

Spontaneous 'regression' of enhanced immune function in a photoperiodic rodent *Peromyscus maniculatus*

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Short days inhibit reproduction and enhance immune function in deer mice (*Peromyscus maniculatus*). Their reproductive inhibition is sustained by an endogenous timing mechanism: after *ca.* 20 weeks in short days, reproductive photorefractoriness develops, followed by spontaneous recrudescence of the reproductive system. It is unknown whether analogous seasonal timing mechanisms regulate their immune function or whether enhanced immune function is sustained indefinitely under short days. In order to test this hypothesis, we housed adult male deer mice under long (16 h light day⁻¹) or short (8 h light day⁻¹) day conditions for 32 weeks or under long day conditions for 20 weeks followed by 12 weeks of short days. Mice under the long day conditions remained photostimulated over the 32 weeks, whereas mice housed under the short day conditions exhibited gonadal regression followed by photorefractoriness and spontaneous recrudescence. Mice transferred to short days at week 20 were reproductively photoregressed at week 32. Total splenocytes, relative splenic mass and mitogen-activated splenocyte proliferation were greater in those mice transferred to short days at week 20 than in those mice housed under either long or short day conditions for 32 consecutive weeks, and immune function in mice exposed to short days for 32 weeks was comparable with that of long day animals. These data suggest that short day enhancement of immune function is not indefinite. With prolonged (≤ 32 weeks) exposure to short days, several measures of immune function exhibit 'spontaneous' regression, restoring long day-like immunocompetence. The results suggest that formal similarities and, possibly, common substrates exist among the photoperiodic timekeeping mechanisms that regulate seasonal transitions in reproductive and immune function.

Keywords: immune function; photoperiodism; seasonal rhythms; photorefractoriness; spontaneous recrudescence; *Peromyscus maniculatus*

1. INTRODUCTION

Annual variations in temperature, rainfall and food availability predominate in non-equatorial environments (Saunders 1977). Mammals inhabiting these regions exhibit seasonal adjustments in their physiology, morphology and behaviour (e.g. fur moult, cessation of reproduction and hibernation) that ultimately restrict energetically expensive activities (e.g. eutheria, reproduction and lactation) to energetically favourable times of year (Bronson 1989). Seasonal changes in day length are relatively 'noise-free' predictors of phase in the annual geophysical cycle and can function as proximate cues for initiating these physiological transitions (Saunders 1977; Bronson 1989).

Seasonal changes in immune function are ubiquitous in mammals (Nelson & Demas 1996) and probably contribute to annual fluctuations in illness and death (Nelson *et al.* 2002). In common with reproductive cycles, seasonal changes in immunocompetence are driven by changes in day length (Nelson *et al.* 1995). For example, in deer mice (*Peromyscus maniculatus*), which are small-bodied, temperate zone rodents, both *in vitro* mitogen-stimulated splenic lymphocyte proliferation and non-specific IgG antibody production are enhanced under short (< 12 h light day⁻¹) relative to long days (Demas & Nelson 1996). Depending on species and immunological-dependent variable, short days enhance (Blom *et al.* 1994; Demas & Nelson 1996,

1998; Yellon *et al.* 1999) or suppress (Di Stefano & Paulesu 1994; Prendergast *et al.* 2001) mammals' immune function.

Photoperiodic mechanisms for explaining how changes in day length drive seasonal transitions in physiology have principally been elaborated from the study of seasonal reproduction in rodents (e.g. mice and hamsters) and artiodactyls (e.g. sheep) (Goldman 2001). The neuroendocrine system transduces day length information into the reproductive axis by varying the duration of nightly pineal melatonin production and the serum melatonin rhythm, which is proportional to the duration of the night (Illnerova *et al.* 1984). The relatively longer nights during autumn and winter result in longer melatonin signals, which are read by the neural substrates that control the secretion of gonadotropins, whereas the converse holds true for the shorter nights of spring and summer. Thus, deer mice undergo testicular regression when exposed to short days (≤ 12 h light day⁻¹) (Stebbins 1977). However, this reproductive inhibition is not permanent. After *ca.* 20 weeks of exposure to short days, deer mice spontaneously revert to a reproductively competent phenotype (Heath & Lynch 1981). This recovery is termed spontaneous recrudescence as it occurs despite continued exposure to photoperiods that were previously non-stimulatory (Watson-Whitmyre & Stetson 1988). Photoperiodic rodents do not exhibit another transition to the short day phenotype after spontaneous recrudescence has occurred unless first exposed to 10–20 weeks of long days, which restores their responsiveness to short days. An individual is considered refractory to short days when a photoperiod

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previously sufficient for sustaining a short day phenotype (e.g. regressed gonads) no longer does so (Watson-Whitmyre & Stetson 1988). Photorefractoriness is a fundamental feature of photoperiodic seasonal timekeeping mechanisms in mammals and, in all photoperiodic traits described to date, invariably ensues after prolonged exposure to short days.

