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# Exposure to short days, but not short-term melatonin, enhances humoral immunity of male Syrian hamsters (*Mesocricetus auratus*)

Abstract: Many non-tropical rodent species rely on photoperiod as the primary cue to co-ordinate seasonally appropriate changes in physiology and behavior. Among these seasonal changes, several rodent species (e.g. deer mice, prairie voles, Siberian hamsters) adjust immune function in response to changes in ambient day lengths. The goals of the present study were to examine the effects of photoperiod on immune function of Syrian hamsters (*Mesocricetus auratus*), and to determine the role of melatonin in mediating photoperiodic changes in immunity. In Experiment 1, male Syrian hamsters were housed in long (LD 14:10) or short days (LD 10:14) for 10 wk. In Experiment 2, hamsters were housed in long days and half of the animals were given 10 consecutive days of i.p. melatonin injections (15 $\mu$g) in the early evening, while the remaining animals received injections of the vehicle alone. After the respective experimental manipulations, animals were injected with the antigen, keyhole limpet hemocyanin (KLH), blood samples were obtained and anti-KLH IgG antibody production was assessed. In Experiment 1, short-day hamsters underwent gonadal regression and reduced serum testosterone as well as displayed increased humoral immune function compared with long-day animals. In Experiment 2, short-term melatonin treatment did not affect gonadal mass, testosterone or humoral immune function. These results confirm previous findings of photoperiodic changes in immunity in rodents and suggest that changes in humoral immunity are not due to short-term changes in melatonin.

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## Introduction

In addition to marked seasonal changes in reproductive, metabolic, and other physiological functions, many mammalian and non-mammalian species undergo seasonal changes in immune function and disease [1–3]. Field studies of seasonal changes in immunity typically report reduced immune function and increased disease susceptibility during winter compared with spring and summer [4–6]. Suppression of immune function during winter probably reflects the physiological 'stress' of harsh environmental conditions (e.g. low ambient temperatures, reduced food availability) [7]. Glucocorticoids, primarily corticosterone in most rodents, are released from the adrenal glands in response to stress and can suppress immune function [8–10]. In contrast to field studies reporting reduced immune function during the short days of winter, the results of several laboratory studies examining photoperiodic changes in immunity report *enhanced* immune function in short as compared with long days [2, 11–13]. One hypothesis to account for this observation is that individuals of some rodent species appear to have evolved mechanisms to bolster immune function in short days in response to the deterioration of environmental conditions [14, 15].

Although the precise mechanisms mediating photoperiodic changes in immune function are not yet known, it is plausible that increased immunity may be the result of the increased duration of melatonin secretion in short days [12, 16, 17]. Melatonin acts both directly and indirectly on target tissue within the immune system [17–21]. Melatonin can act indirectly to affect immunity by altering several other hormones, including gonadal steroid hormones (e.g. estradiol, testosterone), prolactin, and corticosterone [22–24]. In addition, all of these hormones can affect immune function [reviewed in 19]. For example, testosterone typically suppresses immune function [25–27, but see 28]. Animals maintained in short days or receiving long-term melatonin treatment undergo significant reductions in circulating concentrations of androgens. Thus, short-day enhancement of immune function may reflect an indirect effect of melatonin on circulating concentrations of androgenic steroid hormones. As discussed above, glucocorticoids are released in response to stress and often suppress immune function [8, 9]. However, maintaining animals in

short days or treatment with melatonin attenuates the glucocorticoid response among individuals of some species and enhances immunity [17, 29]. These effects are also consistent with an indirect effect of melatonin on immune function.

