Photoperiod, Ambient Temperature, and Food Availability Interact to Affect Reproductive and Immune Function in Adult Male Deer Mice (*Peromyscus maniculatus*)

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Abstract Winter is often stressful. Increased energetic demands in winter and concurrent reductions in energy availability can lead to an energetic imbalance and compromise survival. To increase the odds of surviving winter, individuals of some nontropical rodent species have evolved mechanisms to enhance immune function in advance of harsh winter conditions. Short day lengths provide a proximate cue for enhancement of immune function, an adaptive functional response to counter environmental stress-induced reduction in immune function. In the present study, photoperiod, ambient temperature, and food availability were manipulated and reproductive function and cell-mediated immunity were assessed in adult male deer mice (*Peromyscus maniculatus*). Mice maintained in short days regressed their reproductive systems and displayed enhanced immune function compared to long-day animals. Reduced food availability elevated corticosterone concentrations and suppressed reproductive and immune function, whereas ambient temperature alone had no effect on cell-mediated immunity. The suppressive effect of food restriction on reproductive and immune function was overcome by maintaining animals in short days. However, short-day, food-restricted mice maintained at low ambient temperatures displayed reduced reproductive and immune function compared to animals maintained at mild temperatures. Taken together, these results suggest that short-day enhancement of immune function can counteract some, but not all, of the immunosuppressive effects of winter stressors. These data are consistent with the hypothesis that immune function is enhanced in short days to counteract stress-mediated immune suppression occurring during winter.

Key words seasonality, rhythms, stress, immunity, spleen, corticosterone, adrenal

INTRODUCTION

Individuals of most temperate zone rodent species studied to date rely mainly on photoperiod (day length) as a precise temporal cue to estimate the time of year. Photoperiodic information is used to phase energetically expensive activities to coincide with adequate energy availability (Nelson et al., 1990). For

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example, breeding is energetically demanding, and breeding at inappropriate times of the year can compromise survival of both parents and offspring (Bronson, 1989). Consequently, individuals of many species have evolved to restrict breeding to specific seasons of the year when energy availability is high.

Previous studies of seasonal changes in mammalian physiology and behavior have placed considerable emphasis on seasonal fluctuations in reproduction and energetics (Bronson and Heideman, 1994; Wunder, 1992). Many studies have demonstrated striking seasonal patterns of mating and birth (Bronson and Heideman, 1994; Bronson, 1995). Although less extensively studied, there also exist salient cycles of infectious disease and death (John, 1994; Lochmiller et al., 1994; Nelson and Demas, 1996). Disease prevalence is typically increased during the fall and winter compared to spring and summer for individuals of many nontropical vertebrate species (John, 1994; Lochmiller et al., 1994). Many of these animals presumably become sick and die from exposure to extreme weather or starvation; however, many animals die from opportunistic diseases that seem to overwhelm immunological defenses, presumably at times when these defenses are compromised.

Several clinical studies have provided evidence for reduced immune function and increased death rates from infectious disease during the winter (Afoke et al., 1993; Boctor et al., 1989). Additionally, seasonal changes in immune parameters reported in field studies of nonhuman animals have typically reported reductions in lymphoid tissue mass and immune function during winter compared to summer (John, 1994; Lochmiller et al., 1994; Nelson and Demas, 1996). For example, reductions in white blood cells in cotton rats (*Sigmodon hispidus*) (Lochmiller et al., 1994) and splenic lymphoid tissue in European ground squirrels (*Citellus citellus*) (Shivatcheva and Hadjioloff, 1987) occur in winter compared to summer. Ground squirrels also display lower levels of hemagglutinins raised against sheep red blood cells in winter (Sidky et al., 1972). In short-tailed voles (*Microtus agrestis*), both splenic mass and splenic reticular cell counts are reduced in winter as compared to spring and summer (Newson, 1962).