Spontaneous recrudescence of the reproductive axis is of considerable functional significance. Reproductive hormone secretion and gametogenesis require several weeks in order to be fully reactivated from a state of photoperiod-induced quiescence (e.g. Simpson *et al.* 1982). Photorefractoriness in mid-winter initiates the recovery of reproductive function many weeks in advance of permissive (long) day lengths (Gorman & Zucker 1995a) and permits early spring breeding (Clarke 1981). Surprisingly, the issue of photorefractoriness has not been addressed with regard to immune function. It has been established in deer mice that the seasonal changes in their immune function are not simply secondary consequences of changes in their reproductive condition or gonadal hormone secretion (Demas & Nelson 1998), yet it remains unknown whether photoperiodic adjustments in their immune function are regulated by seasonal timekeeping mechanisms analogous to those that govern their reproductive transitions. Specifically, it is unknown whether any components of the immune system become refractory to short days. It may be the case that, unlike in the reproductive system, transition from the short day to the long day immune phenotype can be accomplished in a matter of hours or days. If this were so, there would be no obvious advantage associated with photorefractoriness mechanisms governing the immune system: animals could simply wait for permissive spring photoperiods to initiate the transition to a seasonally-appropriate immune status. Alternatively, photorefractoriness may be a feature common to all physiological systems regulated by day length, irrespective of the trait's capacity for rapid phenotypic change.

Here, we tested the hypothesis that immune function becomes refractory to short days. Short days enhance cell-mediated immune function in male deer mice (Demas & Nelson 1998). The short-day immune phenotype may be sustained for several months under chronic exposure to short days and then exhibit spontaneous recrudescence, returning to the long-day state despite continued maintenance in short days. Alternatively, the short day immune phenotype may persist indefinitely under short photoperiods. Age-matched groups of male deer mice were exposed to short days for 12 weeks or 32 weeks. The latter group exhibited regression and spontaneous recrudescence of the gonads and accessory sex organs, which is indicative of photorefractoriness of the reproductive system, whereas the former group exhibited gonadal regression, which is indicative of short day photoresponsiveness. We then assessed features of the immune system in order to determine whether reproductively photorefractory mice had become immunologically refractory to short days as well.

2. METHODS

(a) *Animals*

Adult (50–70 days of age) male deer mice (*P. maniculatus*) were obtained from the *Peromyscus* Stock Centre at the University

of South Carolina. The mice were housed singly in polypropylene cages in a room illuminated for 16 h day⁻¹ with incandescent light (lights on at 23.00 Eastern Standard Time). Food (LabDiet 5001, PMI Nutrition, Brentwood, MO, USA) and water were provided *ad libitum* and the ambient temperature and relative humidity were held constant at 21 ± 2 °C and 50 ± 5%, respectively. Mice acclimated for 3 weeks prior to the start of the experiment.

(b) *Photoperiod manipulations*

At the beginning of the experiment (week 0), a randomly selected group of deer mice was transferred to a short day photoperiod (8 h light day⁻¹ with lights on at 07.00) where they remained for the next 32 weeks (treatment SD32). Another group remained under the long day (16 h light day⁻¹) photoperiod for the entire 32 week experiment (treatment LD32). A third group was housed under 16 h light day⁻¹ for 20 weeks, at which time (week 20) they were transferred to 8 h light day⁻¹ for the remaining 12 weeks of the experiment (treatment SD12). Due to a computer failure, SD12 and SD32 mice were exposed to 12 h light day⁻¹ for 1 week beginning at week 28, after which the experimental 8 h light day⁻¹ photoperiod was restored. This resulted in a transient increase in day length, though it remained a short day (< 12 h light day⁻¹) photoperiod. Nevertheless, the majority of SD12 mice sustained gonadal regression by week 32.

(c) *Somatic and reproductive measures*

The mice were weighed (± 0.1 g) at predetermined intervals beginning at week 0, and the lengths and widths of their left testes were measured (± 0.1 mm) under light methoxyflurane vapours. The product of testis width squared multiplied by testis length provided a measure of estimated testis volume that is highly correlated with testis weight (Gorman & Zucker 1995b). All mice were killed by cervical dislocation at week 32 and their seminal vesicle, testis and epididymal white adipose tissue weights were determined (± 0.1 mg) at autopsy.