Recent evidence supports a direct action of melatonin on immune function [20, 30–32]. For example, exogenous melatonin enhances both humoral and cell-mediated immunity in mammalian species that are generally reproductively non-responsive to melatonin (i.e. laboratory rats, house mice, humans) [33, 34]. Further, high-affinity melatonin receptors have been identified on circulating lymphocytes, thymocytes, and splenocytes in these same species [35]. Short days enhance splenocyte proliferation in both male and female deer mice; splenocyte proliferation, however, is unaffected by either castration or ovariectomy, and circulating corticosterone concentrations do not differ significantly between the sexes [30]. In vitro melatonin administration enhances splenocyte proliferation in male and female prairie voles (*Microtus ochrogaster*) [36, 37], the mitogenic response of peripheral blood T lymphocytes from chickens [38], and the activity of interleukin production [39, 40]. In addition, in vitro melatonin enhances the ability of splenocytes to proliferate in house mice, and, importantly, this enhancement is attenuated with the addition of the high-affinity melatonin receptor antagonist, luzindole [20]. Taken together, these results suggest that short-day enhancement of cell-mediated immune function is independent of steroid hormones.

The primary goals of the present experiment were to confirm and extend the early work on photoperiod and immunity in Syrian hamsters and to test the hypothesis that increased immunity in short days is due to a direct effect of extended melatonin secretion. To accomplish these goals, animals were either housed in long or short days (Experiment 1) or *exogenous* melatonin was administered to long-day animals (Experiment 2). In both experiments, humoral immunity was determined in response to antigenic stimulation. If melatonin affects immune function *independent* of gonadal steroid hormones, then both short-day housed hamsters and long-day hamsters receiving short-term melatonin treatment should display increased specific antibody concentrations. However, if the biochemical action of exogenous melatonin is indirect, via a 'release' from the immunocompromising effects of testosterone, then short-day hamsters should display enhanced humoral immunity, whereas exogenous injections of melatonin to long-day animals should have no effect on immune function.

## Materials and methods

### Animals and housing conditions

Adult (>60 days of age) Syrian hamsters (*Mesocricetus auratus*) were obtained from a commercial supplier (Charles River) and were housed individually in polypropylene cages (40 × 20 × 20 cm) in colony rooms with a 24-h LD 14:10 cycle (lights on 14:00 hr EST). Temperature was kept constant at 20 ± 2°C and relative humidity was maintained at 50 ± 5%. Food (Purina Rat Chow) and tap water were available ad libitum throughout the experiment. All animals were treated in accordance with the Georgia State University Institutional Animal Care and Use Committee.

### Experiment 1

Twenty male hamsters were used in Experiment 1. At the start of the experiment, all animals were housed in a colony room with a long day (LD 14:10 photoperiod). After 1 wk, a random subset of animals (n = 10) was selected and transferred to a colony room with a short-day (LD 10:14) photoperiod. The remaining animals (n = 10) were maintained in long days for the duration of the experiment. Animals were kept in their respective photoperiod for 10 wk. At this time (Day 0), all hamsters received a single subcutaneous injection of 100 μg of the antigen, keyhole limpet hemocyanin (KLH) (to which all animals were previously naive), suspended in 0.1 mL sterile saline and were then returned to the colony room. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*). KLH was used because it generates a robust antigenic response in rodents, but does not make the animals ill (e.g. little or no inflammation or fever) [41]. Blood was drawn from the retro-orbital sinus at two different sampling periods (Days 5 and 10 post-immunization). These sampling periods were chosen in order to capture peak IgG production during the course of the immune response to KLH [42]. On each sampling day, animals were brought into the surgery room individually, lightly anesthetized with methoxyflurane vapors (Metofane, Mundelein, IL, USA), and blood samples (500 μL) were drawn from the retro-orbital sinus between 10:00 and 12.00 hr EST. Samples were allowed to clot for 1 hr, the clots were removed, and the samples centrifuged (at 4°C) for 30 min at 1430 g. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at −80°C until assayed for anti-KLH IgG. On the last day of sampling (Day 10) animals were killed by cervical dislocation. Paired testes and spleens were removed and cleaned of connective tissue at necropsy. All tissue was weighed to the nearest 0.001 g by laboratory assistants naive to the experimental hypotheses and treatment assignments.