Photoperiod appears to play a critical role in mediating seasonal changes in immune function. Several laboratory studies have reported photoperiodic changes in splenic mass in deer mice (*Peromyscus maniculatus*) (Vriend and Lauber, 1973), Syrian hamsters (*Mesocricetus auratus*) (Brainard et al., 1987; Brainard et al., 1988; Vaughan et al., 1987), and laboratory strains of rats (*Rattus norvegicus*) (Wurtman and Weisel, 1969). In contrast to field studies, virtually all laboratory studies of photoperiodic changes in immune parameters demonstrate enhanced immune function in short, winter-like photoperiods. For example, deer mice significantly increase lymphocyte, neutrophil, and white blood cell counts in short photoperiods (Blom et al., 1994). Increased lympho-proliferative activity and changes in spleen morphology occur in hamsters maintained on short day lengths (Champney and McMurray, 1991). Additionally, maintenance in short days can enhance both cell-mediated and humoral immunity in deer mice (Demas and Nelson, 1996; Demas et al., 1997; Demas and Nelson, 1998).

Recently, the interactive effects of photoperiod and ambient temperature on immunity were examined in deer mice (Demas and Nelson, 1996). Briefly, animals were maintained in long or short days and in mild or low ambient temperatures. Total serum immunoglobulin (Ig) G concentrations were elevated in short-day mice maintained at mild temperatures compared to long-day mice. However, long-day mice kept in low ambient temperatures had reduced IgG, whereas short-day mice maintained in low temperatures had IgG concentrations comparable to long-day animals maintained in mild ambient temperatures. Taken together, these results appear to resolve the discrepancy between field studies demonstrating reduced immune function in winter compared to summer and laboratory studies demonstrating enhanced immune function in short, winter-like photoperiods compared to long, summer-like day lengths. The net effect of elevated immune function in short days appears to be to counteract the suppressive effects of environmental stressors, such as low ambient temperatures, on immune function.

Although photoperiod is the primary cue for seasonally breeding animal species, many other environmental factors, including temperature, humidity, rainfall, and food availability, vary on a seasonal basis, and some of these factors may be perceived as stressful (Bronson, 1989; Sapolsky, 1992). The present experiment was designed to examine another potential seasonal stressor, reduced food availability, and its interaction with photoperiod and ambient temperature to affect reproductive and immune function in deer mice. In common with exposure to low ambient temperatures, reduced food availability evokes increased glucocorticoid secretion in mammals (Murphy and Wideman, 1992). Previous research (Demas and Nelson,

1996) examined total antibody concentrations; however, this measure does not necessarily reflect immune function per se because the immune system remains unchallenged. To assess immune function directly, splenocyte proliferation to the T-cell mitogen, concanavalin A (Con A), was measured in the present study. It was predicted that food restriction, in common with reduced ambient temperatures, acts as an environmental stressor leading to increased glucocorticoid secretion and immune suppression.

MATERIALS AND METHOD

Animals and Housing Conditions

Eighty adult (> 60 days of age) male deer mice (*Peromyscus maniculatus bairdii*) were obtained from our laboratory breeding colony. This colony is derived from animals from the Peromyscus Genetic Stock Center at the University of South Carolina. These animals are descendants of animals originally trapped near East Lansing, Michigan (latitude = 42° 51′ N).

Deer mice were weaned at 21 days of age and housed with same-sex siblings. Two weeks prior to the initiation of the experiment, all animals were individually housed in polypropylene cages in colony rooms under a cycle of 16 h light and 8 h dark (L16:D8; lights on at 0600 h EST). Relative humidity was maintained at 50 ± 5%. Food (Agway Prolab 1000, Syracuse, NY) and tap water were provided ad libitum throughout the experiment unless otherwise noted. Prior to the start of the experiment, animals (> 60 days) were weighed to the nearest 0.1 g daily for 2 weeks to establish baseline body mass. Food consumption was also assessed daily by weighing the food blocks remaining in the hopper, and an average daily food intake was determined for each animal.

Experimental Conditions

All of the experimental animals were randomly assigned to one of four experimental groups: 1) LD / 20°C animals (n = 20) were housed in a long-day photoperiod (L16:D8) with colony room temperature kept constant at 20 ± 1°C; 2) LD / 8°C animals (n = 20) were also housed in long days, but the ambient temperature of the colony room was maintained at 8 ± 1°C; 3) SD / 20°C animals (n = 20) were housed in a short-day photoperiod (L8:D16) with the colony room temperature set at 20 ± 1°C; and 4) SD / 8°C, animals (n = 20) were housed in short days, but in a room with temperature kept constant at 8 ± 1°C. Animals were maintained in their respective conditions for 6 weeks. At this time, animals in each of the four experimental groups were sub- divided further into food restricted groups or ad libidum fed groups. Food restricted animals received 70% of their original baseline food intake while ad libidum fed animals continued to have free access to food. Food restriction continued for 4 weeks.

