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# Sustained melatonin treatment blocks body mass, pelage, reproductive, and fever responses to short day lengths in female Siberian hamsters

Abstract: Winter imposes physiological challenges on individuals including increased thermoregulatory demands, risk of infection, and decreased food availability. To survive these challenges, animals living outside the tropics must appropriately distribute their energetic costs across the year, including reproduction and immune function. Individuals of many species use the annual cycle of changing day lengths (photoperiod), which is encoded by the nightly duration of melatonin secretion, to adjust physiology. Siberian hamsters exposed to short days (SD) (long nights/prolonged endogenous melatonin secretion) enhance some aspects of immune function, but curtail other energetically expensive immune functions including the febrile response. The purpose of this study was twofold. First, we determined whether sustained melatonin treatment would inhibit the development of the SD phenotype in female hamsters as it does in males. Second, we examined whether the SD attenuation of fever would be blocked by continuous exposure to exogenous melatonin. Hamsters were implanted with melatonin or empty capsules, housed in either long days (LD) or SD for 8–9 weeks, and then challenged with lipopolysaccharide; body temperature and locomotor activity were recorded. Unlike hamsters with empty capsules, hamsters with melatonin implants did not respond to SD and maintained a LD phenotype including summer-like spleen, uterine and body masses, and pelage characteristics. Further, sustained melatonin treatment blocked the SD attenuation of febrile responses and prolonged the behavioral components of the sickness response. These results suggest that the daily fluctuations in endogenous melatonin may be masked by continuous exposure to exogenous melatonin, thus inhibiting functional photoperiodic responses to SD.

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## Introduction

Animals that experience seasonal adjustments in ambient temperature, food availability, and pathogen prevalence require physiological flexibility to cope with such changes. Many animals have evolved the ability to predict seasonal changes by monitoring and responding to the annual cycle of changing day lengths (photoperiod) [1–4]. Siberian hamsters (*Phodopus sungorus*) are photoperiodic rodents that experience dramatic alterations in body mass, reproductive capacity, and immune function in response to both naturally occurring and laboratory photoperiods. In long days (LD), mimicking the summer season, Siberian hamsters have relatively high body and gonad mass, reduced circulating leukocytes, and display a prolonged sickness response following lipopolysaccharide (LPS) injections compared to hamsters in winter-like short days (SD) [5, 6]. After 8–13 weeks in SD, however, Siberian hamsters reduce body and gonad mass, develop a white winter pelage, and differentially elevate and suppress some measures of immune function. For example, behavioral and febrile responses to LPS are attenuated in SD which may protect against lethal bacterial sepsis [7–11], whereas delay-type hypersensitivity responses are enhanced which may confer increased resistance to fungi and microbes [4, 7, 11–13]. Furthermore, SD hamsters increase the number of circulating leukocytes, including natural killer cells and blood lymphocytes, while suppressing basal lymphocyte cell proliferation [5, 8, 14, 15]. These processes allow for increased immune competence without dedicating physiological resources to energetically expensive processes.

Melatonin has been postulated to be involved in the enhancement of the SD immune response, as it is secreted for a relatively prolonged duration during the long nights of SD [16]. There are two potential ways that melatonin could affect immune function: (i) melatonin could directly influence immune responses [6, 17–23] or (ii) melatonin could indirectly affect immune responses via downstream targets [19–21, 23]. Daily melatonin injections increase spleen cell numbers, blood leukocyte number, natural killer cytotoxicity, antigen presentation to T cells, and production of humoral antibodies including IgG and IgM [18, 21, 24],

suggesting a role for melatonin as an immunoenhancer [17]. The effects of continuous melatonin release on immune function are equivocal depending on the location and duration of treatment. For example, continuous *in vivo* melatonin treatment and 48 h-*in vitro* [14] melatonin treatment increased the antibody response for T-cell-dependent antigen presentation, elevated leukocyte numbers, and increased splenocyte proliferation [6, 21, 25], whereas other *in vivo* studies report no effect or reduced immune activation [6, 26]. These were contingent on where the implants were placed [i.e., subcutaneous or within the suprachiasmatic nuclei (SCN)], as well as the concentrations of melatonin used. Continuous release of melatonin is also confounded by its effects on photoperiod responsiveness. Appropriately timed daily injections of melatonin promote a SD phenotype inducing body mass changes, gonad regression, and molt to a winter-white pelage [19, 27]. Sustained melatonin treatment seems to eliminate responsiveness to a change in photoperiod in male hamsters [28–31] (except in SCN implants [6]), although it promotes SD phenotypes in other species [32].