(d) *Criteria for gonadal regression*

Only mice that manifested testicular regression within the first 12 weeks of exposure to short days (which is indicative of photoresponsiveness to 8 h light day⁻¹) were retained. A criterion for gonadal regression under 8 h light day⁻¹ was established based on the variance in estimated testis volume among mice that remained in 16 h light day⁻¹ over a comparable time-interval (Gorman & Zucker 1995a). Gonadal regression in short day mice was defined as a decrease in estimated testis volume greater than a value equal to the mean change in estimated testis volume among long day mice over the same interval minus one standard deviation. Only mice transferred to 8 h light day⁻¹ at week 0 that exhibited a decrease in estimated testis volume of > 12.2% ($n = 8$ out of 10) during weeks 0–12 were considered to have undergone gonadal regression, and only mice transferred to 8 h light day⁻¹ at week 20 that exhibited a decrease in estimated testis volume of > 16.2% ($n = 12$ out of 22) during weeks 20–32 were considered reproductively photoresponsive.

(e) *Splenocyte proliferation assay*

Spleens were harvested under aseptic conditions and immediately suspended in culture medium (RPMI-1640, Mediatech, Herndon, VA, USA). Splenocytes were extracted from whole tissue under a sterile laminar flow hood. Spleens were gently ground against and pressed through a 70 µm Falcon cell strainer and resuspended in 2 ml RPMI. Splenocytes were then counted and

assessed for viability on a haemocytometer by Trypan blue exclusion. Viable cells (> 95%) were adjusted to 2×10^6 cells ml⁻¹ with supplemented culture medium.

In vitro splenocyte proliferation was assessed in the presence of the mitogen concanavalin A (ConA, Sigma, St Louis, MO, USA). Measurement of mitogen-stimulated lymphocyte proliferation is perhaps the most widespread functional *in vitro* assessment of the cellular arm of the immune system (Herbert & Cohen 1993); the assumption that the greater the *in vitro* proliferation, the more effective the *in vivo* immune response is implicit in the performance of this assay. A polyclonal mitogen such as ConA triggers essentially the same growth response (proliferation) as an antigen (Janeway *et al.* 1999). ConA was serially diluted to concentrations of 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 µg ml⁻¹ and 50 µl aliquots of each mitogen concentration were added to separate wells of a sterile culture plate. An additional well that did not contain ConA was filled with 50 µl of mitogen-free supplemented culture medium (RPMI-1640 with 1% penicillin/streptomycin, 1% L-glutamine, 0.1% 2-mercaptoethanol and 10% heat-inactivated foetal bovine serum). Next, 50 µl aliquots of splenocyte suspensions (containing 10⁵ splenocytes) were added to each well, yielding a volume of 100 µl per well. All samples were assayed in duplicate.

Culture plates were incubated at 37 °C with 5% CO₂ for 48 h. This interval is sufficient for the induction of splenocyte function and substantial proliferation in this and other rodent species (Azcona-Olivera *et al.* 1995; Demas & Nelson 1998). Splenocyte proliferation was assessed using a colorimetric assay based on a tetrazolium salt (MTS) with 0.92 mg ml⁻¹ of phenazine methosulphate (PMS) in sterile Dulbecco's phosphate-buffered saline (Promega, Madison, WI, USA). Tetrazolium salts are incorporated into active mitochondria and the salts are bio-reduced into a coloured formazan product in metabolically active cells. The quantity of formazan product as measured by the amount of absorbance of 490 nm light is directly proportional to the number of living cells in culture (Cory *et al.* 1991). The accumulation of coloured formazan product (i.e. the product of tetrazolium salt-based cell counting techniques) thus provides a measure of cell number (not mitosis *per se*). In the absence of direct measures of apoptosis and cell death, absorbance readings reflect the net sum of proliferation and cell death. However, the accumulation of coloured formazan products is strongly positively correlated with tritiated thymidine uptake (which is a direct measure of blastogenesis) under conditions of mitogen stimulation (Kitamura *et al.* 1989; Gieni *et al.* 1995). The MTS/PMS reagent was added to each incubation well after the 48 h incubation (20 µl per 100 µl incubation volume). The plates were then incubated at 37 °C with 5% CO₂ for an additional 4 h. The optical density (OD) of each well was determined with a microplate reader (Bio-Rad Benchmark, Bioxab, Richmond, CA, USA) equipped with a 490 nm filter. The mean OD values for each set of duplicates were then used in statistical analyses.

(f) Statistical analyses

Gonadal regression within each treatment group was assessed via repeated-measures analyses of variance (ANOVAs). The body, testis, seminal vesicle, epididymal white adipose tissue and spleen weights obtained at week 32 were compared with separate, between-subjects ANOVAs. Data from two animals (both photoresponsive SD12 mice) were not available for the analyses due to a processing problem. The effect of ConA on splenocyte proliferation was assessed within each photoperiod treatment group using a repeated-measures one-factor ANOVA. Because

