Leptin, but not immune function, is linked to reproductive responsiveness to photoperiod

DRAZEN, DEBORAH L., LANCE J. KRIEGSFELD, JILL E. SCHNEIDER, and RANDY J. NELSON. Leptin, but not immune function, is linked to reproductive responsiveness to photoperiod. Am J Physiol Regulatory Integrative Comp Physiol 278: R1401–R1407, 2000.—Energetic demands are high while energy availability is minimum during winter. To cope with this energetic bottleneck, animals exhibit numerous energy-conserving adaptations during winter, including changes in immune and reproductive functions. A majority of individual rodents within a population inhibits reproductive function (responders) as winter approaches. A substantial proportion of small rodents within a species, however, fails to inhibit reproduction (nonresponders) during winter in the field or in the laboratory when maintained in winter-simulated day lengths. In contrast, immune function is bolstered by short day lengths in some species. The specific mechanisms that link reproductive and immune functions remain unspecified. Leptin is a hormone produced by adipose tissue, and several studies suggest that leptin modulates reproductive and immune functions. The present study sought to determine if photoperiodic alterations in reproductive function and leptin concentrations are linked to photoperiod-modulated changes in immune function. Siberian hamsters (Phodopus sungorus) were housed in either long (LD 16:8) or short (LD 8:16) day lengths for 9 wk. After 9 wk, blood samples were collected during the middle of the light and dark phase to assess leptin concentrations. One week later, animals were injected with keyhole limpet hemocyanin to evaluate humoral immunity. Body mass, body fat content, and serum leptin concentrations were correlated with reproductive responsiveness to photoperiod; short-day animals with regressed gonads exhibited a reduction in these measures, whereas short-day nonresponders resembled long-day animals. In contrast, immune function was influenced by photoperiod but not reproductive status. Taken together, these data suggest that humoral immune function in Siberian hamsters is independent of photoperiod-mediated changes in leptin concentrations.

day length; diurnal rhythm; seasonal; immunoglobulin; adipose tissue

ANIMALS HAVE EVOLVED TEMPORAL strategies so that energetically expensive activities such as mating, mating, molting, and caring for offspring are separated in time. Seasonal breeding is part of a complex suite of adaptations that serves to maximize survival and reproductive success (10, 31, 40). Energy availability is generally reduced during winter when the energetic requirements for thermogenesis are high. Small animals cope with this energetic bottleneck by reducing activities that use energy during the winter but that are not critical for immediate survival (10). For example, locomotor activities, growth, or reproductive activities are often curtailed during energy shortages, and energy stores are used during winter for thermoregulation, immune function, and cellular maintenance (48). If energy shortages continue to deplete energy stores, then survival may be compromised. Thus trade-offs among competing energetic demands exist, and strategies for allocation of energy to competing needs vary according to an individual’s life history strategy, age, sex, and other extrinsic and intrinsic factors (10, 40).

Restricted food intake is a potent stressor that depresses immune function (34). Generally, immune function is compromised by winter conditions such as low temperatures and reduced food availability (28, 39). To compensate for these extrinsic immunosuppressant influences, many individuals appear to have evolved mechanisms to bolster immune function in response to the onset of autumnal short day lengths (4, 32, 38, 40, 49). Essentially, short days or melatonin treatment enhance humoral immune function above that observed in long days with otherwise optimal conditions. Indeed, the short-day enhancement of immunity can be suppressed to long-day levels if deer mice are challenged with low ambient temperatures or with restricted food availability (17). Siberian hamsters (Phodopus sungorus) both increase and decrease particular immune parameters in short days (49). Although melatonin appears to be involved in the allocation of energy from reproduction to immune functions (16), the specific mechanisms that link reproductive and immune functions remain unspecified. One potential candidate for this link is the hormone leptin.

