J Comp Physiol B (2003) 173: 171–176
DOI 10.1007/s00360-002-0325-5

ORIGINAL PAPER

G.E. Demas · R.J. Nelson

Lack of immunological responsiveness to photoperiod in a tropical rodent, *Peromyscus aztecus hylocetes*

Accepted: 12 December 2002 / Published online: 12 February 2003

Abstract Non-tropical rodents undergo seasonal changes in immune function and disease. It has been hypothesized that seasonal fluctuations in immunity of non-tropical rodents are due to suppressed immune function during harsh winter conditions. A logical extension of this hypothesis is that seasonal changes in immunity should be reduced or absent in tropical rodents that do not experience marked seasonal fluctuations in environmental conditions; however this hypothesis remains to be tested. The present study tested the effects of photoperiod on humoral and cell-mediated immune function of male Aztec mice (*Peromyscus aztecus hylocetes*). *P. a. hylocetes* were housed in long (L:D 16:8) or short days (L:D 8:16) for 10 weeks. Animals were then immunized with the antigen keyhole limpet hemocyanin (KLH). Serum anti-KLH immunoglobulin G (IgG) concentrations and splenocyte proliferation in response to the T-cell mitogen Concanavalin A were assessed. Short-day *P. a. hylocetes* did not display differences in reproductive or immune measures compared with long-day mice. Collectively, these results suggest that *P. a. hylocetes* are reproductively and immunologically non-responsive to photoperiod. This lack of immunological responsiveness is likely due to the relative seasonal stability of their environment compared with temperate zone species.

Keywords Immune · Spleen · Antibodies · Seasonal · Opportunistic

Communicated by L.C.-H. Wang

G.E. Demas (✉)
Center for the Integrative Study of Animal Behavior,
Department of Biology and Program in Neural Science,
Indiana University, 1001 East 3rd Street,
Bloomington, IN, 47405, USA
E-mail: gdemas@bio.indiana.edu
Tel.: +1-812-8560158
Fax: +1-812-8556705

R.J. Nelson
Departments of Psychology and Neuroscience, Ohio State University, Columbus, OH, 43210, USA

Abbreviations IgG immunoglobulin G · KLH keyhole limpet hemocyanin · OD optical density · PBS-T phosphate buffered saline containing 0.05% Tween 20 · MTS 3-(4,5-demethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Introduction

In addition to marked seasonal changes in reproductive, energetic, and other physiological processes, many non-tropical mammalian species undergo seasonal changes in immune function and disease (Mann et al. 2000; Nelson and Demas 1996; Nelson et al. 2002; Zapata et al. 1992). Field studies of seasonal changes in immunity typically report reduced immune function and increased disease susceptibility during winter compared with spring and summer (Afoke et al. 1993; John 1994; Lochmiller and Deerenberg 2000; Lochmiller et al. 1994; Sinclair and Lochmiller 2000). In addition, laboratory studies have documented photoperiodic changes in immune function for several non-tropical rodent species, including deer mice (*Peromyscus maniculatus*) (Demas and Nelson 1996), prairie voles (*Microtus ochrogaster*) (Nelson et al. 1996), as well as Syrian (*Mesocricetus auratus*) (Brainard et al. 1987; Drazen et al. 2002) and Siberian (*Phodopus sungorus*) (Drazen et al. 2000; Yellon et al. 1999) hamsters. In contrast to field reports, some species (e.g., deer mice, Syrian hamsters), display *enhanced* immune function in the laboratory when exposed to short "winter-like" day lengths compared with long "summer-like" days (Brainard et al. 1987; Demas and Nelson 1996). In other species (e.g., Siberian hamsters, prairie voles), some components of immune function are suppressed in short days (Drazen et al. 2000; Yellon et al. 1999), in accordance with the majority of field studies, whereas other components are elevated (Yellon et al. 1999).