### Experiment 2

Twenty male hamsters were used in Experiment 2. All animals were maintained in long days as described in Experiment 1 for the duration of the experiment. Half of the animals (n = 10) were randomly selected to receive daily subcutaneous injections (0.1 mL per animal) of melatonin (15 μg) (Sigma Chemical, St Louis, MO, USA) dissolved in a 1:10 ethanol:saline solution [43] for 10 days, whereas the remaining animals (n = 10) received injections of the vehicle alone. The melatonin injection protocol that was used in Experiment 2 was chosen for several reasons. First, the precise timing of melatonin injections (i.e. 2 hr before lights off) extends the normal long-day pattern of endogenous melatonin secretion; the resulting extended pattern of melatonin is interpreted by

hamsters as a short day [22]. Thus, the pattern of melatonin generated in experimental animals, rather than being artificial or supra-physiological, accurately reflects typical short-day patterns. Secondly, melatonin was administered on a relatively short-term basis (i.e. 10 days); this time period was chosen because it is not sufficiently prolonged to trigger gonadal regression, and unlike maintenance in short days, leaves gonadal steroid concentrations unaffected [44]. This allows the effects of exogenous melatonin to be tested directly, without subsequent changes in steroid hormones. After 3 days of melatonin treatment, all animals were injected with KLH and blood samples were drawn as in Experiment 1. On Day 10 of melatonin treatment, animals were killed by cervical dislocation. Paired testes and spleens were removed and cleaned of connective tissue at necropsy. Tissue was weighed and handled as in Experiment 1.

### Testosterone assay

Blood serum testosterone concentrations were determined in a single radioimmunoassay (RIA) from a commercially prepared kit (Diagnostic Systems Laboratories, Webster, TX, USA). This assay was validated for use with Syrian hamsters by the Neuroendocrinology Core Facility at the Yerkes Regional Primate Research Center in Atlanta, Georgia. The antiserum used was highly specific for testosterone; cross-reactivity with other steroid hormones was $<0.01\%$. Intra-assay variability was $<10\%$ for all samples (Figs. 1 and 3).

### Humoral immunity

To assess humoral immunity, serum anti-KLH IgG concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with antigen by incubating overnight at 4°C with 0.5 mg/mL KLH in sodium bicarbonate buffer (pH = 9.6), washed with phosphate buffered saline (PBS; pH = 7.4) containing 0.05% Tween 20 (PBS-T; pH = 7.4), then blocked with 5% non-fat dry milk in PBS-T overnight at 4°C to reduce non-specific binding, and washed again with PBS-T. Thawed serum samples were diluted 1:20 with PBS-T, and 150 $\mu$L of each serum dilution was added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled sera from hamsters previously determined to have high concentrations of anti-KLH antibody, similarly diluted with PBS-T) and negative control samples (pooled sera from KLH-naive hamsters, similarly diluted with PBS-T) were also added in duplicate to each plate; plates were sealed, incubated at 37°C for 3 hr, then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated-antimouse IgG diluted 1:2000 with PBS-T; Cappel, Durham, NC, USA) was added to the wells, and the plates were sealed and incubated for 1 hr at 37°C. Plates were washed again with PBS-T and 150 $\mu$L of the enzyme substrate *p*-nitrophenyl phosphate (Sigma Chemical, St Louis, MO, USA: 1 mg/mL in diethanolamine substrate buffer) was added to each well. Plates were protected from light during the enzyme-substrate reaction, which was terminated after 20 min by adding 50 $\mu$L of 1.5 M NaOH to each well. The optical density (OD) of each well was determined using a plate reader (Bio-Rad; Benchmark, Richmond, CA, USA) equipped with a 405-nm wavelength filter, and the mean OD for each set of duplicate wells was calculated. To minimize intra-assay variability, the mean OD for each sample was expressed as a percent of its plate positive control OD for statistical analyses (Figs. 2 and 4).