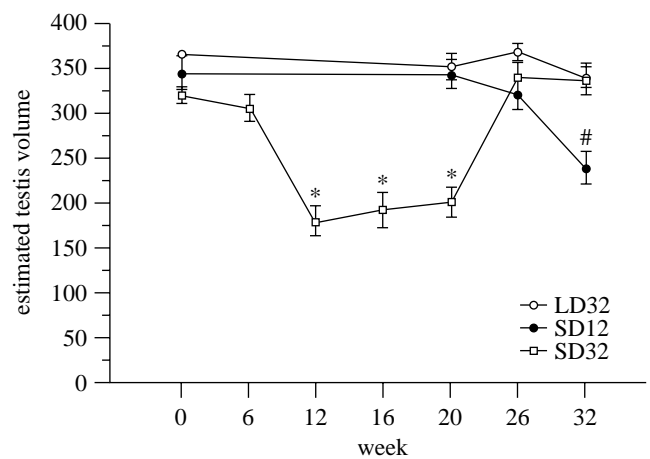


Figure 1. Mean (\pm s.e.m.) estimated testis volumes of adult deer mice housed in long days (16 h light day⁻¹) prior to week 0 and either kept in long days for 32 weeks (LD32) or transferred permanently to short days (8 h light day⁻¹) at week 0 (SD32) or at week 20 (SD12). * $p < 0.05$ versus same group week 0 value and # $p < 0.05$ versus same group week 0 and week 20 values.

the basal rates of splenocyte proliferation differed among the treatment groups, splenocyte responses to mitogen were calculated as a percentage of baseline (0 µg ml⁻¹ ConA). The effect of photoperiod on the proliferative responses to ConA was assessed via a mixed within-subjects factorial ANOVA using the treatment groups (LD32, SD12 and SD32) as between-subjects factors and mitogen concentration as a within-subjects factor. Between-group means were compared using unpaired *t*-tests (ANOVA, STATVIEW5, SAS Institute, Cary, NC, USA) if justified by a significant *F*-value. The mean differences observed were considered significant if $p < 0.05$.

3. RESULTS

(a) Weeks 0–32

(i) Gonadal regression and recrudescence

The testis sizes of the mice did not differ between the three treatment groups prior to the initiation of the photoperiod manipulations ($F = 0.73$, d.f. = 2,14 and $p > 0.40$). Eight out of 10 mice transferred to 8 h light day⁻¹ at week 0 (SD32) exhibited gonadal regression within the first 12 weeks of exposure to 8 h light day⁻¹ and were included in the following analyses. The testis sizes of SD32 mice changed significantly over the 32 weeks of exposure to 8 h light day⁻¹ ($F = 39.8$, d.f. = 7,42 and $p < 0.0001$) (figure 1). Their testes were fully regressed after 12 weeks of exposure to 8 h light day⁻¹ and remained regressed to week 20. Spontaneous gonadal recrudescence was initiated between weeks 20 and 26 and by week 26 the testes of SD32 mice no longer differed from week 0 values. The testes of SD32 mice did not exhibit a second bout of regression subsequent to week 26 and remained fully developed at week 32, which is indicative of reproductive photorefractoriness to short days.

The testes of SD12 mice remained fully developed through to week 20. Twelve out of 22 mice exhibited gonadal regression over the next 12 weeks when transferred to 8 h light day⁻¹ at week 20, and were included in the SD12 group. The estimated testis volume in these

