ORIGINAL ARTICLE

Immune and stress responses in C57BL/6 and C3H/HeN mouse strains following photoperiod manipulation

Michelle L. Gatien, Andrew K. Hotchkiss, Gretchen N. Neigh, Firdaus S. Dhabhar[§], & Randy J. Nelson

Departments of Psychology, Neuroscience, [§]Oral Biology, and [§]Molecular Virology, Immunology, and Medical Genetics, and Institute of Behavioral Medicine Research, The Ohio State University, Columbus, OH, USA

Correspondence to: Randy J. Nelson
Department of Psychology
The Ohio State University
Columbus, Ohio 43210, USA
TEL: +1 (614) 538-9540 FAX: +1 (614) 451-3116
EMAIL: rnelson@osu.edu

Submitted: February 4, 2004 *Accepted: April 23, 2004*

Key words: **day length; photoperiodism; seasonality; glucocorticoids; leukocytes**

Neuroendocrinol Lett 2004; ***25****(4):267–272 NEL250404A04 Copyright © Neuroendocrinology Letters* ***www.nel.edu***

Abstract

OBJECTIVES: The purpose of this study was to determine whether two different strains of mice, that are reproductively unresponsive to photoperiod, adjust immune function in response to photoperiod.

SETTING AND DESIGN: Adult male C3H/HeN (C3H) and C57BL/6 (C57) mice were each placed into either a long (LD 16:8) or short (LD 8:16) photoperiod for 10 weeks (n=15/ group).

METHODS: Blood was collected for flow cytometry and radioimmunoassay analysis of leukocyte numbers and corticosterone concentrations, respectively. In addition, all mice were sensitized to, and challenged with the antigen, 2,4-dinitro-1-fluorobenzene. Pinnae measurements were obtained for 7 days following challenge to determine the magnitude of the inflammatory response.

RESULTS: Photoperiod did not affect leukocyte cell numbers in either C3H or C57 mice. C3H mice displayed higher neutrophil numbers than C57 mice ($p < 0.0001$), whereas C57 mice displayed higher lymphocyte numbers than C3H mice ($p < 0.01$). C3H mice housed in LD 16:8 had higher corticosterone concentrations than those housed in LD 8:16 ($p < 0.005$) and C57 mice housed in either photoperiod ($p < 0.05$). Photoperiod did not affect the inflammatory response, though C57 mice displayed an overall higher magnitude of response than C3H mice ($p<0.05$).

MAIN FINDINGS: Our results suggest that photoperiod does not alter immune function in C3H and C57 mice. Strain differences in immune function, however, were observed.

CONCLUSIONS: Immune function is unresponsive to photoperiod in these laboratory mice, possibly indicating; a link between immune and reproductive responsiveness to photoperiod, the decreased predictive value of this annual cue to domesticated animals, or both.

Abbreviations and units

C57	C57BL/ 6 inbred mouse strain
C3H	C3H/ HeN inbred mouse strain
LD	light/ dark cycle
RIA	radioimmunoassay
DNFB	2,4-dinitro-1-fluorobenzene
DTH	delayed-type-hypersensitivity
IL-1α	interleukin-1 alpha
EST	Eastern Standard Time
IP	intraperitoneal
ANOVA	analysis of variance

Introduction

Seasonal environmental perturbations such as low temperatures and food availability present energetic challenges for individuals of many nontropical animal species [1]. Increased energetic demands during winter, when energy availability is generally low, can create an energetic bottleneck, requiring adjustments in energetic allocation. Inhibition of reproductive function is a well-known adjustment in winter energy re-partitioning among rodents [2, 3]. Although many environmental factors change seasonally, in the laboratory, manipulation of only day length (photoperiod) is sufficient to evoke the seasonal adjustments in reproductive function observed in nature [4].