It is currently not known why rodent species differ in their immune responses to short days in the laboratory, but these differences are likely due to differences in the

environments in which these animals evolved. For example, it has been hypothesized that seasonal fluctuations in immune function in non-tropical rodents are due, at least in part, to suppressed immune function during the harsh environmental conditions of winter (Demas and Nelson 1996; Nelson and Demas 1996). Specifically, high thermoregulatory requirements for small mammals during winter coincide with low environmental food availability; this energetic "bottleneck" has led to the evolution of specific adaptations that allow individuals to cope with winter (Bronson and Heideman 1994). Among these adaptations, non-tropical animals may have evolved photoperiodic changes in immune function to counteract immune suppression during harsh winter conditions (Nelson and Demas 1996). Recent evidence supports this idea (Demas and Nelson 1996, 1998a; Lochmiller and Deerenberg 2000; Sinclair and Lochmiller 2000). For example, deer mice display enhanced immune function in short compared with long days and this enhanced immunity appears to counteract immune suppression caused by low ambient temperatures or reduced food availability (Demas and Nelson 1996, 1998a). A logical extension of this hypothesis is that photoperiodic changes in immunity should be reduced or absent in rodent species that do not experience marked seasonal fluctuations in their environment. Consistent with this idea, some non-tropical populations of the genus *Peromyscus* inhabiting low latitudes (e.g., Georgia, Texas) of the temperate zone demonstrate year-round breeding due to suitable environmental conditions (Carlson et al. 1989; Demas et al. 1997) and these animals also fail to demonstrate photoperiodic changes in immunity (Demas et al. 1997). In contrast, individuals of *Peromyscus* species inhabiting relatively high latitudes (e.g., Connecticut), where environmental fluctuations can be more pronounced, display marked photoperiodic changes in immunity (Demas et al. 1997).

Many tropical rodents display seasonal changes in population densities which are due, at least in part, to changes in disease susceptibility (Vázquez et al. 2000). Surprisingly, virtually nothing is known about the effects of ambient photoperiod on immune function of tropical rodents. Several studies, however, have demonstrated that tropical mammals are generally *reproductively* non-responsive to photoperiod (e.g., Bronson and Heideman 1992; Demas and Nelson 1998b; Heideman et al. 1992). Thus, the goal of the present study was to examine photoperiodic changes in humoral and cell-mediated immunity of at least one tropical rodent species, *Peromyscus aztecus hylocetes*, which inhabit the humid, mountainous regions of southeastern Mexico through the highlands of Guatemala and to Honduras and Northern El Salvador (Carleton 1989; Sullivan et al. 1997). Annual temperature is relatively stable in these regions, although there can be pronounced seasonal variation in rainfall (Vázquez et al. 2000). In addition, population densities vary seasonally in this species (Vázquez et al. 2000), likely due to changes in disease prevalence and/or susceptibility. Although some *Peromyscus* species inhabiting relatively low latitudes may cease reproductive activities during the winter in the field (Findley 1987), the results of a recent laboratory study suggest that reproductive function of *P. a. hylocetes* appears more responsive to social cues rather than photoperiod (Demas and Nelson 1998b). Consistent with these results, we predicted that *P. a. hylocetes* would also be *immunologically* non-responsive to photoperiod due, in part, to the presence of relatively mild year-round conditions in this species' natural environment. To test this hypothesis, mice were maintained on long or short days for 10 weeks and then we measured antibody concentrations in response to an antigenic challenge and lymphocyte proliferation in response to a mitogenic challenge.