Leptin is produced by adipose tissue, and circulating leptin concentrations are positively correlated with fat mass in a variety of mammals (3, 11, 12, 36, 41). In addition, fasting decreases circulating leptin concentra-
tions (2, 23, 37). Recently, a link was established between leptin and immune function; mice that are deficient in leptin (ob/ob) or in leptin receptors (db/db) are obese and also display impaired T cell immunity (34). Leptin has a specific effect on T lymphocyte responses, differentially regulating the proliferation of naive and memory T cells. Leptin also regulates tumor necrosis factor in proinflammatory immune responses (21, 22, 33, 46). Importantly, treatment with leptin counteracts the immunosuppressive effects of acute inflammation (34). Thus leptin is a likely candidate to mediate the interactions among energy allocation, immune function, and reproduction.

Many rodents undergo seasonal changes in body mass, primarily by altering the amount of body fat stored (30). The seasonal cycles of body mass (and of specific fat pad mass) fluctuations are regulated in these animals, for the most part, by day length (photoperiod) in addition to gonadal steroids (5–7, 47). Individuals either decrease or increase body mass before winter. Reduced body mass decreases absolute energy requirements, foraging time, and exposure to inclement conditions (13, 27). In contrast, autumnal fattening provides a readily available energy store and presumably provides insulation (15). The onset of short days triggers a decrease in body fat stores in Siberian hamsters that precedes a decrease in ad libitum food intake (47).

Most rodent species, including Siberian hamsters, exhibit variation in reproductive responses to photoperiod (24, 25, 38, 43). A majority of Siberian hamsters inhibits reproduction during the short days of winter or when maintained on short days in the laboratory (26, 48). A substantial minority of individuals in a population, however, does not respond to changes in photoperiod (25, 35, 38). These individuals are termed “nonresponders” (44).

The present study sought to determine whether humoral immunity changes with day length in Siberian hamsters. By comparing humoral immunity in responders to that of nonresponders, we were able to determine whether humoral immunity is affected by photoperiod independent of reproductive responsiveness to photoperiod. To our knowledge, serum leptin concentrations have not been measured in this species. Serum leptin concentrations were measured to determine whether changes in circulating concentrations of this protein were correlated with humoral immune function, reproductive responsiveness to photoperiod, or both. A positive correlation between serum leptin concentrations and humoral immunity would be consistent with the hypothesis that changes in serum leptin concentrations mediate photoperiod-induced changes in immune function.

**MATERIALS AND METHODS**

Forty-five adult (>60 days of age) male Siberian hamsters (Phodopus sungorus) were obtained from a colony maintained at the University of Connecticut. Hamsters were weaned at 21 days of age and housed with same-sex siblings. Two weeks before the onset of the experiment, all animals were individually housed in polypropylene cages (27.8 × 7.5 × 13 cm) in colony rooms. They were maintained on a light-dark cycle with 16 h of light and 8 h of darkness per day (LD 16:8; lights on at 0600 Eastern Standard Time). Rooms were maintained with an ambient temperature of 21 ± 2°C and relative humidity at 50 ± 5%. Food (Agway Prolab 1000, Syracuse, NY) and tap water were provided ad libitum throughout the study.

At the onset of the experiment, animals were weighed to the nearest 0.1 g to establish baseline body mass. Fifteen of the animals were randomly assigned to 11 wk of long photoperiod (LD 16:8), and 30 of the animals were assigned to 11 wk of short photoperiod (LD 8:16) (to ensure an adequate number of reproductive responders). To assess serum leptin concentrations, two blood samples were obtained from each animal. After 9 wk, animals were brought into the surgery room during the middle of the light period (~1300 for short-day animals, 1500 for long-day animals) one at a time and lightly anesthetized with methoxyflurane vapors (Metofane, Schering-Plough, Union, NJ). Blood samples (500 µl) were drawn into capillary tubes from the retroorbital sinus (45). Two days later, another blood sample was taken from each animal in the middle of the dark period (~2300 for short-day animals and 0100 for long-day animals). Samples were allowed to clot at 4°C for 30 min at 2,500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at ~80°C until assayed.