In addition to inhibition of reproduction, short days also affect growth, body mass, and immune function. For example, Siberian hamsters (*Phodopus sungorus*) maintained in short day lengths (light:dark (LD) 8: 16) enhance skin-antigen specific delayed-type-hypersensitivity (DTH) responses and increase immune cell numbers, as compared to animals housed in long day lengths (LD 16:8) [5]. Short days also increase natural killer cell cytotoxicity and circulating T-cell and B-cell numbers [5–7]. However, not all components of the immune system are augmented by short days. For example, exposure to short photoperiods suppresses phagocytosis, granulocyte numbers and oxidative burst activity, T-cell dependent humoral immunity, as well as *in vitro* basal lymphocyte proliferation and lymphocyte interleukin-1 alpha (IL-1α) production [5, 7–9].

Reproductive photoperiod-responsiveness can be revealed in species traditionally considered reproductively "nonresponsive" to photoperiodic manipulation, such as laboratory rats (*Rattus norvegicus*) and mice (*Mus musculus*), suggesting that the physiological mechanisms for these adaptations remain extant [10]. For example, exposure of olfactory bulbectomized laboratory rats to short photoperiods reduces reproductive function, as compared to animals housed in long days and sham-operated rats housed in both photoperiods. In addition, perinatal exposure to testosterone delays testicular development in animals exposed to short photoperiods. Presumably, the reproductive response to short days was selected against during the domestication of laboratory rodents when breeding colonies were illuminated only during work hours. Animals that failed to breed under short days were culled. However, selection against reproductive responsiveness to short days is not necessarily linked to the photoperiodic responsiveness of non-reproductive functions such as body mass and pelage color [11]. The uncoupling of photoperiodic responsiveness between nonreproductive and reproductive traits suggests that the lack of reproductive photoperiodic responsiveness in laboratory strains of mice and rats does not predict immunological responsiveness to photoperiod. Illustrating this point, immune function is not linked to reproductive responsiveness to photoperiod in Siberian hamsters [12], or to steroid hormone concentrations in deer mice (*Peromyscus maniculatus*) [13].

The mechanisms by which short days alter components of immune function remain largely unknown, although *in vivo* [7, 14–16] and *in vitro* [9] results indicate immunomodulatory roles for both photoperiod-dependent changes in melatonin secretion and melatonin-dependent changes in secretion of other hormones (e.g., cortisol, prolactin, and testosterone). Photoperiod information is transduced into a physiological signal by the duration of the nightly secretion of melatonin [17]. Thus, during short days, the duration of melatonin secretion is relatively extended as compared to the duration of melatonin secretion during long days (short nights). Many strains of laboratory mice do not display strong rhythms of melatonin secretion [18, 19]. In contrast, C3H/HeN (C3H) mice display robust circadian melatonin rhythms [18, 20], raising the possibility that they may retain immunological responsiveness to photoperiod. Supporting this hypothesis, recent work has shown that C3H mice display some circadian alterations in immune cell expression [21], which may forecast photoperiodic changes. The present study investigated the photoperiodic responsiveness of immune function of C3H mice as compared to C57BL/6 (C57) mice, thus comparing two strains of mice that display either a robust circadian melatonin rhythm or virtually no circadian melatonin rhythm, respectively.

Materials and methods

Animals

Adult (>2 months of age) male C3H/HeN and C57BL/6 mice were single-housed in poly-propylene cages (27.8 x 7.5 x 13 cm) in colony rooms held under constant temperature (21 ± 4° C) and relative humidity (50 ± 10%), and were given *ad libitum* access to food (Harlan Teklad 8640 Rodent Diet, Indianapolis, IN) and filtered tap water. Animals were housed in either a long photoperiod room (n=15, for each strain) with a reverse light/dark cycle (16 h of light per day (LD 16:8); lights illuminated at 23:00 h Eastern Standard Time (EST)), or a short photoperiod room (n=16, for each strain; 8 h of light per day (LD 8:16); lights illuminated at 0700 h EST).