Materials and methods

Animals and housing conditions

Twenty adult (>60 days of age) male Aztec mice (*P. a. hylocetes*) were obtained from the *Peromyscus* Genetic Stock Center at the University of South Carolina. These animals are descendants of animals originally trapped on Sierra Chincua near Michoacan, Mexico (latitude = 20.0°N) and breeding pairs were housed in a L:D 16:8 photoperiod with an ambient temperature of 22 °C and ~50% humidity. Upon arrival in our laboratory, animals were group-housed as four animals per cage. Two weeks prior to the initiation of the experiments, animals were housed individually in polypropylene cages (27.8×7.5×13.0 cm) in colony rooms with a 24-h L:D 16:8 cycle (lights on 0300 hours EST). Temperature wase kept constant at 22 °C and relative humidity was maintained at 50 ± 5%. Food (Prolab 2000) and tap water were available ad libitum throughout the experiment.

Animals were randomly selected and assigned to one of two photoperiodic conditions. Half of the animals ($n = 10$) were transferred to a short-day photoperiod (L:D 8:16), whereas the remaining animals ($n = 10$) were maintained under long days (L:D 16:8). The animals were weighed after group assignment to ensure that animals in both groups had comparable body mass at the start of the experiment. Animals were maintained in their respective photoperiods for 10 weeks. At this time, all mice received a single subcutaneous injection of 100 μg of the 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 does not make the animals sick (e.g., inflammation or fever; Dixon et al. 1966). All mice were naïve to KLH (i.e., they lacked measurable antibodies to KLH) and therefore we tested a primary antibody response to this novel antigen. Blood was drawn from the retro-orbital sinus at two different sampling periods (day 5 and day 10 post-immunization). These sampling periods were chosen in order to capture peak immunoglobulin G (IgG) production during the course of the immune response to KLH (Demas et al. 1997). On each sampling day, animals were brought into the surgery room individually, lightly anesthetized with methoxyflurane vapors (Metofane, Mundelein, Ill.), and blood samples (500 μl) were drawn from the retro-orbital sinus between 1000 hours and 1200 hours EST. Samples were allowed to clot for 1 h, the clots were removed, and the samples were centrifuged (at 4 °C) for 30 min at 2,500 rpm. Serum aliquots were aspirated and stored in microcentrifuge tubes at −80 °C until assayed for IgG. On the last day of sampling (day 10) animals were killed by cervical dislocation. Spleens were removed under aseptic

conditions and immediately suspended in culture medium (RPMI-1640/Hepes). Paired testes and epididymides also were removed and cleaned of connective tissue at necropsy. All tissue was weighed to the nearest 0.1 mg by laboratory assistants naïve to the experimental hypotheses and treatment assignments. Both anti-KLH IgG and splenocyte proliferation were assessed in the same set of animals; we have previously demonstrated that injections with KLH and blood sampling does not affect subsequent splenocyte proliferation in *Peromyscus* species (G.E. Demas, R.J. Nelson, unpublished data).

IgG enzyme-linked immunosorbant assay

Humoral immunity was assessed by measuring serum anti-KLH IgG concentrations using an enzyme-linked immunosorbant assay (ELISA) via the method of Drazen et al. (2000). Briefly, 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 phosphate buffered saline (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 300 µl of each serum dilution was added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled sera from mice previously determined to have high levels of anti-KLH antibody, similarly diluted with PBS-T) and negative control samples (pooled sera from KLH-naive mice, similarly diluted with PBS-T) were also added in duplicate to each plate; plates were sealed, incubated at 37 °C for 3 h, then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated-anti-mouse IgG diluted 1:100 with PBS-T; Cappel, Durham, N.C.) was added to the wells, and the plates sealed and incubated for 1 h at 37 °C. Plates were washed again with PBS-T and 150 µl enzyme substrate p-nitrophenyl phosphate (Sigma Chemicals, 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, which was terminated after 20 min by adding 50 µl 1.5 M NaOH to each well. The optical density (OD) of each well was determined using a plate reader (Bio-Rad Benchmark; Richmond, Calif.) equipped with a 405-nm wavelength filter, and the mean OD for each set of duplicate wells were 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. Intra-assay variability for the positive control wells was 4.5%.