One week later, animals received a single subcutaneous injection of 100 µg of the novel antigen keyhole limpet hemocyanin (KLH) suspended in 0.1 ml sterile saline (day 0) 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 it does not make the animals ill (e.g., inflammation or fever) (20). Blood was drawn at three different sampling periods (days 5, 10, and 15 postimmunization). These dates were chosen to capture peak IgG production during the course of the immune response to KLH (20). On each sampling day, animals were brought into the surgery room individually, lightly anesthetized with methoxyflurane vapors, and blood samples (500 µl) were drawn from the retroorbital sinus between 1000 and 1200. Handling time was kept consistent and to a minimum; the time from initial removal from the cage to the end of bleeding was ~3 min. Samples were allowed to clot for 1 h; thereafter the clot was removed, and the samples were centrifuged at 4°C for 30 min at 2,500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at ~80°C until assayed. On the last day of sampling (day 15), animals were killed by cervical dislocation.

Paired testses and epididymides, seminal vesicles, epididymal white adipose tissue (EWAT), interscapular brown adipose tissue (IBAT), and spleens were removed and cleaned of connective tissue at autopsy. 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.

Humoral immunity. To assess humoral immunity, serum anti-KLH IgG concentrations were assayed using an ELISA. Microtiter plates were coated with antigen, incubated overnight at 4°C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH 9.6), washed with PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T; pH 7.4), then blocked with 5% nonfat dry milk in PBS-T overnight at 4°C to reduce nonspecific binding, and washed again with PBS-T. Thawed serum samples were diluted 1:20 with PBS-T, and 150 µl 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 levels 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 h, and then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG diluted 1:2,000 with PBS-T; Cappel, Durham, NC) was added to the wells, and the plates were sealed and incubated for 1 h at 37°C. Plates were washed again with PBS-T, and 150 µl of the enzyme substrate p-nitrophenyl phosphate (Sigma Chemical, St. Louis, MO: 1 mg/ml in diethanolamine substrate buffer) was added to each well. Plates were protected from light during the enzyme-substrate reaction that was terminated after 20 min by adding 50 µl 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) equipped with a 405-nm wavelength filter, and the average OD for each set of duplicate wells was calculated. To minimize intra-assay variability, the average OD for each sample was expressed as a percentage of its plate positive control OD for statistical analysis.

Serum leptin. Blood serum leptin concentrations were assayed by RIA using the Linco (St. Louis, MO) 


definitions Kit. The leptin assay was highly specific, cross-reacting at <1% with other hormones. Serum leptin values were determined in a single RIA. The coefficients of variation were all <10%, and intra-assay variation was <1%.

Statistical analyses. Serum anti-KLH IgG concentrations were analyzed using a 2 (reproductive condition) \times 2 (day) analysis of variance. Leptin concentrations were analyzed using a 2 (reproductive condition) \times 2 (time of day) analysis of variance. Reproductive organ weights and other tissue weights were analyzed using a one-way analysis of variance. All pairwise comparisons of mean differences were conducted using Tukey’s test (honestly significant difference) comparisons. Differences between group means were considered statistically significant if \textit{P} < 0.05.

RESULTS

Overall, reproductive organ masses were reduced in short-day compared with long-day Siberian hamsters (\textit{P} < 0.05 in all cases). Short-day hamsters had smaller testes (\textit{P} < 0.05), epididymides (\textit{P} < 0.05), seminal vesicles (\textit{P} < 0.05), and EWAT (\textit{P} < 0.05) than their long-day counterparts (Fig. 1; Table 1). Two distinct testicular responses were observed among short-day males: 1) testes mass was either indistinguishable from long-day males (\textit{P} > 0.05) ("nonresponders"; \textit{n} = 16) or 2) testes mass was <4 SE of long-day males ("responders"; \textit{n} = 14). This strategy for classifying male rodents on the basis of testes mass is similar to that used in past studies, and the methodology is validated as representative of reproductive function in many species, including Siberian hamsters (9, 19, 24).