Leukocyte Analysis

Following 9 weeks of housing in their respective light cycles, animals were lightly anesthetized with isoflurane vapor, and blood was drawn (120μl) from the

retro-orbital sinus at 0900 h EST. Animals received an intraperitoneal (ip) injection of sterile isotonic saline (0.5 ml) post blood-draw to prevent dehydration. Handling time was kept to a minimum for all animals (<2 min), and animals were quickly returned to their home cages in the colony rooms. Approximately 100 µl of blood was collected into heparinized polypropylene microcentrifuge tubes. The remaining 20 μl were collected into unheparinized polypropylene tubes for radioimmunoassay (RIA) analysis of corticosterone concentrations.

Total leukocyte counts were obtained on a hematology analyzer (F800, Sysmex, McGraw Park, IL). Lymphocyte, neutrophil, and monocyte subpopulations were identified using forward- vs. side-scatter parameters on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA). Data were analyzed by using CELLQUEST software (Becton Dickinson).

RIA procedures

Blood collected for RIA analysis, as described above, was allowed to clot for 30 min. The clot was then removed, and the samples were centrifuged at 4° C for 30 min at 2500 rpm. Serum aliquots were aspirated and stored in re-sealable polypropylene microcentrifuge tubes at –70° C until assayed for corticosterone concentrations using an ^{125}I RIA kit (ICN Biomedicals; Costa Mesa, California). The intra-assay coefficient of variation was < 20% in all cases.

DTH Sensitization and Induction

After 10 weeks in their respective photoperiod, one week following the blood-draw, DTH sensitization and induction began using a protocol reported previously in the literature [22]. Animals were again anesthetized under light isoflurane vapor, weighed, and sensitized to the antigen 2,4-dinitro-1-flourobenzene (DNFB; Sigma) by shaving a 2x3 cm patch of skin on the dorsum and applying 25 µl DNFB (0.5%(wt/vol) in 4:1, acetone: olive oil). This process was repeated 24 hrs later, at the same time of day (0900h EST). The thickness of both of the pinnae was measured using a constant-loading dial micrometer (Mitutoyo America, Aurora, IL) for later comparison during DTH induction.

One week following sensitization, baseline pinnae thickness was again measured, the animals weighed, and DTH induction was achieved by applying 20 µl DNFB (0.2% (wt/vol) in 4:1, acetone: olive oil) directly to the skin on the dorsal surface of the right pinna. The left pinna was treated in the same manner with vehicle solution only. Pinnae thickness was measured every 24 hours for the next seven days, at approximately the same time of day (0900h–1000h EST), during the light cycle. All measurements were obtained from the same relative region of the pinnae.

Data Analysis

Hormone concentrations and leukocyte numbers were analyzed between groups using analysis of variance (ANOVA). DTH reactions were analyzed as percentage increases over average baseline pinnae thickness for each animal and compared between groups using repeated measures ANOVA. All differences were considered statistically significant if $p \leq 0.05$.

Results

DTH

Neither the magnitude nor duration of the DTH response differed in the C3H or C57 strain, as a result of photoperiod manipulation. Mice of the C57 strain responded with a larger magnitude of response than mice of the C3H strain ($p < 0.05$), though both groups displayed a significant increase from the baseline pinnae measurement in response to challenge with DNFB ($p < 0.0001$; Figure 1).

Immune Cell Numbers

No differences were detected between mice housed in long or short photoperiods in the number or percentage of blood leukocytes for either the C3H or C57 strain, though there were strain differences in these