The aims of this experiment were to (i) determine whether 8 weeks of sustained melatonin treatment would inhibit the development of the SD phenotype in female hamsters as it does in males and (ii) establish whether the SD attenuation of fever would be blocked by continuous exposure to exogenous melatonin.

## Materials and methods

### Animals

Adult (2–4 months) female Siberian hamsters (*Phodopus sungorus*) from our breeding colony were used in this study. Animals were individually housed in polypropylene cages at constant temperature (21 ± 4°C) and humidity (50 ± 10%) for the duration of the study and received *ad libitum* food (Harlan Teklad 8640 rodent diet; Indianapolis, IN, USA) and filtered tap water. Prior to the experiment, all animals were maintained under a 16:8 light/dark cycle (lights on at 23:00. Eastern Standard Time [EST]). Body mass measurements and pelage assessments were obtained weekly. Pelage color was rated on a scale of 1 (LD coloration, brown) to 4 (SD coloration, white) [33]. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee at Ohio State and complied with NIH Guidelines for the Care and Use of Animals.

### Melatonin treatment

At time point designated Week 0, a 14-mm-long Silastic capsule (1.47 mm ID, 1.95 mm OD; Fisher Scientific, Pittsburgh, PA, USA) filled with 10-mm crystalline melatonin (Sigma Chemical Co., St. Louis, MO, USA) (n = 20) [34] or an empty Silastic capsule (n = 18) was implanted subcutaneously into hamsters under deep anesthesia (2.5% Isoflurane vapors; Abbott, Chicago, IL, USA). Concentrations of melatonin were modeled after other long-term studies with melatonin in which low (μg/h or ng/h) doses were delivered [35, 36]. The capsules used in this study delivered 4.2 ± 0.4 μg/h (0.71 mg/week) of melatonin. Capsules were sealed on either end with 2-mm 100% silicone. Immediately following surgery, hamsters were either returned to the long-day condition (LD; 16-h light/day) (n = 10; 10-melatonin, 10-empty) or placed in a SD condition (SD; 8-h light/day) (n = 8–10; 10-melatonin, 8-empty) for the duration of the experiment. Constant-release capsules were used for 8–9 weeks in accordance with previous studies indicating that it requires at least 6 weeks for most Siberian hamsters to fully respond to a change in photoperiod, but beyond 19 weeks will cause a spontaneous revival of the LD condition [37].

### Peripheral immune stimulus

Hamsters were maintained in their respective LD or SD light cycles for 8–9 weeks. During Week 8–9, radio transmitters to monitor locomotor activity and body temperature were implanted intraperitoneally (i.p.) into randomly selected hamsters (10 LD and 10 SD) under deep anesthesia (2.5% Isoflurane vapors). All hamsters were allowed 5 days to recover. After recovery, hamsters' cages were placed on receiver boards, and hamsters were injected i.p. with 100–150 μL of sterile saline commensurate with body mass just before lights off (1500 EST). Body temperature and locomotor activity were recorded for 17 h at 15-min intervals. The following day, hamsters were again injected with sterile saline, and body temperature and locomotor activity were recorded. On the third day, hamsters were injected with 100–150 μL of 400 μg/kg LPS immediately before lights off, and body temperature and locomotor activity were recorded. During Week 9, this procedure was repeated with the remaining hamsters (10 LD and 8 SD).

### Tissue collection

After completion of the immune challenge with LPS (17 h), hamsters were anesthetized with 5% isoflurane vapors and euthanized by rapid cervical dislocation. Spleen, uterus, and axillae brown adipose tissue (BAT) were collected and weighed.

### Statistical analyses

Main effects of photoperiod condition (LD, SD) and capsule condition (melatonin, empty), and interactions thereof were assessed using a two-way analysis of variance (ANOVA). Post hoc statistical analyses were performed using two-tailed *t*-tests to further distinguish among groups. Baseline body temperatures and locomotor activity for the active and inactive phases were determined for each animal using the mean values for the 2 days of saline injections before LPS. We defined fever as temperatures significantly ($P < 0.05$) higher than active-phase baseline for each 15-min interval using two-tailed *t*-tests. These values were averaged into hours, and the total duration (h) of temperatures above baseline was compared between groups using a two-way ANOVA. Fever duration was additionally analyzed using area under the curve. Mean total locomotor activity following LPS treatment was compared with baseline total locomotor activity using a

two-way ANOVA. Mean differences were considered statistically significant when $P \leq 0.05$. All statistical analyses were conducted using Statistical Analysis Systems (SAS) statistical software, Cary, NC, USA.