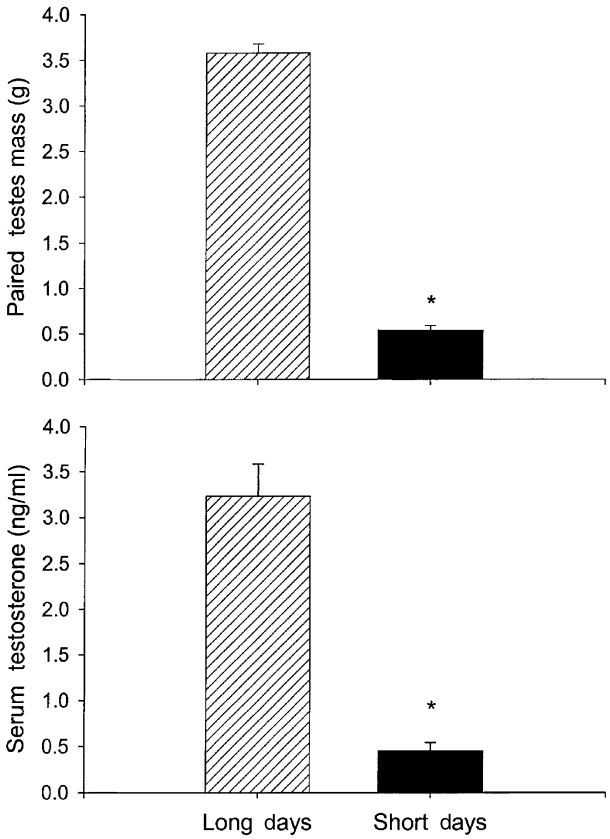


*Fig. 1.* (A) Mean (±S.E.M.) paired testes mass of male Syrian hamsters that were housed in either long (LD 14:10) or short (LD 10:14) days for 10 wk. (B) Mean (±S.E.M.) serum testosterone concentrations of male Syrian hamsters that were housed in either long (LD 14:10) or short (LD 10:14) days for 10 wk. *Significantly different from long days ($P < 0.05$).

### Statistical analyses

In Experiment 1, hamsters that did not display the typical gonadal regression after 10 wk in short days (n = 1) were considered to be photoperiodic non-responders and were not included in subsequent statistical analyses. The data from Experiments 1 and 2 were analyzed using independent student's *t*-tests (Sigma Stat, Jandel Scientific, San Rafael, CA, USA). Day 10 serum samples were chosen for anti-KLH IgG analyses. Differences between group means were considered statistically significant if $P < 0.05$. Exact probabilities and test values have been omitted for simplification and clarity of the presentation of results.

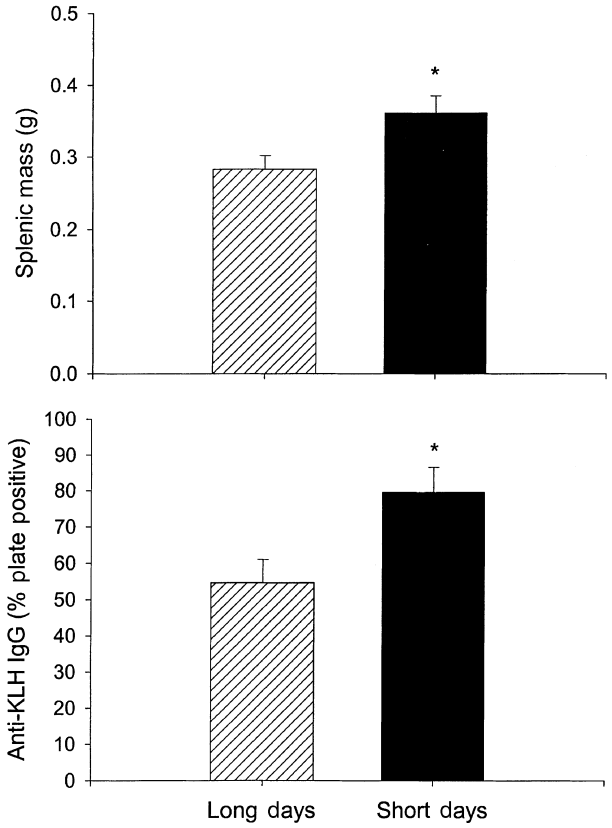


*Fig. 2.* (A) Mean (±S.E.M.) splenic mass of male Syrian hamsters that were housed in either long (LD 14:10) or short (LD 10:14) days for 10 wk. (B) Mean (±S.E.M.) serum anti-KLH immunoglobulin G concentrations (represented as percentage of plate positive) of male Syrian hamsters that were housed in either long (LD 14:10) or short (LD 10:14) days for 10 wk. *Significantly different from long days ($P < 0.05$).