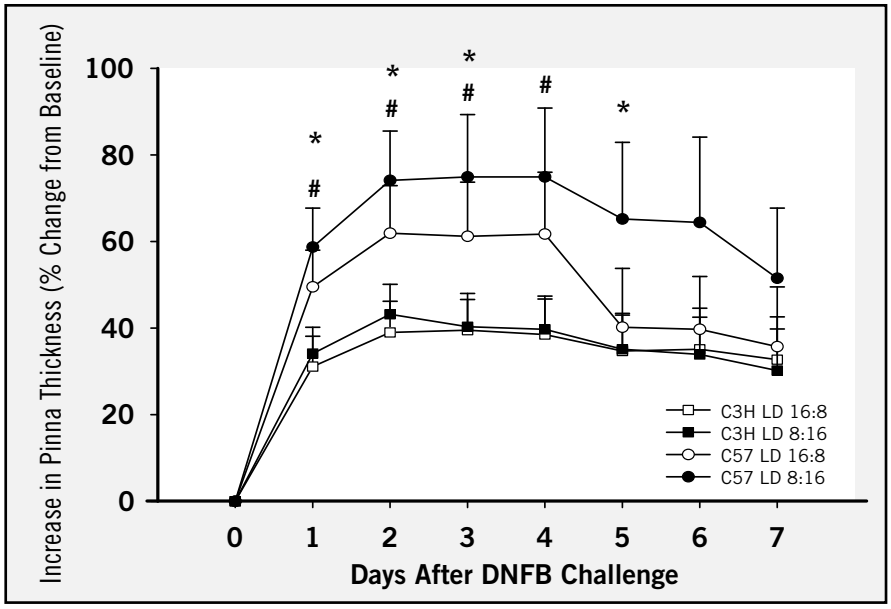

Figure 1. Photoperiod did not affect the inflammatory response in C3H or C57 mice. C57 mice displayed a higher magnitude of inflammatory response than C3H mice overall ($p<0.05$; data not shown), and C57 mice housed in LD 8:16 displayed greater inflammatory response than C3H mice housed in either photoperiod, at several time points ($p<0.05$). Both the C57 and C3H mice displayed a significant increase from the baseline pinna measurement as a result of challenge with DNFB ($p<0.0001$). *, C57mice housed in LD 8:16 different from C3H mice housed in LD 16:8; #, C57 mice housed in LD 8:16 different from C3H mice housed in LD 8:16; $p<0.05$.

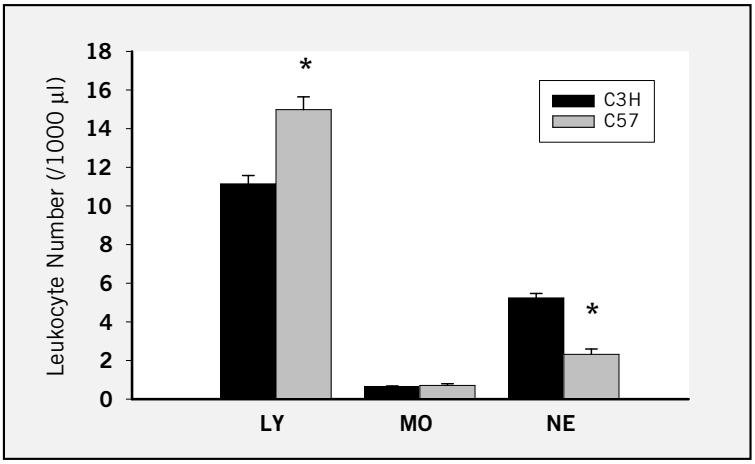

Figure 2. Photoperiod did not affect leukocyte number in either C57 or C3H mice. Monocyte numbers were not different in C57 and C3H mice, whereas C57 mice had a greater number of lymphocytes, and C3H mice had a greater number of neutrophils. *, Significant strain difference in leukocyte number; p<0.01.

