J Comp Physiol A (1996) 179: 819–825

ORIGINAL PAPER

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Reproductive and immune responses to photoperiod and melatonin are linked in *Peromyscus* subspecies

Accepted: 5 July 1996

Abstract The effects of photoperiod and melatonin treatment on reproductive and immune function were assessed in two subspecies of *Peromyscus maniculatus* from different latitudes of origin. In experiment 1, *P. m. bairidii* (latitude = 42°51′ N) and *P. m. luteus* (latitude = 30°37′ N) were housed in either long (LD 16:8) or short days (LD 8:16) for 8 weeks. Short-day *P. m. bairdii* displayed reproductive regression and elevated splenocyte proliferation in response to the T-cell mitogen concanavalin A, as compared to long-day mice. In contrast, *P. m. luteus* did not undergo reproductive regression or exhibit any increase in lymphocyte proliferation in short days. In experiment 2, individuals of both *P. m. bairdii* and *P. m. luteus* were implanted with empty capsules or capsules that contained melatonin. Individual *P. m. bairdii* implanted with melatonin underwent reproductive regression. Individuals of this subspecies also displayed elevated lymphocyte proliferation compared to control mice. Conversely, *P. m. luteus* implanted with melatonin did not undergo reproductive regression and displayed no significant changes in lymphocyte proliferation. These results suggest that reproductive responsiveness to melatonin mediates short-day enhancement of immune function in deer mice. These data also imply that melatonin may not possess universal immunoenhancing properties. Rather, the effectiveness of melatonin to influence immune responses may be constrained by reproductive responsiveness to this indole-amine.

Key words Seasonality · Rhythms · Breeding · Pineal · Immunity

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Introduction

Animals inhabiting non-tropical latitudes must adapt to potentially large fluctuations in environmental energy availability and physiological energy expenditures (Bronson 1989; Goldman and Nelson 1993). Breeding is an energetically demanding activity that can compromise survival of both parents and offspring if it occurs at inappropriate times of the year, i.e., when energy availability is low (Bronson and Heideman 1994). Consequently, individuals of many species have evolved to restrict breeding to specific times of the year to ensure producing offspring at a time when environmental energy availability is high (Bronson 1989; Bronson and Heideman 1994). Individuals that restrict breeding to times when environmental conditions are optimal presumably increase their fitness relative to individuals breeding at inappropriate times (Fairbairn 1977). Thus, the curtailment of breeding appears central among the suite of winter coping strategies among non-tropical rodents (Blank 1992; Wunder 1992).

Individuals of most animal species studied to date rely mainly on photoperiod as a precise temporal cue to estimate the time of year (Bartness et al. 1993). The extent to which this observation demonstrates the importance of photoperiod in mediating reproductive processes or the predominance of high latitude species being studied remains unspecified (Bronson 1989). Photoperiodic information is used to phase energetically expensive activities, such as breeding, to coincide with adequate energy availability [reviews: Goldman and Darrow (1983); Nelson et al. (1990); Bartness and Goldman (1989)]. The physiological signal encoding photoperiod appears to be the duration of nightly melatonin secretion (Bartness et al. 1993). Prolonged secretion of melatonin (> 12 h) results in short-day, or winter, adaptations; limited duration of melatonin secretion (< 8 h) results in long-day, or summer, adaptations (Carter and Goldman 1983; Bittman et al. 1983).

Virtually all previous studies of seasonal changes in mammalian physiology have placed considerable emphasis on seasonal fluctuations of reproduction and energy balance (Bronson and Heideman 1994). Although photoperiodic responsiveness can affect several physiological processes [reviews: Sullivan and Lynch (1986); Heldmaier et al. (1989); Moffatt et al. (1993)] it has been demonstrated primarily within the context of reproduction (Bronson 1989). Reproduction, however, is only one of many physiological processes that undergo seasonal changes. Although less commonly studied, there also exist salient cycles of disease and death (John 1994; Lochmiller et al. 1994). Disease and death rates typically are highest during the winter as compared to summer for individuals of many vertebrate species (John 1994; Lochmiller et al. 1994). Many of these animals presumably become sick and die from exposure to energetically-challenging ambient conditions, including reduced food availability. However, many animals die from opportunistic diseases that seem to overwhelm immunological defenses, presumably at times when these defenses are compromised by energetically-challenging stressors [review: Nelson and Demas (1996)]. Many epidemiologial studies have implicated reduced immune function and increased mortality rates from infectious disease during the winter (Afoke et al. 1993; Boctor et al. 1989; John 1994; Lochmiller et al. 1994). Field studies examining immune parameters across seasons have reported reductions in lymphatic tissue size and immune activity during winter [review: Nelson and Demas (1996)].