Splenocyte proliferation

Splenocyte proliferation in response to the T-cell mitogen, Concanavalin A (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; Cory et al. 1991). Splenocytes were separated from tissue by compressing the whole spleen between sterile frosted glass slides; separated cells were suspended in 4 ml culture medium RPMI-1640/Hepes supplemented with 1% penicillin (5000 U/ml)/streptomycin (5000 µl/ml), 1% L-glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5×10^{-2}M/ml), and 10% heat-inactivated fetal bovine serum). Splenocyte counts and viability were determined with a hemacytometer using 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 Chemicals, St. Louis, Mo.) was diluted with culture medium to concentrations of 40, 20, 10, 5, 2.5, 1.25, and 0.6 µg/ml; 50 µl 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 37 °C with 5% CO_2 for 48 h prior to addition of 20 µl MTS/PMS solution [Promega; 0.92 mg/ml phenazine methosulfate (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 OD of each well was determined with a microplate reader (Bio-Rad: Model no. 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.

Table 1 Mean (±SEM) body mass, paired testes mass and epididymal mass of adult male *Peromyscus aztecus hylocetes* housed on either long (L:D 16:8) or short days (L:D 8:16) for 10 weeks

	Body mass (g)	Paired testes mass (mg)	Epididymal mass (mg)
Long days	40.90 ± 1.44	328.0 ± 33.0	100.3 ± 16.0
Short days	44.37 ± 1.42	369.0 ± 49.0	137.5 ± 20.0

Statistical analyses

An a priori power analysis on previous data in this species allowed us to determine that $n = 20$ (10/group) was required in order to achieve sufficient statistical power (i.e., ≥80%) to test our experimental hypothesis. A larger number of animals were not used in the present experiment due to the limited availability of this species at the *Peromyscus* Stock Center. Reproductive and IgG data were analyzed using independent student's *t*-tests (Sigma Stat, Jandel Scientific, San Rafael, Calif.). Splenocyte proliferation was analyzed using a two-way (photoperiod×Con A concentration) mixed model analysis of variance (ANOVA). Differences between group means were considered statistically significant if $P < 0.05$.

Results and discussion

Photoperiod had no effect on body mass, paired testes mass, or epididymal mass ($P > 0.05$ in all cases; Table 1). In addition, photoperiod had no effect on serum anti-KLH IgG production (Fig. 1) or T cell proliferation in response to Con A ($P > 0.05$; Fig. 2). Con A, however, did elicit a typical dose-dependent effect on proliferation, with optimal concentrations (e.g., 2.5 µg/ml) causing greater absorbance values compared with either sub-optimal (e.g., 0.625 µg/ml) or super-optimal (e.g., 40 µg/ml) Con A concentrations (Fig. 2).

The results of the present study suggest that photoperiod did not affect either humoral or cell-mediated immunity in *P. a. hylocetes*. These results provide the first report of immune function in *P. a. hylocetes* and support the hypothesis that photoperiodic changes in immunity are blunted or absent in at least one tropical rodent species. It is not possible to determine from the present data, however, whether the observed lack of photoperiodic responsiveness is due to a failure of individuals to evolve such a response or due to a secondary loss of this response. At present, there is insufficient phylogenetic information within the genus *Peromyscus* to separate these alternative hypotheses. Because interactions between the endocrine and immune