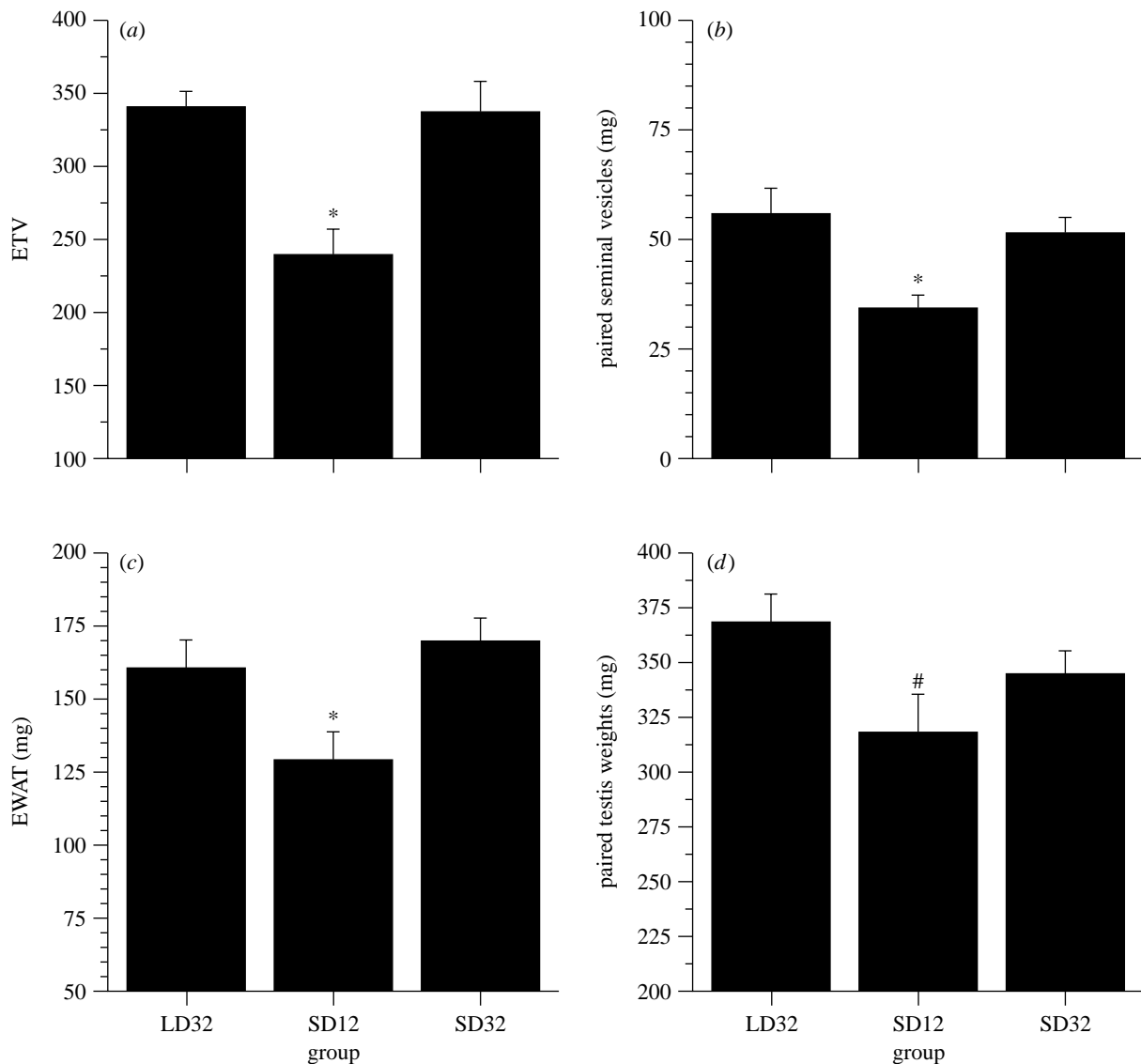


Figure 2. (a) Estimated testis volume (ETV), (b) paired seminal vesicle weights, (c) paired epididymal white adipose tissue weights (EWAT) and (d) paired testis weights of deer mice after exposure to long days (16 h light day⁻¹) for 32 weeks (LD32), short days (8 h light day⁻¹) for 12 weeks (SD12) or short days for 32 weeks (SD32). Values are means + s.e.m.s. * $p < 0.05$ versus all other groups and # $p < 0.05$ versus LD32 mice.

animals changed significantly between weeks 20 and 32 ($F=21.3$, d.f. = 9,18 and $p < 0.0001$) (figure 1). The estimated testis volume of SD12 mice was significantly smaller at week 32 as compared with week 20. The testis sizes of LD32 mice remained fully developed throughout the 32 week experiment (figure 1).

(b) Week 32

(i) Somatic and reproductive measures

The photoperiod treatments significantly affected the estimated testis volumes as measured at week 32 ($F=12.4$, d.f. = 2,22 and $p < 0.0005$) (figure 2a). The estimated testis volume of SD12 mice was significantly smaller than that of LD32 mice at week 32 ($p < 0.0005$). In contrast, the testes of SD32 mice were fully developed (which is indicative of complete recrudescence) and did not differ from those of LD32 animals ($p > 0.90$), but were significantly larger than those of SD12 mice ($p < 0.0005$). The accessory gland weights of the mice revealed identical patterns (seminal vesicles: $F=10.1$, d.f. = 2,22 and

$p < 0.001$, and epididymal white adipose tissue: $F=5.16$, d.f. = 2,22 and $p < 0.05$) (see figure 2b,c, respectively). The seminal vesicles weights of LD32 and SD32 mice did not differ ($p > 0.40$), but both significantly exceeded those of SD12 mice in weight ($p < 0.005$ for both comparisons). The paired epididymal white adipose tissue weights of SD12 mice weighed less than those of both LD32 ($p < 0.05$) and SD32 ($p < 0.01$) mice, which did not differ ($p > 0.50$). The testes of LD32 mice were significantly heavier than those of SD12 mice at autopsy ($p < 0.05$) and did not differ from those of SD32 mice ($p > 0.20$). However, the testis weights of SD12 and SD32 mice did not differ at week 32 ($p > 0.20$) (figure 2d). The body weights of the mice did not differ significantly among SD12, SD32 and LD32 mice at week 32 ($F=0.18$, d.f. = 2,22 and $p > 0.80$) (table 1).