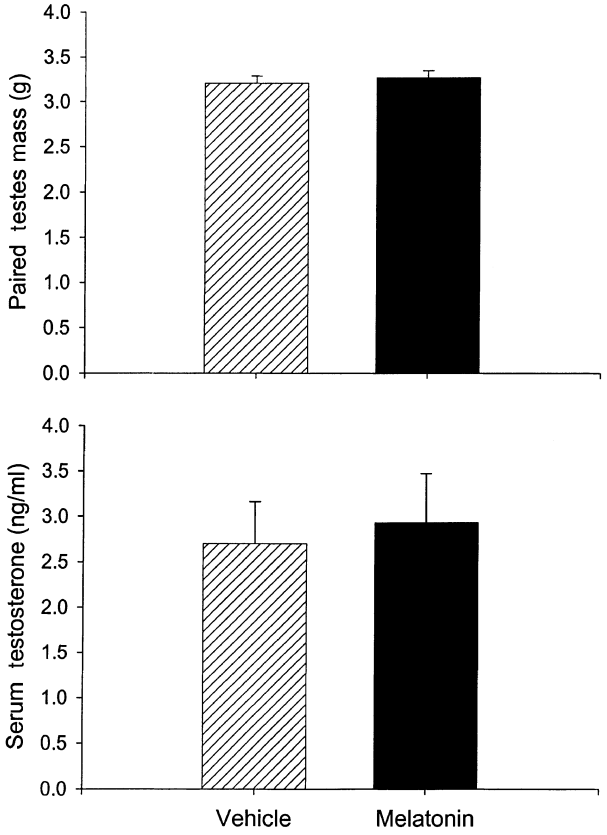


*Fig. 3.* (A) Mean (±S.E.M.) paired testes mass of male Syrian hamsters that were housed in long days (LD 14:10) and received either 10 days of vehicle or melatonin treatment. (B) Mean (±S.E.M.) serum testosterone of male Syrian hamsters that were housed in long days (LD 14:10) and received either 10 days of vehicle or melatonin treatment.

## Results

In Experiment 1, hamsters maintained in short days had significantly reduced paired testes (Fig. 1) and significantly increased body (data not shown) and spleen masses compared with long-day animals ($P < 0.05$). In addition, serum anti-KLH antibody concentrations were significantly higher in short- compared with long-day hamsters ($P < 0.05$) (Fig. 2). Short-day hamsters had significantly reduced serum testosterone concentrations compared with long-day animals ($P < 0.05$) (Fig. 1).

In Experiment 2, melatonin-treated hamsters displayed statistically equivalent anti-KLH antibody concentrations compared with vehicle-treated animals ($P > 0.05$) (Fig. 4). In addition, there were no significant differences between melatonin- and vehicle-treated hamsters in body (data not shown), paired testes (Fig. 3), or splenic masses (Fig. 4), or serum testosterone concentrations ($P > 0.05$) (Fig. 3).

## Discussion

The results of the present study suggest that Syrian hamsters housed in short days display increased humoral immunity compared with long day-housed hamsters. These results are consistent with previous reports of increased immunity in short days in this species [11], as well as the increases in immunity reported in other rodent species [reviewed in 2]. Short-term melatonin treatment, however, did not enhance immune function in the present study. These results contrast with previous studies demonstrating increased immune function in animals receiving short-term melatonin treatment [reviewed in 34, 35]. Collectively, these results suggest that short-day enhancement of humoral immunity in Syrian hamsters may not be due to a direct effect of a short-term increase in duration of melatonin secretion; rather, increased immunity may be the result of other physiological and hormonal changes (e.g. decreased testosterone) associated with short day lengths or direct effects that require additional time to develop.

