Effects of photoperiod and 2-deoxy-D-glucose-induced metabolic stress on immune function in female deer mice

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Demas, Gregory E., A. Courtney DeVries, and Randy J. Nelson. Effects of photoperiod and 2-deoxy-D-glucose-induced metabolic stress on immune function in female deer mice. *Am. J. Physiol.* 272 (*Regulatory Integrative Comp. Physiol.* 41): R1762–R1767, 1997.—Nontropical rodents may experience large fluctuations in both food availability and energetic demands. The energy required for thermoregulation is high during the winter when energy availability is usually low. Winter conditions can induce a state of energetic stress that elevates circulating glucocorticoid levels and compromises immune function. Exposure to short days enhances immune function; the adaptive function of short-day enhancement of immune function may be to counteract the effects of stress-induced immunocompromise. To examine the role of energy availability in immune function, female deer mice were housed in either long (16:8-h light-dark cycle) or short (8:16-h light-dark cycle) days for 8 wk and then injected with either saline or 2-deoxy-D-glucose (2-DG), a glucose analog that inhibits cellular utilization of glucose and induces energetic stress. Long-day mice injected with 2-DG exhibited elevated corticosterone levels and reduced splenocyte proliferation compared with control mice. Short days buffered the animals against glucoprivation stress. Neither corticosterone levels nor splenocyte proliferation differed between 2-DG injected and control mice housed in short days. These data are consistent with the hypothesis that short days provide a buffer against metabolic stress.

seasonal; energetics; corticosterone; glucocorticoid; food restriction

NONTROPICAL RODENTS often experience large seasonal fluctuations in both food availability and energy demands. The energy required for thermoregulation is highest during the winter when food availability is typically reduced. Animals require a balanced energy budget (i.e., energy intake $\geq$ energy expended) to maximize survival (10, 35). Animals maintain a relatively constant flow of energy to the body despite fluctuating energy supplies and demands. Energy must also be partitioned where it is most needed (32). Reproduction is energetically costly, and it has been suggested that a temporary cessation of breeding provides a mechanism for energy conservation at a time when energetic availability is low (27). The conservation of energy preserves the energetic balance required for attaining maximal reproductive success. Thus the curtailment of winter breeding is central among winter-coping strategies in temperate rodent species (22, 35). Individuals of many animal species rely primarily on photoperiod (day length), transduced by a melatonin signal, as a precise temporal cue to ascertain the time of year. Photoperiodic information can be used to time energetically expensive processes, such as reproduction, to coincide with optimal energy availability (reviewed in Ref. 1).

The vast majority of studies of seasonal changes in mammalian physiology have emphasized seasonal fluctuations in reproduction (10). Seasonal reproduction, however, is only one among many winter-coping strategies utilized by individuals of many rodent species. Changes in body mass, pelage, thermoregulation, and immunity also occur on a seasonal basis (reviewed in Refs. 22 and 25). Although less well studied, there also exist prominent seasonal fluctuations of illness and death (reviewed in Ref. 24). Disease and death rates typically are highest during the winter compared with summer for individuals in a wide range of species (24). Animals most likely succumb to opportunistic diseases at times when their immune systems are most vulnerable. Consistent with this idea, impaired immune function in winter compared with summer has been reported for many of these species (25).

An energetic perspective has proven valuable in understanding seasonal changes in reproduction (12). Similarly, an energetic perspective may help explain seasonal fluctuations in immune function (25); mounting an immune response requires substantial energy (Ref. 29; Demas and Nelson, unpublished data). It is currently hypothesized that winter suppression of immune function commonly reported in field studies is caused in part by environmental stressors such as reduced ambient temperature and reduced food availability, which reduce energy availability (14). The term "stressor" is operationally defined here as a stimulus that evokes prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis (17, 28, cf 26). For example, either low ambient temperatures or restricted food intake elevates glucocorticoid concentrations in the laboratory (6, 23). Winter environmental stressors could create an energetic shortage by either decreasing energetic availability (e.g., reduced food availability) or by increasing energetic demands (e.g., increased thermoregulation; Refs. 7 and 34). The net result of an energetic shortage would likely be a state of metabolic stress.