## Results

Body mass was recorded from Week 1–8. Treatment with LPS activates the innate immune system resulting in sickness responses (decrease in food intake and body mass) [11]. Therefore, body mass measurements are not reported following surgery and LPS treatment. At the beginning of the experiment, all groups were statistically equivalent with respect to both body mass and pelage color ($P > 0.05$) (data not shown). After 8 weeks in photoperiod treatment, there was a main effect of photoperiod on body mass ($F_{1,38} = 21.59$, $P < 0.0001$) and pelage color ($F_{1,37} = 17.29$, $P < 0.002$). LD hamsters increased their body mass and maintained a pelage color rating of $1.2 \pm 0.07$ independent of melatonin treatment (Fig. 1A–C), whereas SD hamsters treated with empty capsules (SD-Empty) lost body mass and changed pelage color from a rating of 1 to $2.7 \pm 0.3$ consistent with a SD-provoked response [12, 38, 39]. There was also a significant photoperiod × melatonin treatment interaction in body mass ($F_{1,38} = 7.85$, $P < 0.009$) and pelage color ($F_{1,37} = 24.40$, $P < 0.0001$). In contrast to SD-Empty hamsters, SD hamsters treated with melatonin (SD-melatonin) did not respond to photoperiod. Instead, these hamsters resembled the LD groups with increased body mass over the 8-week treatment period (Fig. 1A,B) and maintained a LD pelage rating of $1.5 \pm 0.09$ (Fig. 1C).

Because of the photoperiod effects on body mass, all tissues were standardized as a percentage of body mass. To evaluate reproductive competence, uterus weight was compared between groups. There was a significant effect of photoperiod on uterine mass ($F_{1,37} = 4.30$, $P < 0.05$); LD hamsters had higher uterine mass than SD hamsters independent of melatonin treatment (Fig. 2A). There was also a tendency for a photoperiod × melatonin treatment interaction ($F_{1,37} = 3.65$, $P = 0.06$). SD-Empty hamsters reduced uterine mass compared to all other groups following post hoc analysis ($P < 0.05$). As a gross indicator of immune function, spleen mass was also measured following LPS treatment. SD-Empty hamsters decreased spleen mass compared to LD-Empty hamsters ($P < 0.05$), but did not significantly differ from either of the melatonin-treated groups ($P > 0.05$; Fig. 2B). BAT was measured as a component of photoperiod response. There was a significant effect of photoperiod ($F_{1,37} = 6.24$, $P < 0.02$) as SD-Empty hamsters increased BAT mass compared to LD hamsters independent of melatonin treatment (Fig. 2C). In contrast, SD-melatonin hamsters did not significantly differ from any other group ($P > 0.05$).

Body temperature and locomotor activity measurements for hamsters in each group following LPS treatment were compared with the responses to saline injections performed on the previous day. After treatment with LPS, all groups increased body temperature and reduced activity with similar latencies ($P > 0.05$) (Fig. 3A, data not shown). Fever duration was calculated as a measure of immune function. SD hamsters attenuate fever duration resulting in a decreased sickness response [5, 11]. Consistent with previous findings, SD-Empty hamsters shortened fever.

responses in comparison with LD-Empty hamsters ($P < 0.05$). Additionally, there was a significant photoperiod x melatonin treatment interaction ($F_{1,36} = 4.43$, $P < 0.05$) as both melatonin-treated groups showed intermediate fever duration in response to LPS (Fig. 3A,B). SD-Empty hamsters also showed a tendency to be more active following LPS treatment compared to SD-melatonin-treated hamsters ($P = 0.06$; Fig. 3C). LD hamsters re-