Photoperiod, in addition to providing a temporal cue for mammalian reproduction, also appears to play an important role in seasonal changes in immune status. Virtually all photoperiodic studies of immune function conducted to date report *increased* immune function in short as compared to long days [review: Nelson and Demas (1996)]. Laboratory studies have reported photoperiodic changes in gross splenic mass in deer mice *Peromyscus maniculatus* (Vriend and Lauber 1973), Syrian hamsters *Mesocricetus auratus* (Brainard et al. 1988; Vaughan et al. 1987), and laboratory strains of rats *Rattus norvegicus* (Wurtman and Weisel 1969). In addition, deer mice significantly increase lymphocytes, neutrophils, and counts (Blom et al. 1994) and a lower number of animals exhibit tumorigenesis in response to the chemical carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA) in short as com-pared to long days (Nelson and Blom 1994) or after melatonin treatment (Tamarkin et al. 1981; Blask et al. 1992). Presumably, short-day enhanced immunologic function represents an adaptation to permit physiological coping with energetically challenging stressors that would otherwise compromise survival (Demas and Nelson 1996).

Photoperiodic responsiveness, however, is not a species-typical trait. Individuals of many rodent species differ in their responsiveness to photoperiod depending on their latitude of origin (Lynch et al. 1981; Dark et al. 1983; Carlson et al. 1989). For example, individuals of the genus *Peromyscus* display a range of reproductive responsiveness to photoperiod that corresponds to their latitude of origin (Desjardins and Lopez 1980; Lynch et al. 1980; Bronson 1989). In general, a higher proportion of mice from high latitudes inhibit reproduction in short days as compared to mice from low latitudes [review: Bronson (1989)]. Reproductive responsiveness to day length is mediated primarily by differences in melatonin target-tissue responsiveness (Petterborg and Reiter 1981; Weaver et al. 1989; Weaver et al. 1991). Again, individuals of some rodent species may have evolved to respond to short day lengths by bolstering immune function in advance of harsh winter conditions (Nelson and Demas 1996; Demas and Nelson 1996). The present study was conducted to address whether reproductive responsiveness to photoperiod, and more specifically, reproductive responsiveness to melatonin, mediates short-day enhancement of immune function in deer mice.

Materials and methods

Thirty adult (> 60 days of age) male deer mice (*Peromyscus maniculatus bairdii*) were obtained from our laboratory breeding colony. This colony was originally derived from the *Peromyscus* Genetic Stock Center at the University of South Carolina, Columbia, S.C., USA. These animals are descendants of the *P. m. bairdii* subspecies originally trapped near East Lansing, Michigan (latitude = 42°51′ N). Another 40 adult (> 60 days) male deer mice (*Peromyscus maniculatus luteus*) were generously provided by Dr. Ira F. Greenbaum and Chance Stavinoha, Department of Biology, Texas A&M University. These animals were descendants of animals originally trapped near College Station, Texas (latitude 30° 37′ N).

Deer mice were weaned at 21 days of age and housed with same-sex siblings. Two weeks prior to the initiation of the experiments, all animals were individually housed in polypropylene cages (27.8 × 7.5 × 13.0 cm) in colony rooms with a 24 h LD 16 : 8 light cycle [lights on 0600 hours Eastern Standard Time (EST)]. Temperature was kept constant at 20 °C and relative humidity was maintained at 50 ± 5%. Food (Agway Prolab 1000, Syracuse, N.Y., USA) and tap water were provided *ad libitum* throughout the experiment.