To increase the odds of surviving the energetic demands of winter, individuals of some rodent species may have evolved mechanisms to enhance immune function before environmental conditions deteriorate (13, 14, 24). Previous laboratory studies suggest that immune function can be enhanced by short, winter-like photoperiods or melatonin treatment (see Ref. 24 for a review). These findings contrast with the results of several field studies reporting suppressed immune

function during winter (e.g., Ref. 30). To resolve this discrepancy, environmental stressors present in field studies have been proposed to counteract the short-day enhancement of immune function reported in laboratory studies (14, 24). Winter conditions may induce a state of metabolic stress in deer mice, reflected by elevated glucocorticoid secretion and subsequent reduction of immune function (e.g., Ref. 14). Maintaining animals on short days or administration of melatonin or the melatonin agonist S-20304 may attenuate the glucocorticoid stress response (9) and thus enhance immune function (20).

Mounting an immune response likely requires utilizing resources that could otherwise be allocated to other biological functions (29). Immune function should be "optimized" so that individuals can tolerate small infections if the energetic costs of mounting an immune response outweigh the benefits (2). Recent research has suggested an energetic trade-off exists between immune function and the costs of reproduction (15). For example, lactating bighorn ewes display increased parasitic infection (presumably due to reduced immune function) compared with nonlactating ewes (15). Thus optimal resource allocation between reproductive and immune function depends on competing energetic demands and their associated costs and benefits (29).

Although energetic availability can affect an animal's ability to mount an immune response (e.g., Ref. 16), virtually no studies have adopted an energetic perspective in seasonal changes in immunity. To examine the role of energetic availability in seasonal changes in immune function in female deer mice, the chemical compound 2-deoxy-D-glucose (2-DG) was used to manipulate energy availability at the input end of the energetic equation. 2-DG is a glucose analog that inhibits cellular utilization of glucose, thus inducing a state of glucoprivation (33). 2-DG can act as a metabolic stressor, increasing serum corticosterone levels (18). 2-DG glucoprivation induces anestrus in female Syrian hamsters (*Mesocricetus auratus*) (27) and torpor in female Siberian hamsters (*Phodopus sungorus*) (11). 2-DG administration inhibits splenic T lymphocyte proliferation in a dose-dependent fashion in laboratory strains of rats (*Rattus norvegicus*) (18) and mice (*Mus musculus*) (21). If 2-DG acts as a metabolic stressor in deer mice, then it is expected that animals receiving 2-DG will display increased serum levels of corticosterone. Furthermore, if 2-DG-induced metabolic stress compromises immune function, then animals treated with 2-DG should also display reduced splenocyte proliferation in response to concanavalin A (Con A). If, however, exposure to short days reduces the stress response to 2-DG, then it is expected that animals maintained in short days will demonstrate reduced corticosterone levels and greater splenocyte proliferation than their long-day cohorts.

MATERIALS AND METHODS

Animals. Forty-eight adult (>60 days of age) female deer mice (*Peromyscus maniculatus bairdii*) were obtained from the breeding colony within our laboratory. This colony was originally derived from animals from the Peromyscus Stock Center at the University of South Carolina (Columbia, SC). These animals are descendants of animals originally trapped near East Lansing, MI (latitude 42° 51′ N). Deer mice were weaned at 21 days of age and housed with same-sex siblings. Two weeks before the initiation of the experiments, all animals were individually housed in propylene cages (27.8 × 7.5 × 13.0 cm) in colony rooms with a 16:8-h light-dark (L-D) cycle [lights on at 0600 Eastern Standard Time (EST)]. Relative humidity was held constant at 50 ± 5%. Food (Prolab 1000; Agway, Syracuse, NY) and tap water were provided ad libitum throughout the course of the experiment.

Procedure. Deer mice were randomly selected and assigned to one of two photoperiodic conditions. One-half of the animals (n = 24) were housed in a short-day photoperiod (8:16-h L-D cycle, lights on at 0600 EST), whereas the other one-half (n = 24) were housed in long days (16:8-h L-D cycle, lights on at 1000 EST). All animals were maintained in their respective photoperiodic conditions for 8 wk.