Procedure

After 10 weeks, animals were brought into the surgery room one at a time and lightly anesthetized with methoxyflurane vapors (Metofane, Pitman-Moore, Mundelein, IL). Handling time was kept consistent and to a minimum; the time from initial removal from the cage to the end of bleeding was less than 3 min. Blood samples (500 µl) were drawn into capillary tubes via retro-orbital bleeding (Riley, 1960) between 1000 and 1200 h EST, a time when corticosterone values have been shown to be consistently low (Taymans et al., 1997). All samples were allowed to clot for 1 h. The clot was removed, and the samples were centrifuged (at 4°C) for 1 h at 3500 rpm. Serum aliquots were extracted and stored at –80°C until assayed.

After blood sampling, all animals were killed by cervical dislocation. Paired testes, epididymides, seminal vesicles, brown adipose tissue (BAT), and epididymal white adipose tissue (EWAT) were removed and cleaned of connective tissue. Spleens were removed under aseptic conditions and immediately suspended in culture medium (RPMI). Seminal vesicles were compressed with a glass vial to remove seminal fluid. All organs were weighed by laboratory assistants naive to the experimental hypotheses and treatment assignments. Tissue masses were corrected for total body mass, and both absolute and relative masses were used in subsequent statistical analyses.

Splenocyte Proliferation

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

[5000 U/ml]/streptomycin [5000 µl/ml], 1% L-glutamine [2 mM], 0.1% 2-mercaptoethanol [5 2×10^{-2} M], and 10% heat-inactivated fetal bovine serum). Splenocyte counts and viability were determined with a hemacytometer and trypan blue exclusion. Viable cells (which exceed 95%) were adjusted to 2 × 106 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 Co., St. Louis, MO) 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 was 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 37ºC with 5% CO_2 for 48 h prior to addition of 20 µl of MTS/phenazine methosulphate solution (0.92 mg/ml of PMS in sterile Dulbecco's phosphate-buffered saline; Promega, Madison, WI) per well. Plates were then incubated at 37ºC with 5% CO_2 for an additional 4 h. The optical density (OD) of each well was determined with a microplate reader (model 3550, Bio-Rad, Richmond, CA) 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 concentrations were assayed by radioimmunoassay (RIA) using the ICN Biomedicals Inc. (Costa Mesa, CA) 125I kit. The deer mouse dilution was prepared according to the guidelines furnished by ICN. The corticosterone assay was highly specific, cross-reacting at less than 3% with other steroid hormones. Serum corticosterone values were determined in a single RIA. The coefficients of variability were all below 10, and intra-assay variation was less than 1%.

Statistical Analyses

Reproductive organs and other tissue masses, as well as serum corticosterone concentrations, were analyzed using a 2 (photoperiod) × 2 (temperature) × 2 (food availability) between-subjects analysis of variance (ANOVA). Splenocyte proliferation was analyzed using a 2 (photoperiod) × 2 (temperature) × 2 (food availability) × 8 (mitogen concentration) mixed-model ANOVA. Significant interactions were probed using individual two-way ANOVAs, and all pairwise comparisons of mean differences were conducted using Tukey honestly significant difference comparisons. Differences between group means were considered statistically significant if $p < .05$.

RESULTS

Splenocyte proliferation was significantly elevated in mice maintained in mild ambient temperatures in short- compared to long-day deer mice averaged across feeding conditions ($p < .05$) (Fig. 1). Food-restricted animals demonstrated reduced splenocyte proliferation compared to ad libidum fed animals ($p < .05$) (Fig. 1). Food restriction reduced splenocyte proliferation in long- but not short-day mice housed in mild ambient temperatures. However, food restriction reduced splenocyte proliferation in both long- and short-day mice housed in low ambient temperatures ($p < .05$ in both cases) (Fig. 1).