In experiment 1, *P. m. bairdii* (n = 10) and *P. m. luteus* (n = 20) were randomly selected and assigned to one of two photoperiodic conditions. Half of the animals of each subspecies were housed under a short-day photoperiod (LD 8 : 16), while the others were housed under long days (LD 16 : 8). In experiment 2, the remaining *P. m. bairdii* (n = 20) and *P. m. luteus* (n = 20) were implanted s.c. with either a 15 mm long empty Silastic capsule (1.47 mm i.d., 1.95 mm o.d., Silicone Medical Grade Tubing, American Scientific Product, McGraw Park, Ill., USA) or a 15-mm capsule filled with melatonin crystals (10 mm) (Sigma, St. Louis, Mo., USA). Animals were lightly anesthetized with methoxyflurane vapors (Metofane, Pitman-Moore, Inc., Mundelein, Ill., USA). A 70% alcohol solution was applied to the intrascapular surface and a 5-mm incision was made perpendicular to midline. Capsules were implanted and the incision was closed with a 9-mm autoclip (Clay Adams, Parsippany, N.J., USA). Nitrofurazone antibacterial ointment (Phoenix Pharmaceutical, St. Joseph, Mich., USA) was applied to the skin surface to prevent infection. Animals were then returned to the colony room.

Animals in both experiments were maintained in their respective conditions for 8 weeks. At such time, they were brought into the surgery room, lightly anesthetized with methoxyflurane vapors, and then killed by cervical dislocation. Spleens were re-

moved under aseptic conditions and immediately suspended in culture medium (RPMI-1640/Hepes). Paired testes and epididymides, seminal vesicles, and epididymal fat pads were removed and cleaned of connective tissue and fat. 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.

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-dimethylthiazol-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 of culture medium: RPMI-1640/Hepes supplemented with 1% penicillin (5000 U ml^{-1})/streptomycin (5000 µl ml^{-1}), 1% L-glutamine (2 mmol l^{-1}), 0.1% 2-mercaptoethanol (5×10^{-2} µl ml^{-1}), 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^{-1} by dilution with culture medium, and 50-µl aliquots of each cell suspension (i.e., 100 000 cells) were added in duplicate to the wells of sterile flat-bottom 96-well culture plates. Con A (Sigma, St. Louis, Mo., USA) was diluted with culture medium to concentrations of 40, 20, 10, 5, 2.5, 1.25, and 0.60 µg ml^{-1}; 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 per 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^{-1} of phenazine methosulphate (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.

Reproductive organ mass data were analyzed using independent two-tailed *t*-tests. Analyses on ranked sums were conducted in cases where a violation of normality occurred. Splenocyte proliferation data were analyzed using a mixed model analysis of variance (ANOVA). Differences between group means were considered statistically significant if $P < 0.05$.

Results

Experiment 1

Short-day *P. m. bairdii* had significantly smaller testes compared to long-day mice ($P < 0.05$) (Fig. 1). Photoperiod did not affect epididymides, seminal vesicles, or epididymal fat pad masses ($P > 0.05$ in all cases) (Table 1). Short-day *P. m. bairdii* also displayed a significant elevation in splenocyte proliferation to Con A compared to long-day mice ($P < 0.05$) (Fig. 2). In contrast, photoperiod did not significantly affect reproductive masses in *P. m. luteus* ($P > 0.05$ in all cases) (Table 1, Fig. 1). Splenocyte proliferation to Con A was also unaffected by photoperiod in *P. m. luteus* ($P > 0.05$) (Fig. 2). Body masses of both *P. m. bairdii* and *P. m. luteus* were unaffected by photoperiod ($P > 0.05$ in both cases) (Table 1).

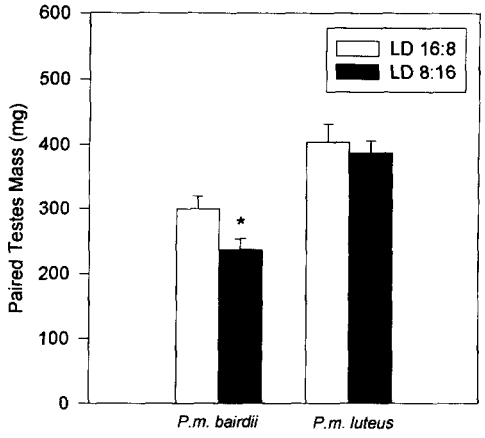

Fig. 1 Mean (± SEM) paired testes mass (mg) of *P. m. bairdii* and *P. m. luteus* housed in long (LD 16:8) or short (LD 8:16) days. Statistically significant differences between means are indicated by an *asterisk*