(ii) Immune system measures

The spleen weights of the mice did not differ between photoreponsive, photorefractory and photostimulated

Table 1. Body weights, spleen weights, splenic cellularity and basal (unstimulated) splenocyte proliferation values in reproductively photostimulated (LD32), photoinhibited (SD12) and photorefractory (SD32) male deer mice.

(Values are means \pm s.e.m. * $p \leq 0.05$ versus LD32 and SD32 groups.)

treatment	<i>n</i>	body weight (g)	spleen weight (mg)	total splenocytes ($\times 10^6$)	splenocytes per milligram of spleen ($\times 10^6$)	splenocytes per gram of body weight ($\times 10^3$)	basal splenocyte proliferation (OD units)
LD32	7	20.1 \pm 0.8	12.0 \pm 3.4	13.1 \pm 1.6	2.9 \pm 1.3	6.7 \pm 1.0	0.815 \pm 0.09
SD12	10	20.5 \pm 0.6	9.6 \pm 2.4	17.5 \pm 1.1*	4.9 \pm 1.8	8.6 \pm 0.6*	0.609 \pm 0.03*
SD32	8	20.0 \pm 0.3	10.1 \pm 2.0	12.9 \pm 0.8	2.1 \pm 0.7	6.4 \pm 0.4	0.805 \pm 0.08

mice at week 32 ($F=0.23$, d.f. = 2,21 and $p=0.80$) (table 1). The number of splenocytes per spleen differed significantly between groups ($F=5.32$, d.f. = 2,22 and $p=0.01$) (table 1): SD12 mice had more splenocytes than either LD32 ($p=0.01$) and SD32 mice ($p < 0.01$), which did not differ ($p > 0.90$). Corrected for spleen weight, the splenocyte counts did not differ as a function of photoperiod treatment ($F=1.12$, d.f. = 2,21 and $p > 0.30$) (table 1). However, SD12 mice had more splenocytes per gram of body weight than both LD32 and SD32 mice ($F=3.45$, d.f. = 2,22 and $p < 0.05$) (table 1).

The *in vitro* proliferation of splenocytes of the mice also differed as a function of the photoperiod treatment ($F=3.66$, d.f. = 2,22 and $p < 0.05$) (table 1). The OD values of splenocytes not treated with ConA, which indicate basal blastogenesis, were lower in the splenocytes of SD12 mice relative to those of LD32 ($p < 0.05$) or SD32 ($p < 0.05$) mice (table 1). Within-group comparisons indicated that, relative to basal ($0 \mu\text{g ml}^{-1}$ ConA) rates of blastogenesis, ConA treatment only stimulated the proliferation of cells from SD12 mice and did so significantly at all concentrations except $20 \mu\text{g ml}^{-1}$ (figure 3a). The proliferative responses of the splenocytes from LD32 and SD32 mice did not exceed basal ($0 \mu\text{g ml}^{-1}$ ConA) values at any concentration of ConA and were inhibited by $20 \mu\text{g ml}^{-1}$ ConA (figure 3a).

Because the basal rates of splenocyte proliferation differed significantly between the groups, the proliferative responses at each mitogen dose were expressed as a percentage of the basal ($0 \mu\text{g ml}^{-1}$) values in order to facilitate between-group comparisons of the effects of ConA on splenocyte function. The increase in proliferation was higher in the splenocytes of SD12 mice relative to both LD32 and SD32 mice at ConA concentrations of 0.3125, 0.625, 1.25 and $2.5 \mu\text{g ml}^{-1}$ (figure 3b). At no concentration of ConA did the change in proliferation differ between the splenocytes of SD32 and LD32 mice ($p > 0.15$ for all comparisons) (figure 3b).

4. DISCUSSION

Male deer mice showed enhancement of their immune function following 12 weeks of exposure to short days, but after 32 weeks in short days reproductively photorefractory deer mice exhibited diminished proliferative responses and reduced splenocyte counts that were comparable with the values of mice housed in long days. These data confirm previous reports that short photoperiods enhance immune function in this photoperiodic species (Demas & Nelson 1996, 1998), but indicate that

prolonged (*ca.* 8 months) exposure to short days is accompanied by a restoration of the long day immune phenotype.