Responders had significantly smaller testes (\textit{P} < 0.05), epididymides (\textit{P} < 0.05), seminal vesicles (\textit{P} < 0.05), body mass (\textit{P} < 0.05), and EWAT (\textit{P} < 0.05) than nonresponders in short days or hamsters housed in long days.

Table 1. Spleen and specific fat pad masses of hamsters held on short and long days

<table>
<thead>
<tr>
<th>Measure</th>
<th>LD 16:15 (15)</th>
<th>LD 8:16 (NR) (16)</th>
<th>LD 8:16 (R) (14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>4.95 ± 0.63</td>
<td>4.56 ± 0.31</td>
<td>6.16 ± 0.53</td>
</tr>
<tr>
<td>EWAT</td>
<td>15.64 ± 1.29</td>
<td>14.62 ± 1.07</td>
<td>7.07 ± 1.35*</td>
</tr>
<tr>
<td>IBAT</td>
<td>4.57 ± 0.33</td>
<td>5.10 ± 0.49</td>
<td>4.64 ± 0.21</td>
</tr>
</tbody>
</table>

Values are means ± SE in mg/g in epididymal white adipose tissue (EWAT) mass, interscapular brown adipose tissue (IBAT) mass, and spleen mass of male Siberian hamsters housed for 11 wk in long day lengths (LD 16:8) or hamsters maintained on short days (LD 8:16) that either responded (R) or did not respond (NR) to inhibitory day lengths with reproductive regression.* Significantly less than both long-day hamsters and short-day animals that did not undergo reproductive regression (\textit{P} < 0.05). LD, light-dark. Nos. of hamsters in parentheses.
long day lengths (Fig. 1; Table 1). Spleen mass and IBAT mass did not differ among responders and nonresponders housed in short or long-day animals (Table 1).

Immune responses to KLH injection were related to photoperiod but not the reproductive response to short days. Irrespective of reproductive condition in short days, serum anti-KLH IgG concentrations were significantly reduced in short-day hamsters compared with long-day animals ($P < 0.01$) (Fig. 2). Serum anti-KLH IgG concentrations were significantly elevated on days 10 and 15 compared with day 5 across both photoperiods ($P < 0.05$).

Leptin concentrations appeared to be related to body mass, EWAT mass, and reproductive response to photoperiod, but not to humoral immune responses. Leptin concentrations were significantly reduced in short-day responders compared with nonresponders housed in short days or long-day hamsters ($P < 0.05$) (Fig. 3). No diurnal variation in leptin concentrations was detected; leptin concentrations did not differ between night and day samples for any of the groups.

There was a significant positive correlation between serum leptin concentrations and EWAT mass ($r = 0.774; P < 0.0001$) and between serum leptin concentrations and body mass ($r = 0.545; P < 0.00001$) (Fig. 4). There was no significant correlation, however, between serum leptin concentration and humoral immune function ($r = 0.124$) (Fig. 4).

**DISCUSSION**

Humoral immunity was primarily linked to photoperiod and was not associated with reproductive responsiveness. Regardless of reproductive response to photoperiod, short-day hamsters exhibited reduced humoral immunity compared with long-day animals. Taken together, these data suggest that seasonal changes in body mass in Siberian hamsters are linked to reproductive status (and possibly gonadal steroid concentrations), whereas immune function is not linked to reproductive status (18). Thus photoperiod-mediated alterations in immune function are independent of circulating leptin concentrations, suggesting that leptin is not the signal responsible for communicating total energy availability for appropriate allocation of energy to immune function in this species.

As expected, Siberian hamsters exhibited a phenotypic polymorphism in reproductive responsiveness to short day lengths. Short day lengths also led to a reduction in body mass among animals that were reproductively responsive relative to long-day animals, whereas nonresponsive animals did not reduce body mass. Leptin concentrations were positively correlated with body mass and EWAT mass. Thus short-day responders had reduced leptin concentrations compared with long-day animals, whereas short-day nonresponders had leptin concentrations similar to long-day hamsters.