Experiment 2

P. m. bairdii implanted with melatonin capsules had significantly smaller testes and epididymides compared to animals administered empty capsules ($P < 0.05$ in both cases) (Table 2, Fig. 3). Melatonin implants did not affect seminal vesicle or epididymal fat pad mass ($P > 0.05$ in both cases). *P. m. bairdii* implanted with melatonin also displayed a significant elevation in splenocyte proliferation to Con A compared to mice implanted with empty capsules ($P < 0.01$) (Fig. 4). In contrast, melatonin implants did not significantly affect reproductive organ masses in *P. m. luteus* ($P > 0.05$ in all cases). (Table 2, Fig. 3). Splenocyte proliferation was also unaffected by melatonin in this subspecies ($P > 0.05$) (Fig. 4). Body mass of both *P. m. bairdii* and *P. m. luteus* was unaffected by melatonin treatment ($P > 0.05$) (Table 2).

Table 1 Mean(± SEM) body masses and organ masses of *P. m. bairdii* and *P. m. luteus* maintained in long (LD 16:8) or short (LD 8:16) photoperiods

	Body mass (g)	Paired epididymides (mg)	Seminal vesicle (mg)	Epididymal fat (mg)
P. m. bairdii				
LD 16:8	21.1 ± 0.5	70.9 ± 5.5	78.8 ± 7.9	89.6 ± 11.4
LD 8:16	20.0 ± 0.7	57.8 ± 4.3	63.2 ± 15.1	63.0 ± 7.1
P. m. luteus				
LD 16:8	21.0 ± 0.5	128.0 ± 7.4	139.4 ± 12.3	196.1 ± 12.7
LD 8:16	21.7 ± 0.6	108.0 ± 10.5	134.6 ± 9.6	190.1 ± 18.1

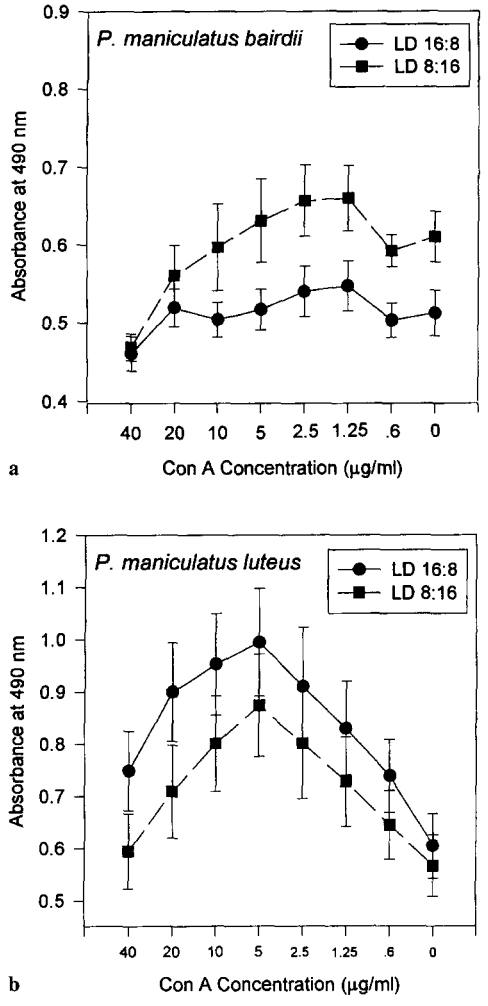

Fig. 2 Mean (± SEM) splenocyte proliferation to Con A (represented as absorbance units) of (**a**) *P. m. bairdii* and (**b**) *P. m. luteus* housed in long (LD 16:8) or short (LD 8:16) days. Higher absorbance values (nm) are indicative of increased splenocyte proliferation in response to mitogenic stimulation