Food restriction elevated corticosterone in short-day animals maintained in low but not mild ambient temperatures compared to ad libidum fed mice ($p < .05$) (Fig. 2). A similar trend was present in long-day animals; however, the results were not statistically significant ($p > .05$). Serum corticosterone concentrations were significantly lower in ad libidum fed mice maintained in low temperatures in short compared to long days ($p < .05$) (Fig. 2).

Food-restricted mice weighed significantly less than ad libidum fed animals ($p < .05$) (Fig. 3). Body mass was not significantly affected by either photoperiod or temperature ($p > .05$). Paired testes masses were smaller in short-day deer mice compared to long-day animals ($p < .05$) (Fig. 4). Similarly, paired testes were significantly smaller in short-day, food-restricted animals maintained in low temperatures relative to short-day animals maintained in mild temperatures ($p < .05$) (Fig. 4). Long-day, food-restricted animals had significantly smaller paired testes compared to ad libidum fed animals ($p < .05$ in both cases) (Fig. 4).

Paired epididymides were smaller in food- restricted compared to ad libidum fed mice maintained in low ambient temperatures in both long and short days ($p < .05$ in both cases) (Fig. 5). Paired epididymides were also significantly smaller in ad libidum fed mice maintained in low ambient tempera-

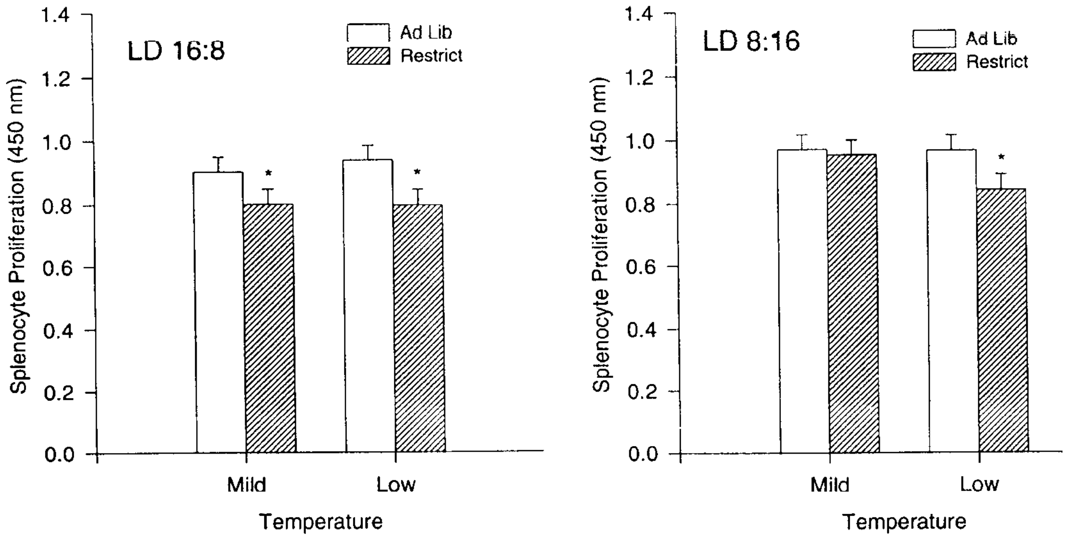

Figure 1. Mean (±SEM) splenocyte proliferation to concanavalin A (Con A) (μg/ml) of food-restricted or ad libidum fed male deer mice housed in long (L16:D8) or short (L8:D16) days and mild (20°C) or low (8°C) temperatures. Splenocyte proliferation is represented as absorbance units. Columns with no symbol or sharing the same symbol are statistically equivalent. Columns with different symbols are significantly different at $p < .05$. Only the optimal concentration of Con A (i.e., the concentration that stimulated the highest amount of cell proliferation) is graphically represented.