In the present study, regardless of photoperiod or reproductive conditions, hamsters did not exhibit diurnal variation in leptin concentrations. Recently, data obtained in rats and mice suggest that serum leptin concentrations and leptin mRNA content in adipose tissue are elevated during the dark phase relative to the light phase (1, 42). In contrast to many other nocturnal rodents (e.g., mice and rats), Siberian hamsters may eat at a relatively constant rate throughout the day and night (8). If this is the case, it is not surprising that there was no diurnal variation observed in serum leptin concentrations. In the present study, however, animals were bled once during the dark phase and once during the light phase. Conceivably, leptin concentrations may only exhibit transient variation throughout the light or dark cycle, and numerous
samples are required to observe diurnal variation in leptin. Future studies where leptin concentrations are sampled at numerous time points throughout the day and night are required to test this possibility.

The data from the present study confirm and extend previous findings of photoperiod and leptin (29). One previous study (29) indicates that either under natural photoperiods or artificial short day lengths, leptin gene expression is reduced in EWAT and IBAT during winter acclimatization or short photoperiods in Siberian hamsters. Klingenspor et al. (29) conclude that the decrease in food intake characteristic of winter-acclimatized and short-day Siberian hamsters is not attributable to elevated ob gene expression. However, they did not measure serum leptin concentrations and did not indicate whether all hamsters responded to winter or short day lengths with reproductive regression. The present study extends their results by 1) providing the first report of serum leptin concentrations in this species and 2) showing that serum leptin concentrations are linked to gonadal responsiveness to short day lengths. Additional studies are required to determine whether gonadal steroid hormones influence changes in leptin in short-day animals or if upstream components of the hypothalamo-pituitary-gonadal axis modulate seasonal alterations in leptin concentrations. There was a significant positive correlation between adiposity and leptin (Fig. 4). A similar correlation between adiposity and leptin is demonstrated in a variety of species (e.g., 3, 11) and does not necessarily imply a causal effect of leptin in mediating short-day responses to photoperiod. Changes in leptin might simply reflect energy intake and storage.

Perspectives

Numerous studies to date suggest that leptin influences immune function (11, 21–23, 33, 41, 46). In general, reductions in leptin cause immunosuppression, whereas leptin administration enhances immune function. Because leptin provides a long-term indicator of total stored energy availability and leptin influences immune function, we speculated that leptin might be the signal by which animals evaluate available energy to be allocated to immune function. In the present study, however, humoral immune function appeared to be independent of leptin concentrations. The effects of hormones on physiology and behavior often involve a complex interaction among receptor numbers/affinity for the ligand as well as levels of binding proteins. Thus additional studies are necessary to determine whether or not immune function in responsive and nonresponsive hamsters is influenced by different leptin concentrations to the same extent because of alterations in leptin receptors or binding proteins. Given the present data, the most parsimonious conclusion is that serum leptin concentrations do not mediate photoperiod-induced changes in immune function.

The results of the present study also suggest that seasonal alterations in immune function are independent of photoperiod-mediated changes in melatonin concentrations. Siberian hamsters that regress their reproductive system in response to short days exhibit the typical, long-duration melatonin pattern characteristic of animals housed in short day lengths (14). In contrast, nonresponsive Siberian hamsters exhibit a long-day pattern of melatonin secretion (i.e., short duration) when housed in either long or short days (44).
Because responders and nonresponders held on short days exhibit different patterns of melatonin secretion, immune changes associated with exposure to short day lengths in Siberian hamsters are unlikely to be the result of direct actions of melatonin on the immune system.

The results of the present study suggest that photoperiod influences leptin concentrations differentially; these changes in leptin concentrations are linked to reproductive responsiveness to short day lengths. In contrast, humoral immune function is influenced by photoperiod independent of leptin concentrations. Additional studies are necessary to determine if different types of immunity (e.g., cellular immunity) are affected by photoperiod independent of leptin concentrations/ reproductive responsiveness. In addition, the testicular dependency of the immune response to day length was not evaluated in the present study; future studies using castration and testosterone replacement are necessary to dissociate these factors.

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