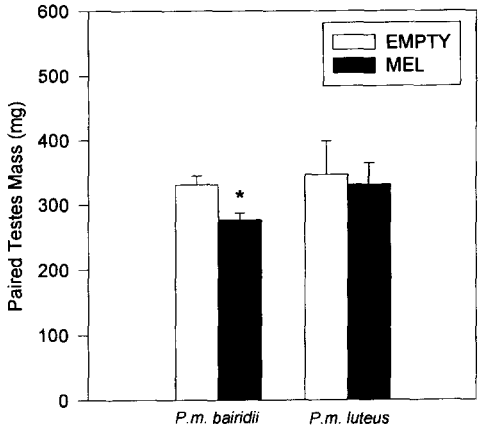

Fig. 3 Mean (± SEM) paired testes mass (mg) of *P. m. bairdii* and *P. m. luteus* implanted with melatonin-filled (*MEL*) or empty capsules (*EMPTY*). Statistically significant differences between means are indicated by an *asterisk*

Discussion

P. m. bairdii originating from high latitudes responded to short days by displaying reproductive regression and elevated splenocyte proliferation in response to Con A. This subspecies displayed a similar pattern of gonadal regression and splenocyte enhancement when implanted with melatonin. In contrast, *P. m. luteus* originating from low latitudes failed to display either reproductive regression or enhanced splenocyte proliferation in short days or after melatonin treatment. Chronic melatonin administration is interpreted by many rodent species, including *Peromyscus*, as a signal for short days (Lynch and Epstein 1976; Petterborg and Reiter 1981). These data demonstrate an important link between reproductive photoperiodic responsiveness and day length mediated changes in immune function.

The present results are consistent with both direct and indirect effects of melatonin on immune function. One possibility is that melatonin acts directly on the immune system, leading to enhanced immune function. Consistent with this direct effect, melatonin treatment reportedly enhances both humoral and cell-mediated immunity (Guerrero and Reiter 1992; Maestroni 1993). Additionally, melatonin receptors have been isolated on circulating lymphocytes (Calvo et al. 1995), as well as on thymocytes and splenocytes (Lopez-Gonzales et al. 1993; Rafii-El-Idrissi et al. 1995). Melatonin can also act indirectly on immune function via its effects on sex and adrenal steroid hormones, as well as prolactin [review: Nelson et al. (1995)]. Sex steroid hormones and prolactin can affect immune function [review: Nelson and Demas (1996)]. Both melatonin administration and maintenance in short days can result in significant changes in circulating levels of these hormones (Bartness et al.

Table 2 Mean (± SEM) body masses and organ masses of *P. m. bairdii* and *P. m. luteus* implanted with melatonin or empty capsules

[a]indicates a statistically significant difference between group means within a subspecies

	Body mass (g)	Paired epididymides (mg)	Seminal vesicle (mg)	Epididymal fat (mg)
P. m. bairdii				
EMPTY	20.9 ± 0.8	89.4 ± 4.5	56.0 ± 7.6	89.6 ± 13.6
MEL	20.4 ± 0.3	74.0 ± 2.5[a]	48.6 ± 2.6	67.2 ± 12.0
P. m. luteus				
EMPTY	22.6 ± 1.1	346.6 ± 51.7	139.4 ± 15.9	104.0 ± 20.3
MEL	19.9 ± 0.9	330.9 ± 33.4	134.6 ± 12.1	95.0 ± 11.6

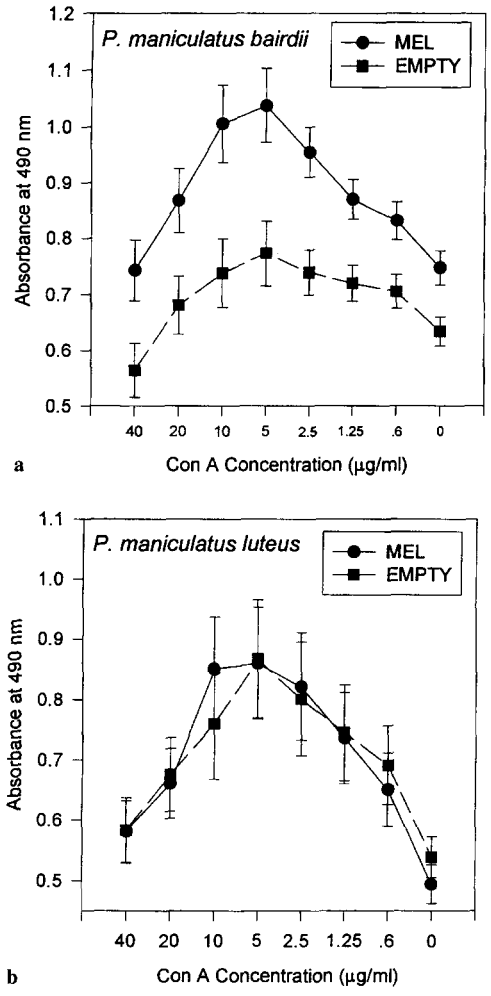

Fig. 4 Mean (± SEM) splenocyte proliferation to Con A of (**a**) *P. m. bairdii* and (**b**) *P. m. luteus* implanted with melatonin-filled (*MEL*) or empty capsules (*EMPTY*)