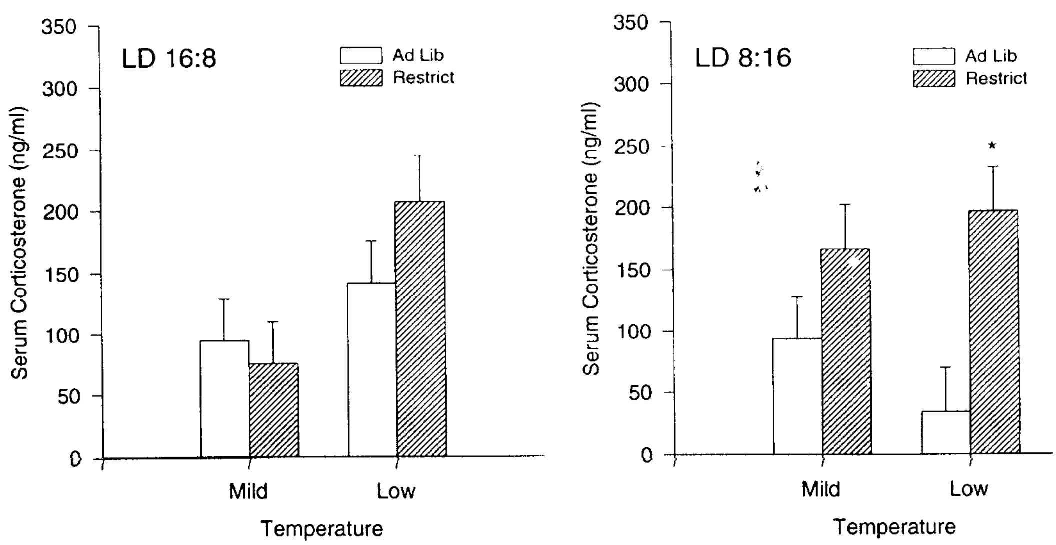

Figure 2. Mean (±SEM) corticosterone levels (ng/ml) of male deer mice housed in long (L16:D8) or short (L8:D16) days and mild (20°C) or low (8°C) temperatures. Splenocyte proliferation is represented as absorbance units. Columns with no symbol or sharing the same symbol are statistically equivalent. Columns with different symbols are significantly different at $p < .05$. Only the optimal concentration of concanavalin A (i.e., the concentration that stimulated the highest amount of cell proliferation) is graphically represented.

tures compared to ad libidum fed mice maintained in mild temperatures. EWAT was significantly reduced in long-day, food-restricted mice compared to long-day mice fed ad libidum ($p < .05$) (Table 1). Food-restricted mice had significantly less BAT than ad libidum fed animals when maintained in low ambient temperatures ($p < .05$) (Table 1). No other statistically significant differences were observed.

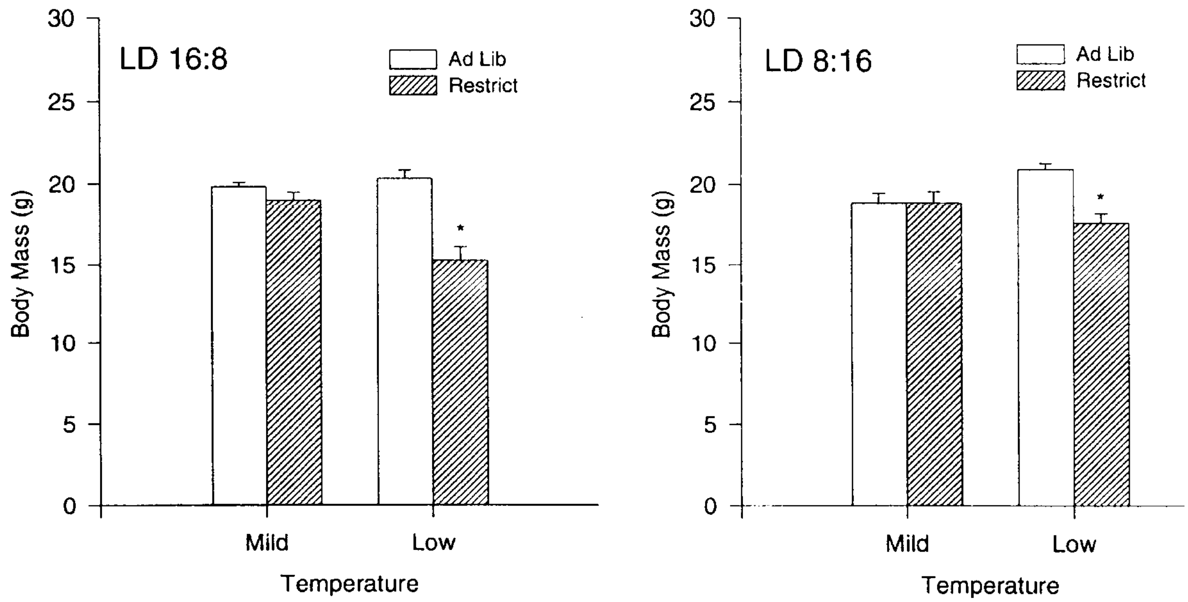

Figure 3. Mean (±SEM) body mass (g) of food-restricted or ad libidum fed male deer mice housed in long (L16:D8) or short (L8:D16) days and mild (20°C) or low (8°C) temperatures.