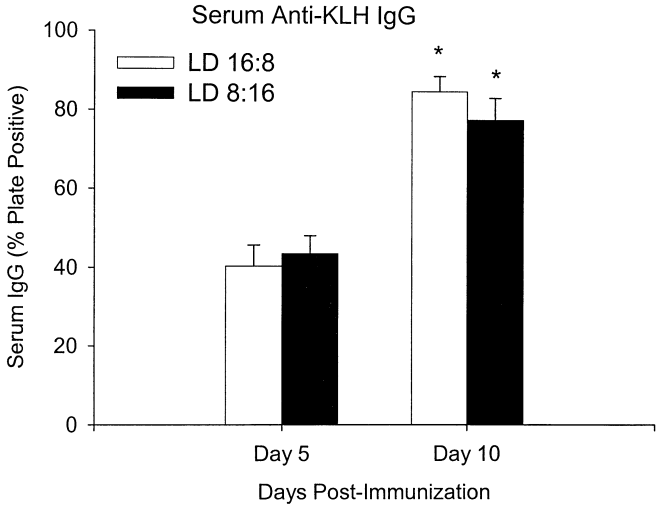

Fig. 1 Mean (±SEM) serum immunoglobulin G (*IgG*) concentrations expressed as a percentage of the plate positive control absorbance values (% plate positive) of adult male *P. a. hylocetes* housed in long days (L:D 16:8) or short days (L:D 8:16) for 10 weeks. Statistical differences in pairwise means are indicated by an *asterisk*

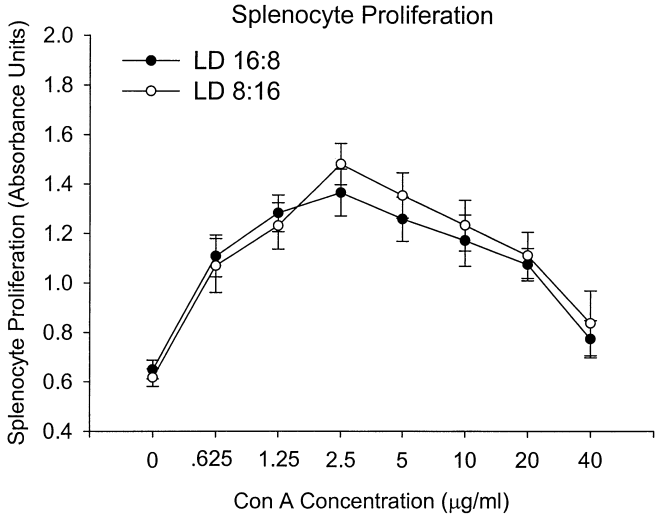

Fig. 2 Mean (±SEM) splenocyte proliferation (represented as absorbance units) in response to different concentrations of the T-cell mitogen Con A of adult male *P. a. hylocetes* housed in long (L:D 16:8) or short (L:D 8:16) days for 10 weeks and treated with varying concentrations of Con A. Higher absorbance values (nm) are indicative of increased splenocyte proliferation in response to mitogenic challenge

systems have become increasingly well-documented and because photoperiodic changes in reproductive hormones can affect immunity, the lack of a photoperiodic effect on immunity in *P. a. hylocetes* is consistent with the lack of a reproductive responsiveness to photoperiod reported previously (Demas and Nelson 1998b).

Many non-tropical rodents experience marked changes in environmental conditions that fluctuate on a seasonal basis (e.g., day length, ambient temperature, rainfall). Because increased thermoregulatory requirements in winter typically coincide with reduced food availability, individuals of many small rodents have evolved physiological adaptations to cope with winter. Among such strategies, some rodents display seasonal changes in immune function that appear to be driven by changes in the ambient photoperiod (Brainard et al. 1987; Nelson and Demas 1996; Yellon et al. 1999). Specifically, individuals of some non-tropical rodent species have evolved to enhance immunity in short days, in advance of harsh winter conditions. It has been suggested that short-day enhancement of immunity has evolved to counteract winter stress-induced immunosuppression (Nelson and Demas 1996; Demas and Nelson 1998a). In contrast, tropical rodents are less likely to experience marked fluctuations in environmental resources compared with non-tropical animals. Because tropical rodents are exposed to relatively mild environmental conditions throughout the year, we predicted that individuals of these species would be less likely to have evolved seasonal/photoperiodic changes in immunity. The present results support this prediction.