1993). Preliminary results with castrated deer mice suggest that elevated mitogen-stimulated splenocyte proliferation in response to short days are mediated by *both* direct and indirect actions of melatonin (G.E. Demas and R.J. Nelson, unpublished data). The extent to which the indirect effects are due to altered levels of gonadal steroids or prolactin requires further testing. The degree of reproductive regression observed in both short-day-exposed and melatonin-treated animals, although statistically significant, was uncharacteristically small for this subspecies. Immune response to short days or melatonin treatment may be greater in individuals exhibiting a more robust reproductive response, although this remains to be tested empirically. Non-reproductive factors were not assessed in the present study. It is possible that individuals of *P. m. luteus* are universally unrespsonsive to photoperiod. In previous studies, traits within a single individual showed differential response to short days (Moffatt et al. 1993). The extent to which immune function is an obligatory or facultative response to short days in unknown.

Individuals of many rodent species differ in their responsiveness to photoperiod and these differences are related to latitude of origin (Lynch et al. 1981; Dark et al. 1983; Carlson et al. 1989). Previous studies have demonstrated a higher proportion of high latitude individuals within the genus *Peromyscus* inhibit reproduction in short days as compared to deer mice from lower latitudes (Lynch et al. 1980; Desjardins and Lopez 1980; Bronson 1989). A likely functional explanation for this observation is that individuals inhabiting high latitude climates encounter more severe winters and thus proper timing of behavioral, physiological, and morphological adaptations is critical; development of winter-coping strategies among low latitude mice is less important because ambient temperatures may support year-long breeding at low latitude climates (Lynch et al. 1981). Individuals of some rodent species may have evolved to bolster immune function in response to short photoperiods (< 12 h) in advance of harsh winter conditions (Nelson and Demas 1996). The results of the present study are consistent with this notion; *P. m. bairdii* display increased immunologic responsiveness to photoperiod compared to *P. m. luteus*. These data suggest that reproductive photoperiodic responsiveness is necessary for photoperiod-mediated changes in immune function.

The duration of nightly melatonin secretion appears to be the physiological signal encoding day length [review: Bartness et al. (1993)]. Reproductive responsiveness to day length, however, is mediated by melatonin target-tissue responsiveness (Petterborg and Reiter 1981; Weaver et al. 1991). Individual deer mice from high latitudes respond to melatonin implants or daily melatonin injections with gonadal regression (Lynch and Epstein 1976; Heath and Lynch 1982). In contrast, individual deer mice from low latitudes fail to display gonadal regression in response to exogenous melatonin treatment (Heath and Lynch 1981). In the present study, *P. m. bairdii* implanted with melatonin display elevated immune function compared to mice implanted with empty capsules. *P. m. luteus*, however, fail to display any melatonin-mediated enhancement of immune function.

In summary, the present findings suggest that reproductive responsiveness to melatonin mediates short-day enhancement of immune function in deer mice. These data also imply that melatonin may not possess universal immunoenhancing properties. Rather, the effectiveness of melatonin to influence immune function may be constrained by reproductive responsiveness to this indole-amine.

Acknowledgements We thank Serena Cardillo, Joyce Hairston, Vinuta Rau, Violette Renard, and Sue Yang for technical support. We also thank Lance Kriegsfeld for valuable comments on the manuscript. We are also greatful to Ed Silverman and Erin Austin for expert animal care, and Dr. Ira F. Greenbaum and Chance Stavinova at Texas A&M University for providing animals. This study was supported by NICHD grant HD 22201. This research protocol conforms with federal NIH and USDA regulations, and was conducted with approval of our local Institutional Animal Care and Use Committee.

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