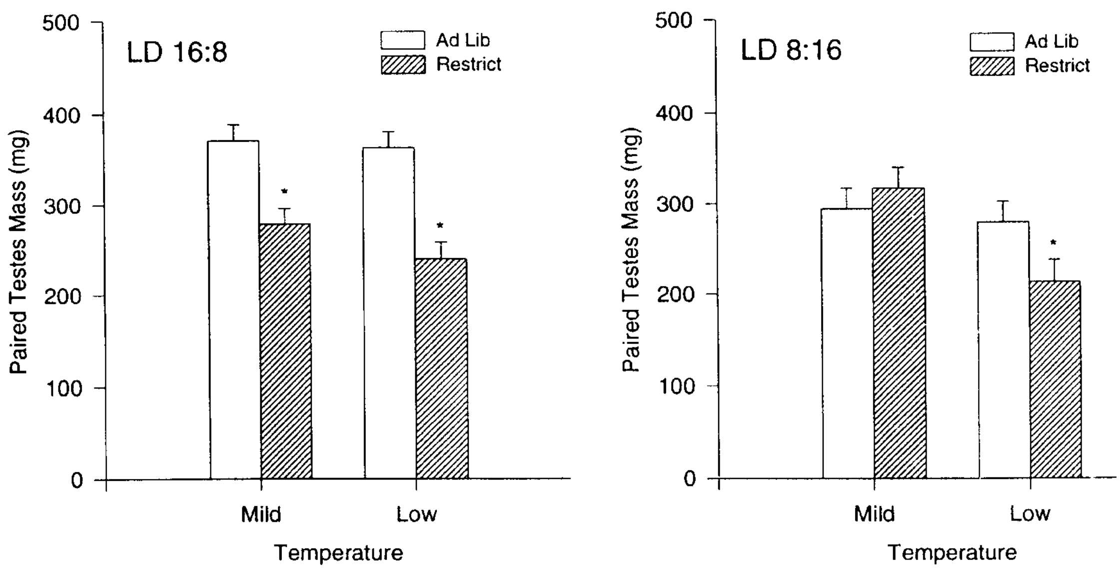

Figure 4. Mean (±SEM) paired testes mass (mg) of food-restricted or ad libidum fed male deer mice housed in long (L16:D8) or short (L8:D16) days and mild (20°C) or low (8°C) temperatures. Splenocyte proliferation is represented as absorbance units. Columns with no symbol or sharing the same symbol are statistically equivalent. Columns with different symbols are significantly different at *p* < .05. Only the optimal concentration of concanavalin A (i.e., the concentration that stimulated the highest amount of cell proliferation) is graphically represented.

DISCUSSION

In general, reproductive physiology was inhibited and immune function enhanced in male deer mice maintained in short compared to long days. The suppressive effects of food restriction on reproductive and immune function in long-day animals were overcome by maintaining animals in short days. Short-day, food-restricted mice maintained in low ambient temperatures demonstrated reduced reproductive and im-