It has been demonstrated that housing animals in simulated winter photoperiods leads to an increase in immunity in several species and for a variety of immune parameters. For example, deer mice (*P. maniculatus*) increase white blood cell (WBC) and lymphocyte counts [45], and increase splenoctye proliferation when housed in short compared with long days. Maintenance in short days also influences immunity of species that are traditionally considered unresponsive to photoperiod; rats increase in

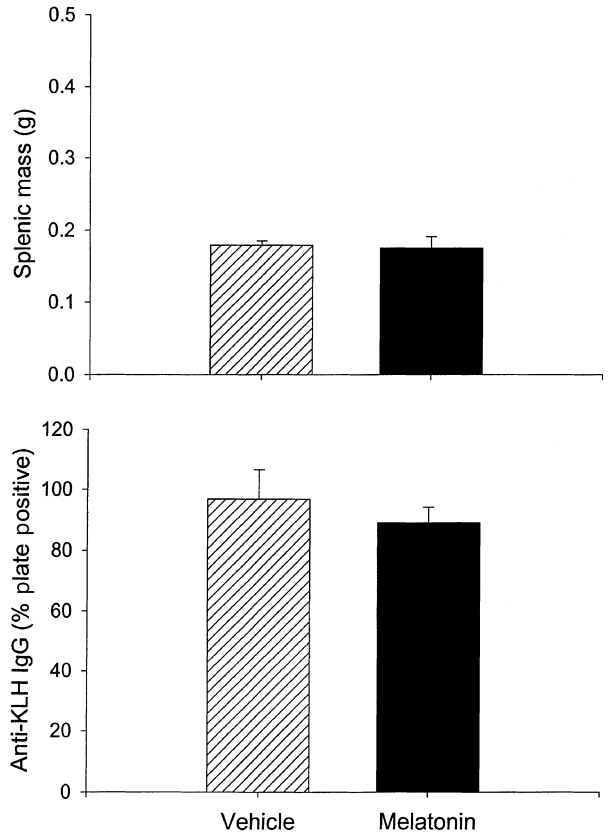


*Fig. 4.* (A) Mean (±S.E.M.) splenic mass of male Syrian hamsters that were housed in long days (LD 14:10) and received either 10 days of vehicle or melatonin treatment. (B) Mean (±S.E.M.) serum anti-KLH immunoglobulin G concentrations (represented as percentage of plate positive) of male Syrian hamsters that were housed in long days (LD 14:10) and received either 10 days of vehicle or melatonin treatment.

splenic and thymic mass when housed in short days [46, 47]. Consistent with the results from the present study, Syrian hamsters elevate splenic mass and enhance lymphoproliferative activity when housed in short days [12, 48].

Melatonin serves as the physiological signal in mammals that 'encodes' day length information, and the majority of studies suggest that the duration of melatonin secretion is the critical parameter mediating physiological responses to photoperiod [22]. Specifically, a short 'summer-like' pattern of melatonin secretion is interpreted as a long-day signal; a long, 'winter-like' pattern of melatonin secretion is interpreted as a short day and elicits short-day physiological responses (e.g. changes in body mass, gonadal regression). Growing evidence suggests that melatonin, in addition to regulating seasonal responses, may also increase immune function. The majority of these studies, however, have treated animals with exogenous melatonin for a short period of time (i.e, several days); this short-term melatonin regimen is not sufficiently prolonged to alter gonadal steroid hormones or trigger reproductive regression. Additionally, these studies have traditionally been conducted in laboratory species (e.g. rats, house mice) that are typically reproductively unresponsive to photoperiod. Many of these studies have demonstrated that short-term treatment with melatonin increases a range of immune parameters [e.g. 49]. For example, short-term melatonin treatment of both normal and immunocompromised house mice increases in vitro and in vivo antibody responses and T helper cell activity [34, 35].