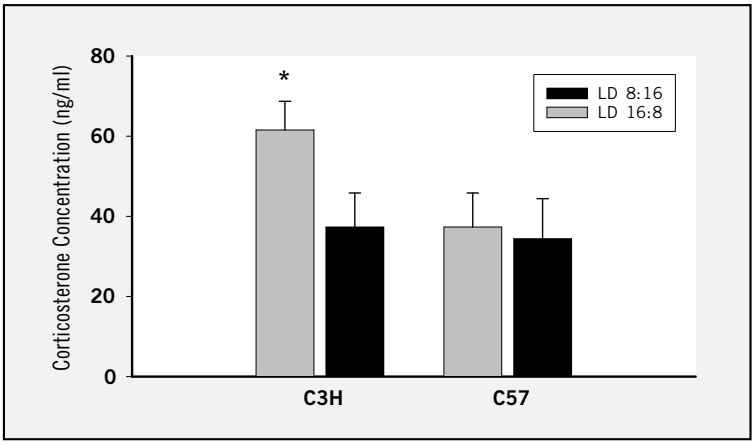

Figure 3. Photoperiod did not affect corticosterone concentrations in C57 mice; whereas C3H mice housed in LD 16:8 displayed higher concentrations of corticosterone than C3H mice housed in LD 8:16. C3H mice housed in LD 16:8 displayed higher corticosterone concentrations than C57 mice in either photoperiod. *, Significant difference between C3H mice housed in LD 16:8 and all other groups; p< 0.05.

measures. Although the number and percentage of monocytes was the same in both mouse strains, mice of the C3H strain displayed a higher overall number and percentage of blood neutrophils than mice of the C57 strain ($p< 0.0001$). Conversely, mice of the C57 strain had a higher overall number and percentage of blood lymphocytes than mice of the C3H strain ($p<0.01$; Figure 2).

Corticosterone RIA

Photoperiod did not affect serum corticosterone concentrations of C57 mice housed in short- and long-photoperiods. In the C3H strain, serum corticosterone concentrations were significantly elevated ($p< 0.005$) in long-day animals; long-day mice displayed nearly double the corticosterone concentrations as compared to short-day animals. In addition, the long-day C3H mice displayed approximately twice the corticosterone concentrations as either long- or short-day C57 mice ($p< 0.05$; Figure 3).

Discussion

We hypothesized that mice of the C3H strain, which possess a circadian melatonin rhythm, in contrast to mice of the C57 strain, which do not, would display an immunological response to photoperiod. The data indicate that the immune function of both strains of mice is relatively unaffected by photoperiod. Strain differences in immune function were observed. The C57 strain of mice displayed an overall increase in the magnitude of the DTH response compared to mice of the C3H strain. This is not an entirely unexpected result given that previous studies have documented increased local inflammatory response in C57 mice as compared to C3H mice to both cutaneous and subcutaneous infections [23–25].

To our knowledge, the increase in corticosterone concentrations in C3H mice housed in long days, as compared to short days, and C57 mice housed in either photoperiod, has not been previously reported, although there is some experimental and anecdotal support for these findings. C3H mice display increased levels of anxiety in long days as opposed to short days, and this effect is mediated by melatonin receptor activation [26]. Increased anxiety is highly correlated with elevated corticosterone concentrations [27], and elevated concentrations of corticosterone decrease the efficacy of anxiolytic drugs [28]. Because mice are nocturnal, it is possible that extended exposure to light might be sufficiently stressful to elicit corticosterone secretion. In addition, rodents maintained in long photoperiods experience abbreviated nights, and might be required to condense all reproductive, foraging, and exploratory behaviors into a reduced time period. Thus, the extended duration of light exposure, and abbreviated night in long days may be perceived by these animals as stressful, and may cause higher circulating corticosterone and increased anxiety. It is important to note however, the elevated corticoste-

rone concentrations observed in the C3H strain did not alter the immune response in these animals, as previously reported [29, 30]. This may reflect that, although corticosterone concentrations for the C3H mice housed in long days were significantly higher than those of mice in each of the other three groups, the mean concentration was approximately 60 ng/ml, a concentration well below that previously reported to be correlated with alterations in immune function in mice [31, 32].

Although not recorded in this study, all animals had large and presumably functional gonads [21], thus immune function may be linked to reproductive responsiveness to photoperiod in both these strains of laboratory mice. It is possible that animal husbandry practices in laboratory animals have selected against reproductive responsiveness to photoperiod, given that animals that failed to breed when housed in relatively short days (i.e. $\leq$ LD 12:12) would have been culled from breeding colonies. If immune responsiveness to photoperiod is indeed linked to reproductive responsiveness, then this laboratory husbandry practice may have resulted in the elimination of immune responsiveness as well. It is also likely that immune responsiveness to photoperiod has not been selected *for* in laboratory animals housed in controlled environments for many generations. These animals would no longer benefit from the predictive value of day length, and thus, the trait may have been eliminated over many generations of breeding under these conditions.