After 8 wk deer mice in each photoperiodic condition were randomly assigned to either one control or three experimental treatment groups. All animals were weighed and their body masses recorded. Control mice (n = 12) received a daily intraperitoneal injection of sterile 0.9% saline across 3 consecutive days. Experimental mice (n = 36) were injected with 2-DG (750 mg/kg body wt; Sigma Chemical, St. Louis, MO). The three experimental groups of animals received a total of three injections comprised of either one, two, or three exposures to 2-DG dissolved in sterile saline. Injections were performed once every 24 h and were administered 4 h into the light phase of the day cycle. A total injection of 0.1 ml/mouse was administered. On injection days in which experimental groups did not receive 2-DG, experimental animals were injected with an equal amount of sterile saline (0.9%). Each control and experimental animal received a total of three injections, and animals were manipulated so that groups of mice differed only in the content of the injection. Some rodent species (e.g., Siberian hamsters) display periods of torpor (e.g., hypothermia, lethargy) in response to 2-DG administration (13). It is possible that torpid animals could display alterations in immune function independent of the effects of glucoprivation. To control for this, all mice were observed daily for signs of torpor.

Twenty-four hours after the last injection, animals were lightly anesthetized with methoxyflurane vapors (Metofane, Pitman-Moore, Mundelein, IL) and weighed. Blood samples (500 μl) were drawn from the retro-orbital sinus between 1000 and 1100 EST. Handling time was kept constant and to a minimum; the time from initial removal from the cage to the end of the bleeding was <3 min. After 1 h at room temperature, the clots were removed from the samples before they were centrifuged at 10°C at 2,000 rpm for 1 h, and the sera were extracted. Serum aliquots were stored in sealable polypropylene microcentrifuge tubes at −80°C until assayed. Animals were then killed by cervical dislocation, and spleens were removed under aseptic conditions and immediately suspended in culture medium [RPMI 1640-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)]. Uterine horns were also removed, cleaned of fat and connective tissue, and then weighed to assess the animals' reproductive responsiveness to short days. Animals that are reproductively responsive to photoperiods should show significant reductions in uterine horn mass.

Immunological assay. Splenocyte proliferation in response to the T cell mitogen Con A was determined using a colormetric assay based on the tetrazolium salt 3-(4,5-demethylthiazol-

2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Splenocytes were separated from tissue by compressing the whole spleen between sterile frosted glass slides; separated cells were suspended in 4 ml of culture medium (RPMI 1640-HEPES) supplemented with 1% penicillin (5,000 U/ml)-streptomycin (5,000 μl/ml), 1% L-glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5×10^{-2} mM/ml), and 10% heat-inactivated fetal bovine serum. Splenocyte counts and viability were determined with a hemacytometer and trypan blue exclusion. Viable cells (which exceeded 95%) were adjusted to 2×10^6 cells/ml by dilution with culture medium, and 50-μl aliquots of each cell suspension (i.e., 100,000 cells) were added to the wells of sterile flat-bottom 96-well culture plates. Con A (Sigma Chemical) was diluted with culture medium to concentrations of 40, 20, 10, 5, 2.5, 1.25, and 0.60 μg/ml; 50 μl of each mitogen concentration were added to the wells of the plate containing the spleen cell suspensions to yield a final volume of 100 μl/well (each in duplicate). Plates were incubated at 37°C with 5% CO_2 for 48 h before addition of 20 μl of MTS-phenazine methosulfate (PMS) solution [Promega; 0.92 mg/ml of PMS in sterile Dulbecco's phosphate-buffered saline] per well. Plates were then incubated at 37°C with 5% CO_2 for an additional 4 h. The optical density (OD) of each well was determined with a microplate reader (Bio-Rad, model 3550) equipped with a 490-nm wavelength filter. Mean OD values for each set of duplicates were used in subsequent statistical analyses. Dose response curves were constructed using group means of the mean OD values at each mitogen concentration and unstimulated cultures.

Serum corticosterone assay. Blood serum corticosterone levels were assayed by radioimmunoassay using the ICN Biomedicals (Costa Mesa, CA) ^{125}I-kit. The deer mouse serum dilution was prepared according to the guidelines furnished by ICN. The corticosterone assay was highly specific, cross-reacting at <0.3% with other steroid hormones. Serum corticosterone values were determined in a single radioimmunoassay. Intra-assay variation was <4.5%.