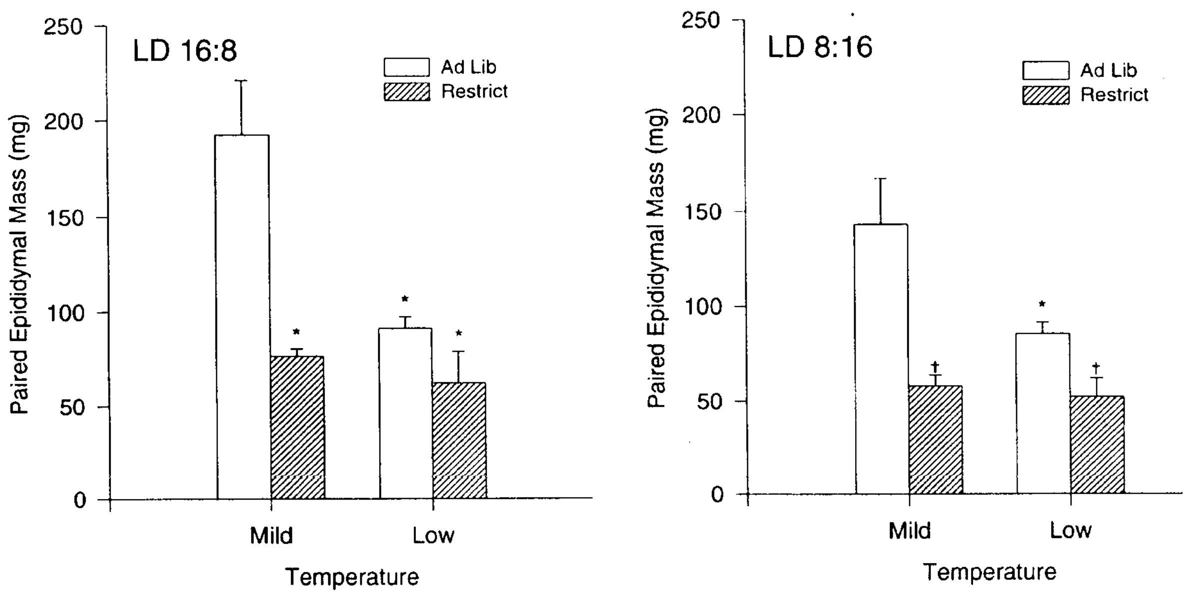

Figure 5. Mean (±SEM) paired epididymal mass (mg) of food-restricted or ad libidum fed male deer mice housed in long (L16:D8) or short (L8:D16) days and mild (20°C) or low (8°C) temperatures. Splenocyte proliferation is represented as absorbance units. Columns with no symbol or sharing the same symbol are statistically equivalent. Columns with different symbols are significantly different at *p* < .05. Only the optimal concentration of concanavalin A (i.e., the concentration that stimulated the highest amount of cell proliferation) is graphically represented.

Table 1. Mean (± SEM), reproductive organ and fat pad mass (mg) in ad libidum fed or food restricted deer mice housed in long (L16:D8) or short (L8:D16) days in mild (20ºC) or low (8ºC) ambient temperatures.

	Temperature	*Food*	*Seminal Vesicles*	*Epididymal White Adipose Tissue*	*Brown Adipose Tissue*
L16:D8	20ºC	Ad libidum	85.6 ± 8.0	128.8 ± 77.0	150.3 ± 11.0
	20ºC	Restricted	71.5 ± 25.0	67.4 ± 10.0	130.4 ± 25.0
	8ºC	Ad libidum	102.1 ± 38.0	140.8 ± 36.0	128.3 ± 6.0
	8ºC	Restricted	69.8 ± 10.0	60.0 ± 7.0	110.7 ± 12.0
L8:D16	20ºC	Ad libidum	21.4 ± 1.5*	80.4 ± 7.0*	154.4 ± 9.0
	20ºC	Restricted	57.7 ± 6.0	75.1 ± 23.0	147.6 ± 16.0
	8ºC	Ad libidum	47.4 ± 4.0	75.9 ± 6.0*	163.7 ± 8.0
	8ºC	Restricted	49.8 ± 9.0	71.7 ± 12.0	133.6 ± 14.0*

*indicates a statistically significant difference between photoperiodic conditions.

mune function compared to either ad libidum fed mice or food-restricted mice maintained in mild ambient temperatures. Taken together, these results suggest that food restriction acts as a stressor and suppresses immune function; maintenance of mice in short days can overcome the immunosuppressive effects of food restriction. Low ambient temperature alone did not affect immune function; however, when low temperature was combined with food restriction, immune function was compromised in short-day animals. Unlike the effect of food restriction alone, reduced reproductive and immune function in long-day, food-restricted mice maintained in low ambient temperatures was not counteracted by maintaining animals in short days. Corticosterone concentrations were generally elevated in food-restricted mice compared to ad libidum fed animals, supporting the hypothesis that reduced food availability acts as an environmental stressor. Also, maintaining animals in short days appeared to reduce corticosterone concentrations in ad libidum fed mice housed in low ambient temperatures. These results suggest that photoperiod, ambient temperature, and food availability interact to affect both reproductive and immune function in deer mice.