In common with several other photoperiodic rodent species (e.g. Siberian hamsters (*Phodopus sungorus*) and Syrian hamsters (*Mesocricetus auratus*)), deer mice exhibit a biphasic reproductive response to short days. Short photoperiods (≤ 12 h light day $^{-1}$) initially inhibit reproductive physiology. Gonadal involution persists for 2–3 months, but prolonged exposure (15–20 weeks) to short days results in the development of photorefractoriness and the reproductive system undergoes 'spontaneous' recrudescence or regrowth, despite prevailing short photoperiods (Heath & Lynch 1981). In the present experiment, 12 weeks of short days caused regression of reproductive (gonads) and accessory tissues (seminal vesicles and epididymal white adipose tissue). However, after 32 weeks of short days, the reproductive system was restored to the long day phenotype, indicating that the reproductive axis had become refractory to short days and had undergone spontaneous recrudescence. Acute (12 weeks) exposure to short days also increased several measures of immune function including the total number of splenocytes, splenocytes per gram of body weight and responsiveness to suboptimal doses of ConA. Chronic (32 weeks) exposure to short days resulted in significant reductions in all of these measures of immune function relative to acute short day exposure. Moreover, the relative and total splenocyte counts and splenocyte proliferative responses to ConA in mice housed under short day conditions for 32 weeks were indistinguishable from those of mice housed under long day conditions for 32 weeks. Our experimental design assessed the effects of acute (12 week) short day treatment on immune function in age-matched controls (i.e. mice that had been under short day conditions for 12 weeks at a time when SD32 mice had been under short day conditions for 32 weeks), thereby permitting the conclusion that the regression of immune function observed in SD32 mice was not a result of age *per se* or interactions between age and photoperiod, but rather a consequence of the duration of exposure to short days. Thus, immune function in this species also appears to exhibit a biphasic, time-dependent response to a short photoperiod, which is characterized by short-term enhancement followed by a long-term loss of responsiveness to short days.

Several studies have assessed photoperiodic effects on immune function in deer mice. Short days and short day-like melatonin treatments enhance splenocyte responsiveness to the T cell mitogen ConA (Demas *et al.* 1996). The present data confirm this enhanced responsiveness in

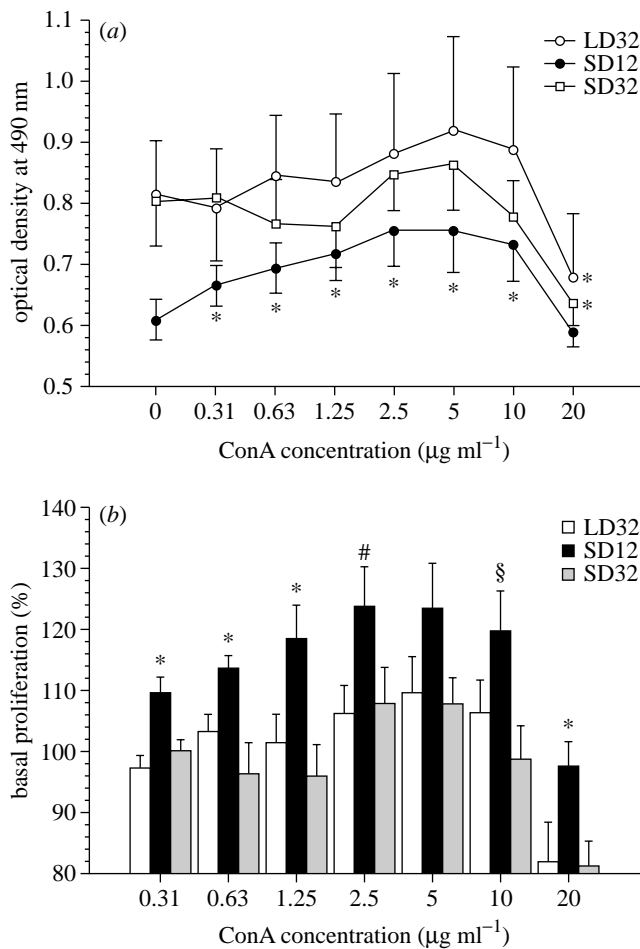


Figure 3. (a) Mean (+s.e.m.) proliferative responses to ConA and (b) mean (+s.e.m.) proliferation expressed as a percentage of basal ($0 \mu\text{g ml}^{-1}$ ConA) proliferation of splenocytes from adult male deer mice. Mice were housed in long days ($16 \text{ h light day}^{-1}$) for 32 weeks (LD32), short days ($8 \text{ h light day}^{-1}$) for 12 weeks (SD12) or short days for 32 weeks (SD32). Higher absorbance values are indicative of increased splenocyte proliferation in response to mitogenic stimulation. (a) $*p < 0.05$ versus same group week 0 value and (b) at a given ConA concentration $*p < 0.05$ versus all other groups, $\#p < 0.05$ versus LD32 mice and $\$p < 0.05$ versus SD32 mice.

short day deer mice, but indicate that the enhancement is transient: after 32 weeks the immunoenhancing effect of short days is no longer apparent. In common with Siberian hamsters (Bertoni *et al.* 1992), but unlike in Syrian hamsters (Brainard *et al.* 1987, 1988), short photoperiods did not increase the spleen weight of deer mice (cf. Demas & Nelson 1996). However, the total number of splenocytes per spleen was significantly increased in short days, an effect that likewise dissipated with prolonged exposure to short days. The increase in total splenic lymphocytes reported in the present study may be one source of the short day-induced increase in circulating lymphocytes previously reported in this species (Blom *et al.* 1994).