Data analyses. Serum corticosterone concentrations, lymphocyte proliferation, and body and uterine mass data were analyzed with a 2 (photoperiod) × 4 (injection) mixed model analysis of variance (Sigmastat, Jandel Scientific). Any pairwise comparisons of mean differences were conducted using planned comparisons. Differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

Deer mice housed in long days (16:8-h L-D cycle) that received injections of 2-DG displayed elevated serum corticosterone levels compared with long-day control animals ($P < 0.05$, Fig. 1). There were no differences among animals receiving one, two, or three injections of 2-DG across consecutive days ($P > 0.05$). Deer mice housed in short days (8:16-h L-D cycle) and receiving 2-DG injections did not differ from control mice in serum corticosterone levels (Fig. 1).

Deer mice housed in long days and receiving injections of 2-DG displayed reduced splenocyte proliferation to Con A relative to short-day control animals (Fig. 2*A*). Animals receiving three consecutive injections of 2-DG displayed reduced splenocyte proliferation compared with control animals ($P < 0.05$). There were no significant differences in proliferation among animals receiving one or two injections of 2-DG ($P > 0.05$). There were no significant differences in splenocyte proliferation between 16:8-h L-D cycle animals receiving either one, two, or three injections of 2-DG across consecutive days ($P > 0.05$). Deer mice housed in short days and receiving injections of 2-DG did not differ from short-day control animals in splenocyte proliferation to Con A ($P > 0.05$, Fig. 2*B*). There were no significant differences in splenocyte proliferation among short-day experimental animals receiving one, two, or three injections of 2-DG ($P > 0.05$). Short-day animals receiving injections of 2-DG showed greater splenocyte proliferation compared with long-day animals injected with 2-DG ($P < 0.05$, Fig. 2).

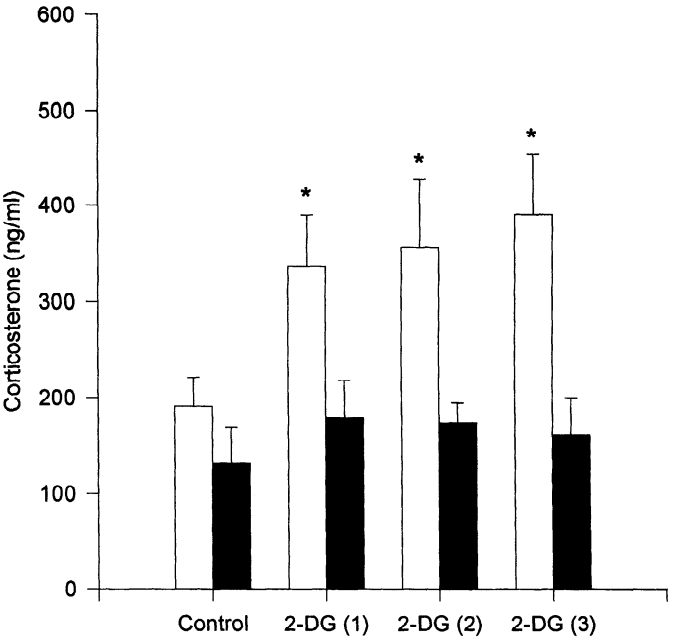

Fig. 1. Means ± SE serum corticosterone (ng/ml) in deer mice housed in long [16:8-h light-dark (L-D) cycle, open bars] or short (8:16-h L-D cycle, solid bars) days. *Statistically significant differences between means; numbers in parentheses correspond to the number of 2-deoxy-D-glucose (2-DG) injections animals received.

Short-day animals had smaller relative uterine masses compared with long-day animals ($P < 0.05$, Fig. 3). There were no differences in uterine mass between experimental and control animals in either photoperiodic condition ($P > 0.05$ in all cases). There were no significant differences in either initial or final body masses between animals receiving 2-DG or saline in long days (18.30 ± 0.37 vs. 18.22 ± 0.25 g, $P > 0.05$) or short days (18.03 ± 0.42 vs. 17.21 ± 0.19 g, $P > 0.05$). None of the deer mice maintained in short days showed any behavioral signs of daily torpor.