In conclusion, the results of the current work demonstrate that mice of the C3H and C57 strains display no alterations in immune function as a result of photoperiod manipulations, as measured by DTH and leukocyte cell counts. C3H mice housed in long photoperiods displayed elevated corticosterone concentrations, as compared to C3H mice housed in a short photoperiod, and C57 mice housed in both photoperiods. Strain differences in immune function were revealed in several immune parameters including the magnitude of the DTH response, lymphocyte number and percentage, and neutrophil number and percentage. These results indicate that photoperiodic responsiveness of immune function in either the C3H or C57 laboratory mouse strains is not extant for the immune parameters assessed in the present study, and that these traits may have been inadvertently selected against over many generations of breeding in laboratory settings.

Acknowledgements

We thank Stephanie Bowers, Jean Tillie, and Rob Rengel for technical support, and Tricia Uhor for expert animal care. This work was supported by National Institutes of Health grants MH57535(RJN), MH66144(RJN), and AI48995(FSD), National Science Foundation grant IBN 00-0854(RJN), and The Dana Foundation (FSD).

REFERENCES

1 Goldman SL, Dhandapani K, Goldman BD. Genetic and environmental influences on short-day responsiveness in Siberian hamsters (Phodopus sungorus). J Biol Rhythms 2000; **15**:417–428.
2 Prendergast BJ, Kriegsfeld LJ, Nelson RJ. Photoperiodic polyphenisms in rodents: neuroendocrine mechanisms, costs, and functions. Q Rev Biol 2001; **76**:293–325.
3 Bronson FH. Mammalian reproductive strategies: genes, photoperiod and latitude. Reprod Nutr Dev 1988; **28**:335–347.
4 Goldman BD. Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 2001; **16**:283–301.
5 Bilbo SD, Dhabhar FS, Viswanathan K, Saul A, Yellon SM, Nelson RJ. Short day lengths augment stress-induced leukocyte trafficking and stress-induced enhancement of skin immune function. Proc Natl Acad Sci U S A 2002; **99**:4067–4072.
6 Prendergast BJ, Hotchkiss AK, Bilbo SD, Kinsey SG, Nelson RJ. Photoperiodic adjustments in immune function protect Siberian hamsters from lethal endotoxemia. J Biol Rhythms 2003; **18**: 51–62.
7 Yellon SM, Teasley LA, Fagoaga OR, Nguyen HC, Truong HN, Nehlsen-Cannarella L. Role of photoperiod and the pineal gland in T cell-dependent humoral immune reactivity in the Siberian hamster. J Pineal Res 1999; **27**:243–248.
8 Yellon SM, Fagoaga OR, Nehlsen-Cannarella SL. Influence of photoperiod on immune cell functions in the male Siberian hamster. Am J Physiol 1999; **276**:R97–R102.
9 Prendergast BJ, Yellon SM, Tran LT, Nelson RJ. Photoperiod modulates the inhibitory effect of in vitro melatonin on lymphocyte proliferation in female Siberian hamsters. J Biol Rhythms 2001; **16**:224–233.
10 Nelson RJ. Photoperiodic responsiveness in house mice. Physiol Behav 1990; **48**:403–408.
11 Nelson RJ, Moffatt CA, Goldman BD. Reproductive and nonreproductive responsiveness to photoperiod in laboratory rats. J Pineal Res 1994; **17**:123–131.
12 Drazen DL, Kriegsfeld LJ, Schneider JE, Nelson RJ. Leptin, but not immune function, is linked to reproductive responsiveness to photoperiod. Am J Physiol Regul Integr Comp Physiol 2000; **278**:R1401–1407.
13 Demas GE, Nelson RJ. Short-day enhancement of immune function is independent of steroid hormones in deer mice (Peromyscus maniculatus). J Comp Physiol [B] 1998; **168**:419–426.
14 Drazen DL, Bilu D, Bilbo SD, Nelson RJ. Melatonin enhancement of splenocyte proliferation is attenuated by luzindole, a melatonin receptor antagonist. Am J Physiol Regul Integr Comp Physiol 2001; **280**:R1476–1482.
15 Bilbo SD, Nelson RJ. Sex steroid hormones enhance immune function in male and female Siberian hamsters. Am J Physiol Regul Integr Comp Physiol 2001; **280**:R207–213.
16 Bilbo SD, Nelson RJ. Melatonin regulates energy balance and attenuates fever in Siberian hamsters. Endocrinology 2002; **143**: 2527–2533.
17 Carter DS, Hall VD, Tamarkin L, Goldman BD. Pineal is required for testicular maintenance in the TURKISH hamster (MESOCRICETUS brandti). Endocrinology 1982; **111**:863–871.
18 Goto M, Oshima I, Tomita T, Ebihara S. Melatonin content of the pineal gland in different mouse strains. J Pineal Res 1989; **7**: 195–204.
19 Reppert SM, Weaver DR. Melatonin madness. Cell 1995; **83**: 1059–1062.
20 Stehle JH, von Gall C, Korf HW. Organisation of the circadian system in melatonin-proficient C3H and melatonin-deficient C57BL mice: a comparative investigation. Cell Tissue Res 2002; **309**: 173–182.
21 Yellon SM, Tran LT. Photoperiod, reproduction, and immunity in select strains of inbred mice. J Biol Rhythms 2002; **17**:65–75.
22 Dhabhar FS, McEwen BS. Stress-induced enhancement of antigen-specific cell-mediated immunity. J Immunol 1996; **156**: 2608–2615.