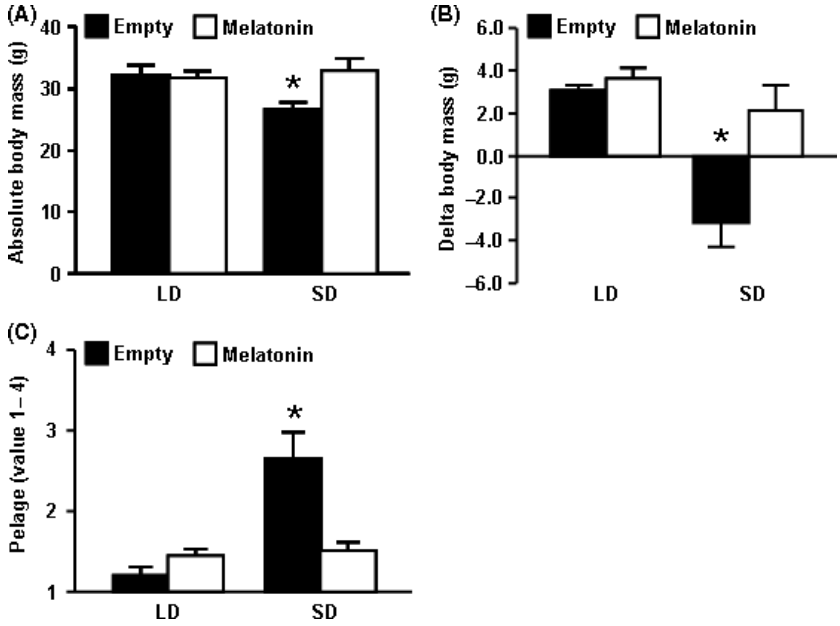


*Fig. 1.* Mean (±S.E.M.) (A) absolute body mass at Week 8, (B) change in body mass from Week 0 to Week 8, and (C) pelage value at Week 8 (n = 8–10). For pelage, values range from 1 (long days [LD] brown coloration) to 4 (short days white coloration). *$P < 0.05$ compared to the LD-Empty group.

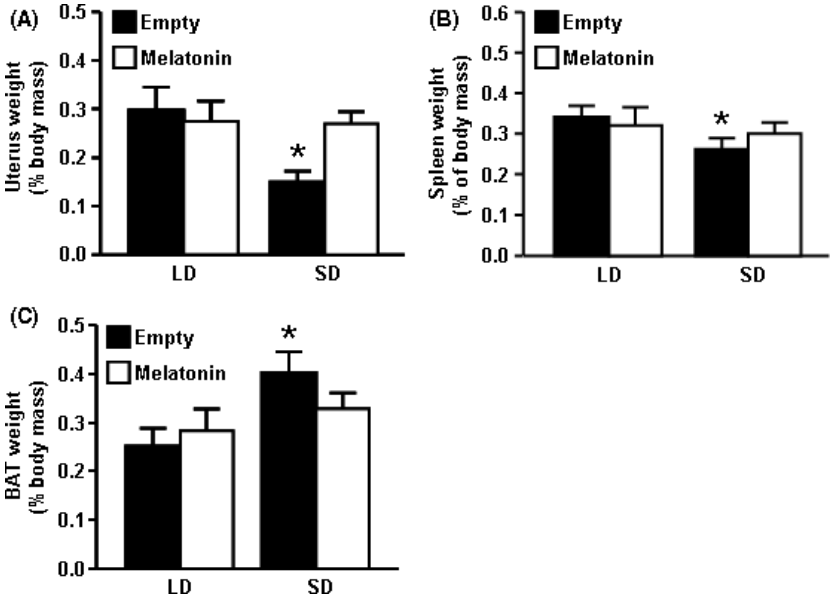


*Fig. 2.* Mean (±S.E.M.) (A) uterus, (B) spleen, and (C) brown adipose tissue mass after 9–10 weeks in photoperiod treatment (n = 8–10). $*P < 0.05$ compared to the long days-Empty group.