DISCUSSION

Short days buffer against metabolic stress in deer mice. Long-day mice injected with 2-DG had increased corticosterone levels, compared with long-day mice injected with saline. Corticosterone levels were not significantly elevated in short-day mice injected with 2-DG. 2-DG-treated long-day mice displayed reduced splenocyte proliferation to Con A compared with control mice. Splenocyte proliferation did not differ among short-day mice regardless of experimental treatment. Consistent with previous findings (i.e., short-day ani-

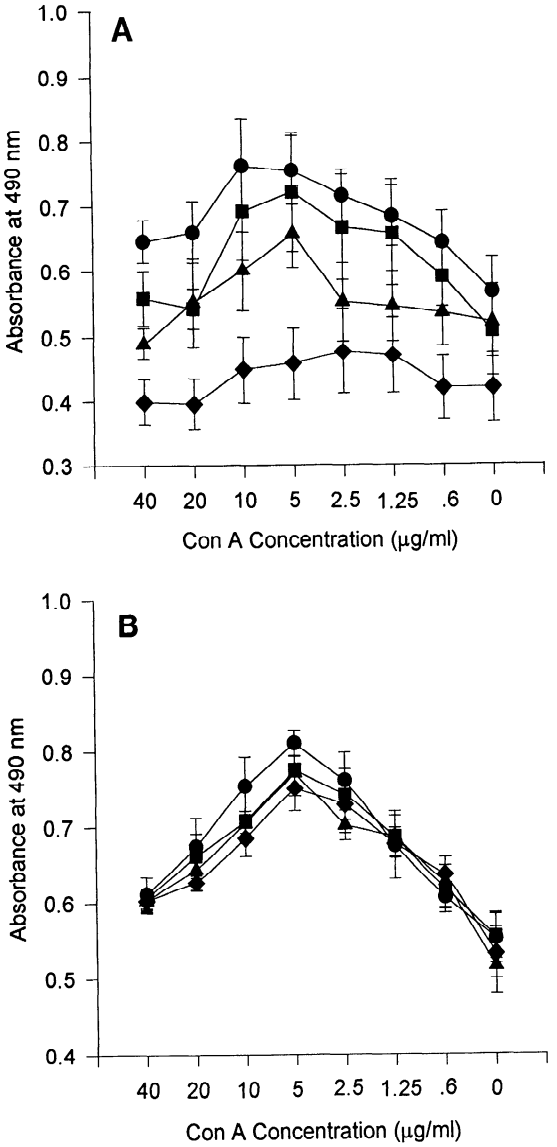

Fig. 2. Means ± SE splenocyte proliferation to concanavalin A (Con A, represented as absorbance units) of deer mice housed in long (*A*) or short (*B*) days (see Fig. 1 legend for day length definition). Higher absorbance values (nm) correspond to increased splenocyte proliferation in response to mitogenic stimulation. ●, Control; ■, 1 injection of 2-DG; ▲, 2 injections of 2-DG; ◆, 3 injections of 2-DG.

mals exhibited enhanced immune function), short-day mice treated with 2-DG displayed higher splenocyte proliferation than long-day mice treated with 2-DG. Thus enhanced splenocyte proliferation appears to compensate for stress-induced immune suppression.

Individuals require a balanced energy budget where energy intake equals energy expended to maximize survival and thus reproductive success (35). Animals must maintain a relatively constant flow of energy to the body despite potentially dramatic fluctuations in both energy supply and demand (32). Winter is stressful; low ambient temperatures and reduced food availability elevate glucocorticoid concentrations in laboratory-housed rodents (6, 23). The environmental stressors present in winter decrease energetic availability by reducing food availability while also increasing physiological energy expenditures through an increase in metabolic rate (7, 34). The net result is a state of energetic imbalance where energy intake is less than energy expended, unless adjustments are made. Mounting an immune response, however, requires energy (29; Demas and Nelson, unpublished data). The suite of cellular processes involved in the acute phase of an immune response (i.e., inflammation, fever, cytokine release) likely requires substantial energetic resources. In a state of energetic shortage, less energy is available for immunological defense and immunocompromise may result.

