level that approximated nearly 60% of the peak nighttime (2400) levels of control rats, which has not been reported previously in rLEN animals. Dietary and water intakes were similar between groups. The lack of a plasma TFA rhythm in rLEN rats during the dark phase, at least at the light intensity we used here, is similar to that reported for rats maintained in constant bright-white light (123 lx; 300 $\mu W/cm^2$).[4,5] The present results suggest that, in the strain of rats used in the present investigation, the SCN-driven and 12:12, light:dark-entrained feeding rhythm became arrhythmic under rLEN conditions. However, that the responses of Sprague–Dawley male rats to rLEN may not be generalizable to other laboratory animals, as such responses may differ among animals of different strains and species and in those with different lighting histories.

The phasing and duration of the daily oscillations in arterial plasma glucose and lactate concentrations and in arterial pO_2 and pCO_2 in rLEN rats closely matched those observed in animals maintained under a normal 12-h dark phase (control group). However, all 4 of these markers were significantly higher in rLEN rats than in control animals. In addition, the overall 24-h integrated levels of these parameters were higher in rLEN as compared with control rats. Lactate, normally a major by-product of glucose metabolism, is no longer considered to be a waste product solely but to have important roles in intermediary metabolism relevant to wound repair and regeneration, anoxia, and dysoxia and as a signaling molecule.[25,49]

Elevated levels of corticosterone have long been associated with various stressors, including anxiety, fear, pain, hemorrhage, infections, low blood glucose, and starvation.[20,28,45,59,61] Corticosterone, a potent glucocorticoid, acts on important metabolic tissues such as muscle, liver, and adipose tissues to alter metabolism and provide the animal with necessary bioenergetics fuels to better cope with stress.[17] The circadian rhythm of circulating plasma corticosterone has been well-documented.[17,18,20,22,28,39,58,64] Corticosterone peak amplitudes vary somewhat depending on strain and sex, with female rats having somewhat higher daily concentrations and male rats demonstrating a more consistent rhythm.[17,18,64] We noted large differences in the circadian rhythm of plasma corticosterone between our 2 groups of rats. Here, in rats maintained under normal 12:12-h light:dark conditions, circulating corticosterone levels revealed a single, major peak in the middle of the dark phase (2400), whereas rLEN evinced a peak near the end of the light phase (1600) followed by a second, slightly lower-amplitude, peak near the end of the dark phase. Whereas the integrated values over a 24-h period were significantly lower in the rLEN group as compared with the control group, rLEN rats showed no extreme fluctuations in peak amplitude during nighttime peak activity period. Interestingly, this finding may suggest that the rLEN rats, over time, developed circadian corticosterone patterns reflective of a lower stress condition throughout the 24-h day, albeit with a somewhat higher metabolic activity.

In addition to corticosterone, the largest changes in hormonal circadian rhythms were observed in plasma insulin and leptin in rats exposed to rLEN conditions. Shifts in insulin and leptin rhythm phasing with accompanying alterations in rhythm amplitude and duration were more prominent in rLEN as compared with control rats. Insulin's important role in energy homeostasis has long been known to involve functionally increasing uptake and metabolism of glucose by several peripheral tissues, including liver, skeletal muscle, and adipose tissue.[6,21,22,38,39,41] The blunted and phase-advanced insulin rhythm in rLEN rats compared with control animals reflects a pattern that may help to explain the circadian-disrupted, elevated glucose levels of the rLEN animals. The higher circadian glucose and lactate levels in the rLEN group may be explained in part by their markedly phase-advanced insulin peak compared with that of the control group.

Under 12:12-h, light:dark-entrained conditions, plasma leptin levels in rats are associated with feeding and insulin release, and the coupling of the 2 provide a meal-timing cue to the brain to accompany information on fat storage as well as energy expenditure.[6,22,38,41,42] Leptin levels, under the control of the SCN, are normally associated with overall body fat content and are generally higher in male compared with female rats.[20,22,37,40] Our findings in male Sprague–Dawley rats revealed 2 distinct high-amplitude peaks in rLEN rats, compared with the single dark-phase peak in controls, consistent with a previous report.[64] The integrated 24-h daily

mean in arterial leptin levels in rLEN rats was significantly higher than that in controls, is consistent with previous reports,[17,18,64] and argues that rLEN conditions significantly alter rhythms of circadian leptin levels in this rat strain.

Leptin and insulin are tightly associated and influenced by both feeding states and lighting conditions.[38,44] The findings we present here demonstrate that rats exposed to dim red light during an otherwise uncompromised dark phase experience major disruptions in circadian rhythms of both leptin and insulin, among other hormonal and metabolic rhythms.[5,53,54] Although insulin stimulates glucose transport and leptin directly inhibits insulin-controlled glucose transport,[43] the mechanisms of action and interactions of these 2 important circadian-regulated hormones in terms of metabolism are not fully understood at this time. Additional study is warranted.

Melatonin exerts regulatory effects on both glucose and lactic acid metabolism and on corticosterone, insulin, and leptin production in both humans[6,41,42,61] and rats.[20,22,44] The marked changes in the circadian amplitude of melatonin, particularly in rats exposed to the rLEN environment, may have influenced at least some of the daily rhythm changes observed in these analytes. Other mechanisms must also be considered, such as melatonin-independent circadian hormonal and neural outputs from the SCN.

To our knowledge, this report is the first to show a direct association between long-term, low-intensity rLEN over the 12-h dark phase, suppression of the normal nighttime melatonin signal, and disruption of the circadian profiles of blood TFA, acid–gases, glucose, lactate, corticosterone, insulin, and leptin in a mammalian species. Our study provides compelling evidence that low-intensity application of polychromatic red 'safe' light (620 nm) during an otherwise unadulterated dark phase can induce almost total melatonin suppression and disrupt important parameters of metabolism and physiology in male Sprague–Dawley rats. Furthermore, the acute suppression of the nighttime melatonin signal by rLEN at light intensities delivered by the controlled red safelight environment occurs after only 30 min of exposure, and complete suppression is rapid (within 90 min). Perhaps briefer exposure to a higher intensity of rLEN or, conversely, prolonged exposure to a lower intensity, would have exerted the same circadian-disruptive effects that we observed in response to 12-h exposure to 8 lx of rLEN (for example, intensity–duration reciprocity).

The findings we presented here, as well as those of others, make a persuasive case that great caution must be exercised when using red-tinted observation windows or red "safety" lighting in animal facilities housing Sprague–Dawley rats and perhaps other rodent strains commonly used in biomedical research. The red 'safelight' designation was originally used in regard to the safety that the device provided for developing photographs in the darkroom. Our investigation clearly demonstrates that the long-term exposure of rats to a red safelight during course of the daily dark phase of a biologic experiment is not, in fact, "safe" with respect to the preservation of 12:12-h light:dark-entrained circadian rhythm in several important neuroendocrine, metabolic, and physiologic parameters. Therefore, scientific investigators and animal care personnel alike should be aware of the variety of disruptions that can occur in the circadian physiology and metabolism of this commonly used laboratory rat strain in response to different types of light exposure and should carefully consider this issue in the development of lighting protocols for laboratory animal facilities, research study design, and the revision of subsequent editions of the *Guide*.

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