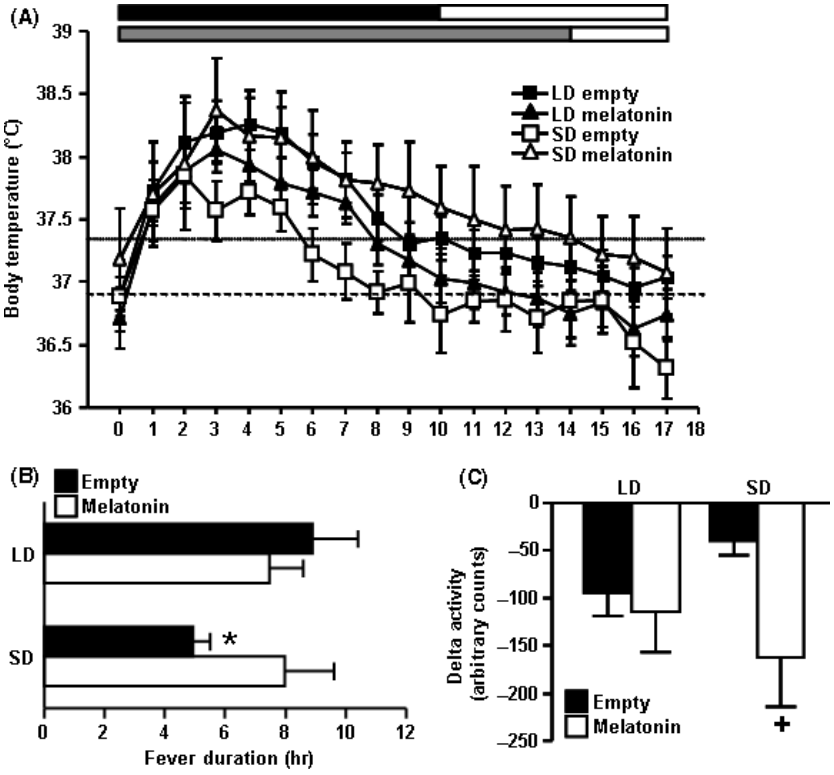


*Fig. 3.* (A) Mean (±S.E.M.) body temperature in long day (LD) and short day (SD) hamsters from 0 to 17 h post-lipopolysaccharide (LPS) injections after 9–10 weeks in photoperiod treatment. Black and gray bars above the graph indicate the active (dark) phase of the light–dark cycle in LD versus SD hamsters, respectively. Horizontal dotted and dashed lines represent mean baseline body temperature during the active versus inactive (light) phases, respectively (n = 8–10). (B) Mean (±S.E.M.) duration (h) of body temperatures higher than the active baseline in hamsters following LPS injections (n = 8–10). (C) Mean (±S.E.M.) locomotor activity changes from 0 to 17 h post-LPS injections compared with baseline after 9–10 weeks in photoperiod treatment. $*P < 0.05$ compared to the LD-Empty group. $+P = 0.06$ compared to the SD-Empty group.

duced locomotor activity following LPS treatment independent of melatonin treatment and were not significantly different from either SD group ($P > 0.05$).

## Discussion

The goal of this study was to establish whether sustained melatonin treatment for 8 weeks would affect photoperiodic responsiveness and febrile activity following a peripheral immune stimulus in female Siberian hamsters. Melatonin treatment altered the immunological and somatic measures in hamsters placed in SD, but had no effect on hamsters maintained in LD. SD uterine involution, body mass reduction, and pelage alterations were prevented by constant-release melatonin treatment. Furthermore, melatonin treatment resulted in an LD-like febrile and locomotor activity response following a peripheral injection of LPS. SD-Empty hamsters treated with LPS showed an attenuated febrile response compared with both LD groups and SD-melatonin hamsters.

Short photoperiods and melatonin administration that mimics SD lengths reduce body mass among Siberian hamsters [38, 40]. Melatonin treatment blocked SD–induced changes in body mass and uterine mass. Siberian hamsters transferred to SD and implanted with empty capsules showed the expected reduction in body mass over the 8-week experiment. Body mass of SD hamsters with exogenous melatonin treatment, however, increased throughout the study similarly to hamsters maintained in LD (Fig. 1). Melatonin mediates photoperiod changes in body mass through binding to MEL1a receptor in brain areas such as the SCN [40, 41]. MEL1a receptor activation drives sympathetic nervous system innervations of white adipose tissue [42], which initiate lipolysis and changes in expression of thermogenic regulators, such as uncoupling proteins [43]. However, in a nonphotoperiodic context melatonin does not directly trigger lipolysis [44], but may reduce body mass in some species through the enhancement of uncoupling protein 1 (for examples see [35, 45]). Our results suggest that constant administration of melatonin in a photoperiodic animal may block the SD–induced alterations of sympathetic nervous system innervations of adipose tissue inhibiting body mass loss (Fig. 1A,B).

In addition to body mass, other somatic measures of SD-melatonin hamsters resembled those of LD hamsters; the pelage of SD-melatonin hamsters remained brown, BAT was not significantly different compared with LD hamsters, and uterine mass was increased compared with SD-Empty hamsters (Figs 1C and 2). In contrast, SD-Empty hamsters showed the expected change in pelage to a white color, and had a reduced uterine mass and increased BAT mass compared with LD- and SD-melatonin-treated groups (Figs 1C and 2). Furthermore, treatment with melatonin in LD conditions did not alter body mass or reproductive tissue mass.