2-DG can mimic the effects of environmental stressors by inducing a state of glucoprivation and decreasing energetic availability without altering total food consumption in rodents (27). 2-DG acts as a metabolic stressor, increasing serum corticosterone levels (18) and inhibiting splenocyte proliferation in rats and mice (18, 21). High glucocorticoid levels compromise cellular immune function (3, 21). Adrenalectomy enhances both lymphatic organ masses and B cell activities (12). The mechanisms by which glucocorticoids affect specific immune responses have not been fully characterized but may involve changes in cytokine release rates or tissue responsiveness to cytokines, increased apoptotic activity among lymphocytes, and alterations in cell trafficking (e.g., Refs. 4 and 5). An alternative hypothesis is that photoperiodic differences in corticosterone secretion are due to changes in negative feedback of glucocorticoids. Reduced androgen levels in short-day rodent species result from increased responsiveness of the hypothalamic-pituitary-gonadal axis to the negative feedback effects of androgens (31). A similar mechanism may underlie photoperiodic changes in glucocorticoid secretion. Short days, leading to a prolonged duration of melatonin secretion, may increase the

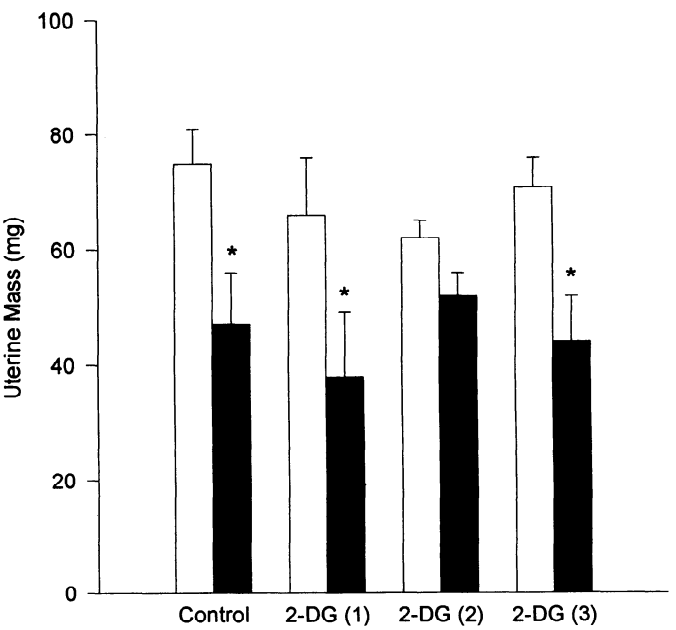

Fig. 3. Means ± SE uterine masses of deer mice housed in long (open bars) or short (solid bars) days (see Fig. 1 legend for day length definition). *Statistically significant differences.

general sensitivity of negative feedback to the HPA axis. Increased sensitivity of the HPA-negative feedback system in short days or after melatonin treatment can account for the reduced blood levels of corticosterone in short days. Reduced glucocorticoid secretion after melatonin treatment or administration of a melatonin agonist is consistent with this hypothesis (9, 20). Furthermore, exogenous melatonin treatment can enhance splenocyte proliferation in male deer mice (13). Whether the immunoenhancing effects of exogenous melatonin treatment are direct (e.g., binding to melatonin receptors on immune tissue) or indirect (e.g., via changes in circulating hormone levels) remains to be determined. Enhanced splenocyte proliferation is only one parameter of immune function. It is possible that other aspects of immunity (e.g., cytokine release, specific antibody production) may not be enhanced by short days. Future studies must be conducted to determine the extent to which photoperiod can affect other aspects of immune function.

Whatever the mechanism, maintenance in short days appears to protect individuals against 2-DG-induced metabolic stress. Reduced corticosterone levels in animals maintained in short days or treated with melatonin are likely due to improved metabolic functions (8). The present study indicates that exposure to short days under otherwise mild laboratory conditions bolsters immune function by reducing glucocorticoid levels. These results provide an adaptive functional hypothesis for the seemingly unconnected reports of immunoenhancing effects of melatonin (reviewed in Refs. 19 and 25). Improved immune function is one part of the complex web of winter coping strategies mediated by photoperiod.

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