Although changes in photoperiod did not affect immune function in *P. a. hylocetes*, it is important to note that *seasonal* changes in immunity were not examined in the present study. One interesting possibility is that *P. a. hylocetes* experiences seasonal fluctuations in the field, but unlike temperate zone rodents, these fluctuations are not entrained by the ambient photoperiod. Although the majority of non-tropical rodent species rely on photoperiod as the primary proximate cue to entrain seasonal breeding, many tropical species use an opportunistic reproductive strategy according to which breeding occurs in the presence of optimal climate and environmental conditions, regardless of the time of year (e.g., Bronson and Heideman 1992; Heideman et al. 1992). Similarly, *P. a. hylocetes* may display seasonal fluctuations in immune function independent of the influences of photoperiod. For example, some tropical mammalian species (e.g., *Anura geoffroyi*) undergo seasonal changes in testes mass in the laboratory *independent* of photoperiod (Heideman et al. 1992). *P. a. hylocetes* examined in the present study were maintained under standard laboratory conditions with ad libitum food and water, and mild ambient temperatures. It is possible that these controlled conditions masked any immunological responsiveness to photoperiod that may be present in the field. For example, individuals of *P. californicus* display seasonal patterns in reproductive function in the field, but fail to respond to photoperiod in a laboratory setting (Nelson et al. 1995). In contrast, California voles (*Microtus californicus*) display seasonal patterns of breeding with reproductive function inhibited during the dry summer months, and constrained to November through May when rain and green vegetation are plentiful (Nelson et al. 1983). In the laboratory, however, California voles inhibit reproductive function in short as compared with long days. Thus, some other environmental factors (e.g., temperature, food availability) must regulate breeding in the field because if they used photoperiod to time reproduction, they would be

reproductively competent 180 degrees out of phase with their optimal breeding season. The effects of these different seasonal factors on immune function remain unspecified.

It is possible that subtle photoperiodic changes in reproduction and immune function may occur in *P. a. hylocetes*, but the effects are too small to be detected with the number of animals used in the present experiment, but could be detected with larger sample sizes. This is not likely, at least for reproductive parameters, given that the present results are consistent with previous studies in this species (Demas and Nelson 1998b). Even if this was true, however, it is reasonable to assume that such small effects (especially those for immune function) would represent statistically, but not *biologically* significant changes in physiology. Thus, a more plausible explanation for the present results and one consistent with previous research is that photoperiod exerts little or no effect on reproductive or immune function in *P. a. hylocetes*. One last possibility is that photoperiodic responsiveness may have been lost due to unintentional selection against this characteristic at the *Peromyscus* Stock Center. This possibility is not likely, given that other traditionally photoperiodic species within the genus *Peromyscus* have retained photoperiodic responsiveness despite ~20–50 years of captive breeding.

Taken together, the present results suggest that individual male *P. a. hylocetes* do not appear to undergo photoperiodic changes in humoral or cell-mediated immunity, at least in controlled laboratory settings. The results are consistent with the hypothesis that tropical rodents may be immunologically non-responsive to photoperiod because individuals of these species do not experience the relatively extreme fluctuations in environmental conditions that likely drive selection for photoperiodic changes in immunity of temperate zone rodents. In addition, these results provide important information regarding the effects of environmental factors on changes in immunity in tropical rodents. Future studies will be required to determine to what extent the present laboratory results generalize to seasonal changes in physiology in more natural field settings.

Acknowledgements The authors acknowledge Brooke Buckley, Joe Casto, Joyce Hairston, Violette Renard, and Sue Yang for technical assistance, and Ed Silverman for expert animal care. We also thank Dr. Wallace Dawson and Janet Crossland from the *Peromyscus* Genetic Stock Center at the University of South Carolina for providing us with mice and information about the source population. This research was supported by USPHS awards NS 10596 to GED and MH 57535 to RJN. The research with animals conforms to federal NIH and USDA regulations, and was conducted with approval of our local Institutional Animal Care and Use Committee (IACUC).

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