The degree of gonadal regression observed in the present study, although statistically significant, was uncharacteristically small for this species. The lack of full gonadal regression likely explains why short-day, food-restricted mice maintained in mild ambient temperatures did not have significantly smaller paired testes compared to long-day mice. Additionally, a small population of deer mice are reproductively nonresponsive to photoperiod (i.e., gonadal regression does not occur in short days). Although some of the short-day deer mice in the present experiment appeared to be reproductively nonresponsive to photoperiod, these animals were included in the statistical analyses in order to ensure sufficient statistical power. Also, no significant correlation existed between paired testes size and splenocyte proliferation, suggesting that enhanced immune function in short days may not be related to the degree of gonadal regression within *P. maniculatus* (cf. Demas et al., 1997).

Animals require a balanced energy budget in which energy availability equals energetic expenditures (Sheldon and Verhulst, 1996). Stressful conditions such as low ambient temperatures and reduced food availability present during winter elevate glucocorticoid concentrations (Bhatnagar et al., 1995; Murphy and Wideman, 1992). Winter environmental stressors decrease energy availability and simultaneously increase energy demands through an increase in metabolic rate (Wunder, 1984). Reproduction is energetically expensive, and both reproductive function and breeding are curtailed during times of reduced energy availability (Bronson, 1989; Wade and Schneider, 1992). Additionally, immunity can be energetically expensive (Demas et al., 1997; Henken and Brandsma, 1982); mounting an immune response requires using resources that could otherwise be allocated to other physiological processes. Thus, it is likely that immune function, like reproduction, is reduced during times of severe energetic shortage, especially winter.

The results of the present experiment suggest that food restriction, in addition to reduced ambient temperature (Demas and Nelson, 1996), can act as an environmental stressor and suppresses both reproductive and immune function in deer mice. Additionally, maintaining animals in short days can counteract some, but not all, of the stress-mediated suppression of reproduction and immunity. The present results appear to reconcile previous findings of suppressed immune function in field studies of seasonal changes in immunity (Lochmiller et al., 1994; Shivatcheva and Hadjioloff, 1987; Sidky et al., 1972) with short-day enhancement of immune function reported in laboratory studies of photoperiodic changes in immunity (Brainard et al., 1987; Vaughan et al., 1987; Vriend and Lauber, 1973). Although short photoperiods enhance immune function (Nelson and Demas, 1996), laboratory studies have typically been conducted with ad libitum access to food and water, mild ambient temperatures, reduced social interactions, and lack of predatory pressures. The present finding that food restriction reduces splenocyte proliferation suggests that several environmental factors mediate seasonal changes in reproductive and immune function in deer mice in the wild. These results demonstrate the importance of manipulating multiple variables when examining seasonal changes in physiological or behavioral processes. Traditionally, most laboratory studies of seasonality have relied solely on manipulations of photoperiod, and in many cases the results of these studies contradict the results of field studies. The present results suggest that a multifactorial approach to the study of seasonality within the laboratory can yield more ecologically relevant results, as well as reconcile some of the apparent discrepancies between laboratory and field data.

In contrast to our previous findings (Demas and Nelson, 1996), reduced ambient temperature alone did not impair immune function in the present experiment. This difference is likely due to differences in immunological assessment; total IgG production was assessed in the previous study, whereas lymphocyte proliferation to Con A was assessed in the present study. For example, it is possible that humoral immunity is more sensitive to changes in ambient temperature compared to cell-mediated immunity, although this possibility remains to be tested. However, reduced ambient temperatures did appear to affect immune function when combined with food restriction. These results provide support for an interactive effect of environmental factors on immune function.

In sum, the present data suggest an important physiological and adaptive functional role of photoperiod-mediated enhancement of immune function. The net effect of elevated immunity in short days appears to be to counteract the suppressive effects of environmental stressors such as low ambient temperatures or reduced food availability. Furthermore, the interaction between environmental factors and immunity must be considered to understand seasonal adaptations in the wild.

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