23 Lovik M, Haugen OA, Closs O. Delayed-type hypersensitivity after immunization with ultrasonicated *Mycobacterium lepraemurium* in C3H and C57BL mice. Scand J Immunol 1984; **20**:227–235.
24 Lagrange PH, Hurtrel B. Local immune response to Mycobacterium lepraemurium in C3H and C57Bl/6 mice. Clin Exp Immunol 1979; **38**:461–474.
25 Darville T, Andrews CW, Jr., Laffoon KK, Shymasani W, Kishen LR, Rank RG. Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response. Infect Immun 1997; **65**: 3065–3073.
26 Kopp C, Vogel E, Rettori MC, Delagrange P, Guardiola-Lemaitre B, Misslin R. Effects of melatonin on neophobic responses in different strains of mice. Pharmacol Biochem Behav 1999; 63: 521–526.
27 Rodgers RJ, Haller J, Holmes A, Halasz J, Walton TJ, Brain PF. Corticosterone response to the plus-maze: high correlation with risk assessment in rats and mice. Physiol Behav 1999; **68**: 47–53.
28 Haller J, Leveleki C, Halasz J, Baranyi J, Makara GB. The effect of glucocorticoids on the anxiolytic efficacy of buspirone. Psychopharmacology (Berl) 2001; **157**:388–394.
29 Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav Immun 1997; **11**: 286–306.
30 Dhabhar FS, McEwen BS. Enhancing versus suppressive effects of stress hormones on skin immune function. Proc Natl Acad Sci U S A 1999; **96**:1059–1064.
31 Azpiroz A, Fano E, Garmendia L, Arregi A, Cacho R, Beitia G, Brain PF. Effects of chronic mild stress (CMS) and imipramine administration, on spleen mononuclear cell proliferative response, serum corticosterone level and brain norepinephrine content in male mice. Psychoneuroendocrinology 1999; **24**:345–361.
32 Cacho R, Fano E, Areso P, Garmendia L, Vegas O, Brain PF, Azpiroz A. Endocrine and lymphoproliferative response changes produced by social stress in mice. Physiol Behav 2003; **78**:505–512.