The splenocytes of the deer mice exposed to short days for only 12 weeks were significantly more responsive to ConA than those of the mice kept under either long or short day conditions for 32 weeks. Enhancement of

proliferation by short days occurred at all but supra-optimal concentrations of ConA and was most evident at suboptimal doses. ConA is a specific mitogen for T cells that has little B cell activity (Palacios 1982; Cogoli 1993). The enhanced proliferation of SD12 mice splenocytes probably reflects increased mitogen-specific responses rather than a general increase in lymphoproliferation. Indeed, baseline lymphoproliferation was lower in SD12 mice. Although the total number of splenocytes per spleen was increased in short days, splenic density (splenocytes per milligram of spleen) was unaffected, suggesting that the spleens of SD12 deer mice were not in a hyperplastic state. The increased proliferative responses of SD12 mice splenocytes may result from either an increase in the relative proportion of T cells in the splenocyte cultures from SD12 mice or an increase in the reactivity of T cells in the SD12 mice cultures.

The regression of immune function after chronic exposure to short days appears inversely analogous to the spontaneous recrudescence observed in the reproductive axis of this and other photoperiodic species (Watson-Whitmyre & Stetson 1988). Of course, no biological process is spontaneous, i.e. without aetiology. However, the physiological mechanisms by which the acute photic enhancement of immune function is lost with chronic exposure to short days are currently unknown. Elevated gonadal steroid hormone secretion coincident with testicular recrudescence is unlikely to be driving the observed changes in immune function as short day enhancement of mitogen-stimulated splenocyte proliferation occurs independently of changes in gonadal hormones in this species (Demas & Nelson 1998). Photoperiodic modulation of prolactin secretion may play a role in the photoperiod-induced and spontaneous changes in splenocyte function described in this study. As in most photoperiodic species, short days suppress circulating prolactin concentrations in deer mice (Blank & Desjardins 1986). Furthermore, inhibition of prolactin secretion enhances some aspects of immune function and suppresses tumorigenesis in deer mice (Nelson & Blom 1994). Although spontaneous recrudescence of prolactin secretion after prolonged exposure to short days has not been documented in deer mice, this phenomenon has been described in Syrian hamsters (Klemcke *et al.* 1981). Spontaneous increases in circulating prolactin after 32 weeks in short days could mask the short day enhancement of immune function, resulting in long day-like splenocyte function in reproductively photorefractory deer mice.

Alternatively, the spontaneous change in splenocyte responsiveness described in the present study may not be a result of masking by peripheral hormone secretion, but instead may result from refractoriness of individual splenocytes to melatonin. *In vivo* melatonin treatments enhance *in vitro* splenocyte responsiveness to mitogens in a manner similar to that of short photoperiods in this species (Demas & Nelson 1998). Spontaneous recrudescence in the reproductive axis under short days is a consequence of acquired unresponsiveness to melatonin (Bittman 1978); refractoriness to melatonin probably occurs at one of several melatonin binding sites in the brain or pituitary via as yet undescribed cellular mechanisms (Lincoln & Richardson 1998; Freeman & Zucker 2001). If the immunoenhancing effects of melatonin in

deer mice are a result of a direct effect of the hormone on immune cells (as previously argued by Nelson & Demas 1996), then it may be the case that the spontaneous regression of immune function observed after chronic exposure to short days is mediated by a 'spontaneous' change in splenocyte responsiveness to melatonin. If true, this would suggest that the eventual development of refractoriness may be a fundamental feature of all melatonin-responsive tissues. Such a concept would have implications for the extended use of melatonin in clinical contexts.

In summary, these data indicate that short days enhance several measures of immune function, but do not do so indefinitely. After 32 weeks of exposure to short days, the immune system reverts to the long day phenotype. Thus, in the same sense that the reproductive system becomes refractory to short days and triggers spontaneous recrudescence of the gonads, so too does the immune system appear to develop photorefractoriness, which is accompanied by a spontaneous regression of splenocyte counts and T cell mitogen responsiveness. The consequences of this seasonal modulation of immune function in the wild are unknown (Lochmiller & Deerenberg 2000; Sinclair & Lochmiller 2000), but the timing of these changes and their independence from the presence of long days would seem to indicate that parallel transitions in reproductive and immune function occur as photorefractoriness develops in mid-late winter. It is important to determine the precise mechanisms by which this photoperiod-independent modulation of immune function occurs. Whether via masking by peripheral hormone secretion, changes in splenocyte responsiveness to melatonin or other mechanisms, immune function appears to change seasonally in a manner formally similar to reproductive function.

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