Unlike previous studies, however, short-term exogenous melatonin treatment did not affect humoral immunity in the present study. One simple explanation for the discrepancy between Experiments 1 and 2 is a difference in photoperiodic responsiveness, which was controlled for in Experiment 1, but not in Experiment 2. Given the general lack of non-responsiveness in our colony (e.g. $n = 1$ in Experiment 1), it is unlikely that responsiveness could account for the discrepant results of Experiments 1 and 2. Alternatively the present data suggest that melatonin may be acting indirectly (e.g. via photoperiod-dependent changes in other circulating hormones) to enhance immune function in Syrian hamsters. These results are also in contrast to previous studies that implicate that melatonin acts directly to influence immunity [e.g. 20, 21]. Another possibility is that melatonin *can* act directly to influence immunity in Syrian hamsters, but that a more prolonged 'short-day' pattern of melatonin (e.g. 10 wk) is required to enhance humoral immunity. For example, in a previous study, 11 wk of melatonin injections to Syrian hamsters influenced interferon-$\gamma$ and IL-4 levels, but had no effect on IL-2 levels or IgG content in the spleen [50]. Because prolonged melatonin treatment also triggers gonadal regression and concomitant changes in a range of hormones, however, the direct versus indirect effects of melatonin cannot be teased apart in this study. Another consideration is that melatonin, which can be produced by immune cells, may mask the direct effects of exogenous melatonin on immune parameters [51, 52]. Studies with specific melatonin inhibitors may be needed to address issues.

If melatonin does not act directly to enhance immunity in Syrian hamsters, then it is likely that other hormones that fluctuate in response to changes in day length may mediate changes in humoral immunity. Short-day exposure leads to a marked reduction in serum testosterone concentrations, as confirmed in the present study. Along with inhibiting reproductive function, testosterone typically suppresses immune function [reviewed in 26, 27]. For example, castration of male rodents *increases* both humoral and cell-mediated immunity, as well as increasing lymphatic organ mass (e.g. spleen, thymus). Importantly, treatment of castrated males with physiological doses of testosterone returns immune function to pre-castration levels [53]. Thus, it is possible that the enhanced humoral immunity observed in short-day Syrian hamsters is because of a reduction in circulating testosterone. This possibility is further supported by the data from the present study showing that melatonin administration that is insufficient for changing testosterone concentrations leaves humoral immunity unaffected.

Another potential hormonal candidate that varies seasonally, and that could influence immunity in short days is the peptide hormone, leptin. Leptin is produced primarily by adipocytes, and circulating leptin concentrations are positively correlated with the percentage of fat mass in a variety of mammals [54]. There is growing evidence that

leptin has both direct and indirect immunoenhancing properties [e.g. 55, 56]. Siberian hamsters show a marked reduction in body fat in short days, and this is correlated with reductions in serum leptin and humoral immunity [42]. Syrian hamsters, in contrast, *increase* body mass in short days, but also display enhanced immunity under this condition. It is plausible that changes in leptin concentrations associated with changes in body mass in different photoperiods could mediate changes in immunity in Syrian hamsters, although this remains to be tested.

Despite the likelihood that a variety of hormones may influence immunity in short-day Syrian hamsters, it is likely that the photoperiod-induced adjustments in immunity are at least indirectly influenced by melatonin, given that splenic mass from pinealectomized short-day Syrian hamsters do not differ from long-day animals [13]. This is consistent with data from Siberian hamsters, in which pinealectomy eliminates the short-day induced reduction in humoral immunity [57]. Taken together, the present data demonstrate that exposure to short day lengths leads to an increase in humoral immunity in Syrian hamsters, results that are consistent with data from other species on a variety of immune parameters. The data suggest that the short-day increase in immunity may be because of an indirect effect of melatonin, although further studies are needed to tease apart what specific hormones may be acting to mediate the change in immunity. Our results are also consistent with the possibility that both direct and indirect effects of melatonin mediate photoperiodic changes in immunity.

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