As previously demonstrated in males, SD reduce the sickness response following a peripheral injection of LPS in Siberian hamsters [11]. SD-Empty hamsters showed both a reduction in fever duration and attenuated suppression of activity level which are both indicative of reduced cytokine response in the brain [46]. In contrast, SD-melatonin hamsters did not diminish fever responses, but rather displayed an intermediary response. Thus, without a clear photoperiodic response and a distinguishable nightly increase in melatonin, the low dose of melatonin used in this study was insufficient to cause gross changes in immune function associated with SD. However, LD-melatonin hamsters also demonstrated an intermediary febrile response. These results suggest that sustained melatonin treatment blocks the SD fever response following injections of LPS, but may also have immunomodulatory activity for hamsters maintained in LD (Fig. 3B). This LD-melatonin intermediate response is consistent with previous results in Syrian hamsters demonstrating that melatonin reduces fever responses in a nonphotoperiodic context [47]. Furthermore, melatonin may have antibiotic and anti-viral properties; daily injections of melatonin protect against bacterial infection by increasing natural killer cell activity [18], enhancing splenocyte antibody-dependent cellular cytotoxicity [48], and inhibiting bacterial growth by curtailing the uptake of growth factors and binding of important metals such as iron [49–51]. In addition, melatonin has also been reported to be a general antioxidant, reducing the amount of nitric oxide produced during bacterial or viral infections [52–54]. Melatonin administration also modifies both pro- and anti-inflammatory cytokine concentrations. Specifically, injections of melatonin 30 min before and 1 h following a dose of LPS capable of inducing sepsis reduce pro-inflammatory cytokines such as TNF-$\alpha$ and INF-$\gamma$ and increase IL-10, the primary anti-inflammatory cytokine [55]. IL-10 is crucial in reducing microglial production of IL-1$\beta$ and TNF-$\alpha$ in the brain which corresponds to a reduction in fever [56]. SD hamsters likely limit energetically expensive sickness responses by inhibiting IL-1$\beta$ and TNF-$\alpha$ production in the brain. The lack of impact on fever of melatonin treatment among LD and SD hamsters in this study may relate to the low dose utilized or reveal a potential ineffectiveness of melatonin treatment if melatonin is delivered continuously.

These results confirm and extend previous research demonstrating that SD melatonin signals are swamped by constant-release peripheral melatonin [36–38]. An increase in the nightly duration of melatonin concentration, resembling a long-night, is necessary and sufficient to induce a SD phenotype [25, 46]. In this study, sustained melatonin presumably resulted in elevated continuous circulating melatonin concentrations thereby obscuring any endogenous melatonin signals. This was evident in the lack of response to a change in photoperiod for hamsters treated with sustained melatonin. In LD, however, natural peak melatonin concentrations are reduced as a result of shorter nights. Therefore, it is possible that sustained melatonin provided an intermediary reduction in febrile response because the sustained melatonin implants delivered higher concentrations than would naturally occur, thereby providing a partial direct signal to attenuate the febrile response.

Taken together, these results support previous findings that sustained exogenous melatonin treatment impairs a photoperiodic response rather than promoting a SD phenotype in Siberian hamsters [28–30]. Additionally, it seems that this photoperiodic response and increase in peak melatonin concentrations is necessary for the immunoenhancing qualities of melatonin for SD hamsters. Continuous melatonin release blocked the attenuated febrile response in SD hamsters and promoted a reduction in locomotor activity following LPS injections. In contrast, sustained melatonin did not appear to alter the morphological phenotype of LD hamsters. LD-melatonin hamsters displayed an intermediate febrile response suggesting a possible role for a direct effect of sustained melatonin treatment in the truncation of fever outside of a photoperiodic context. A more complete understanding of the interaction of melatonin and the immune system could lead to improved treatments of infection.

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## Author contributions

Dr. Randy Nelson developed the idea for this project and was involved in the writing, editing, and final approval for the article. Ashley Fenn acquired half of the reported data, conducted all statistical analyses, and was extensively involved in writing and editing the article. Laura Fonken acquired half of the reported data and was extensively involved in writing and editing the article.

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