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ORIGINAL ARTICLE

Effect of immunization on nocturnal NAT activity in chicken pineal gland

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Abstract

OBJECTIVES: The effect of a single immunization with sheep red blood cells (SRBC) on the nocturnal NAT activity in the pineal gland and serum anti-SRBC agglutinin titer was examined in the young, sexually immature ASTRA S chickens.

METHODS: 3-, 4-, and 5-week-old birds of both sexes, hatched in long (Spring) and short (Autumn) day, were housed from hatching in controlled light (L:D = 12:12) and temperature conditions. Quantity of produced ^{14}C-labeled N-acetyltryptamine was used as a measure of the nocturnal NAT activity in the pineal gland. Serum anti-SRBC agglutinins were determined by microhemagglutination test.

RESULTS: In experimental conditions applied, a sexual dimorphism in both pineal gland function and immune system activity was observed. NAT activity was dependent on the season, sex and age of chickens examined and was modified by the single immunization with SRBC.

CONCLUSION: Results obtained confirmed once again the existence of the reciprocal functional connection between the pineal gland and immune system in chickens and demonstrated that even in well controlled experimental conditions the influence of the seasonal factor(s) on immunity must be taken into consideration.

Abbreviations

SRBC	sheep red blood cells
NAT	serotonin N-acetyltransferase
MEL	melatonin; N-acetyl -5-methoxytryptamine
PBS	phosphate buffered saline
AC	adenylate cyclase
cAMP	adenosine 3′,5′- cyclic phosphate
NA	noradrenaline
APCs	antigen presenting cells
IL-1	interleukin 1
IL-2	interleukin 2
IL-1R	interleukin 1 receptor

Introduction

Immunomodulatory potential of melatonin (MEL), a principal methoxyindole hormone produced in the rhythmical fashion by the pineal gland of all vertebrate species examined to date [1], was demonstrated in several experimental approaches [for review see 2, 3]. In laboratory rodents the immunostimulatory and anti-stress MEL effects seem to be well documented [for review see 4], whereas in other mammalian species, including man, the results are less consistent [for review see 5]. Despite of a huge number of papers published on this topic, the mechanism(s) involved in the immunoregulatory function of MEL is far from understood. Moreover, the experiments indicating the influence of the immune system activity on the pineal gland function are scarce, and, to date, only the effect of some cytokines on MEL level was demonstrated both *in vivo* and *in vitro* [6, 7, 8, 9].

Birds offer a very useful experimental model to examine a reciprocal interdependence between the pineal gland and immune system in both embryogenesis and during the immune response as well [for review see 10]. Actually, it was demonstrated that in the chicken early embryonic pinealectomy caused a decreased humoral immune response accompanied by the significant changes in the content of biogenic amines in the central nervous system and in the spleen [11]. On the other hand, early postnatal pinealectomy abolished the diurnal rhythm of both peripheral blood granulocyte number and serum lysozyme concentration in 5-week-old-cockerels, and both rhythms were restored by daily evening injections of MEL (but not vehicle) for 5 weeks in very low, physiological doses [12].

It is well known, since the classical experiments of Besedovsky and co-workers [13, 14], that immunization itself is recognized not only by the immune cells but also by the different structures within the body, leading to the changes in the spontaneous electrical activity of the central nervous system (hypothalamus) as well as in some blood hormone content (thyroxine, glucocorticoids). Therefore, it seemed worthwhile to examine whether the activation of the chicken immune system could evoke any functional changes within their pineal gland, i.e. in the activity of serotonin N-acetyltransferase (NAT), a key regulatory enzyme in MEL biosynthetic pathway [15]. The effect of a single immunization with the standard non-pathogenic thymo-dependent antigen (sheep red blood cells, SRBC) on the nocturnal level of pineal NAT activity was examined in the young chicken of both sexes, hatched at the spring and autumn, and housed from hatching under controlled light conditions L:D=12:12. Serum anti-SRBC agglutinin level and the number of antibody producing birds were used as a measure of the immune system activation.

Material and Methods

Animals

Experiments were performed on ASTRA S chickens of both sexes in two seasons: the longest day period (May/June, designated later as Spring) and the shortest day period (November/ December, Autumn). Birds were housed from hatching under controlled light (L:D = 12:12, lights on at 08.00) and ambient temperature (temperature of 32° $\pm$ 2°C during first week and a temperature of 24° $\pm$ 2°C thereafter), with free access to standard food and water. The experiments were carried out in accordance with the Polish regulations concerning the experiments on animals.

Experimental protocol

Two-, three-, and four-week-old chickens (5–8 birds per group), respectively, were immunized ip with SRBC at the beginning of the light period, as described previously [16]. The dose of SRBC was 0.5 ml of a 10% suspension per 100 g of BW. Six days later, chickens were sacrificed at midnight in dim-red light conditions. Intact, non-immunized birds at the respective ages were used as control groups. Blood from the jugular vein was taken for preparing serum, stored at –20°C, and used thereafter for the determination of anti-SRBC agglutinins by the microhemagglutination test [17]. Pineal glands were isolated, quickly frozen in liquid nitrogen and stored at –80°C before the dosage of NAT activity.

Pineal NAT activity assay

NAT activity was determined using a method of Deguchi and Axelrod [18] with the modifications described by Nowak et al. [19]. Pineal glands, pooled by 2–3 glands, were homogenized in 100 μL of ice-cold

0.05 sodium phosphate buffer (pH 6.8) under dim-red light. Aliquots of the homogenate were added to the substrate mix containing 1.5 nM of tryptamine-HCl, 152 μM of acetyl-coenzyme A both from Sigma Co., St. Louis, MO and 0.038 μCi of [^{14}C]acetyl coenzyme A (specific activity 50μCi/mmol; DuPont-NEN, Bad Hamburg, Germany). After 30 min. of incubation at 37°C, reaction was stopped by addition of 100 μl ice-cold 0.5 M borate buffer (pH 10.0) and 500 μL of water-saturated chloroform, which partitioned ^{14}C-labeled N-acetyltryptamine into organic layer. The chloroform extract was washed twice with 0.05 M phosphate buffer (pH 6.8) and 500 μL samples were transferred to the scintillation vials and evaporated at 40°C. Radioactivity was counted after addition of scintillation fluid in the Beckmann-Packard β-counter. NAT activity was expressed as μmoles of tryptamine acetylated per hour per pineal gland (μmoles/h/pineal).

Statistical analysis

Data were expressed as mean ± SEM and analyzed for statistical significance by nonparametric Mann–Whitney two-tailed U-test.

Results

Nocturnal NAT activity in the chicken pineal gland

Fig. 1. The highest NAT activity was found in spring in youngest males examined (9.96 ± 0.14 μmol/h/ pineal in 3-week-olds). In older males in the same season as well as in all male groups in autumn, the NAT activity was lower (from 2.11 ± 0.17 μmol/h/pineal to 3.9 ± 0.14 μmol/h/pineal), and did not differ significantly. In females the highest NAT activity was found in 4-week-old birds in both seasons (3.375 ± 0.77 μmol/h/pineal in spring and 8.76 ± 0.9 μmol/h/pineal in autumn, respectively), but only in autumn the differences between age groups were statistically significant. In general, the NAT activity in females was lower in the spring than in autumn.

Effect of immunization on NAT activity

Fig. 2. The best pronounced effect of immunization on pineal NAT activity was observed in the spring. An increase up to 390% and to 520% of control level was found in 3-week-old females and in 5-week-old males, respectively. In older female groups (4- and 5-week-olds) NAT activity was dimin-

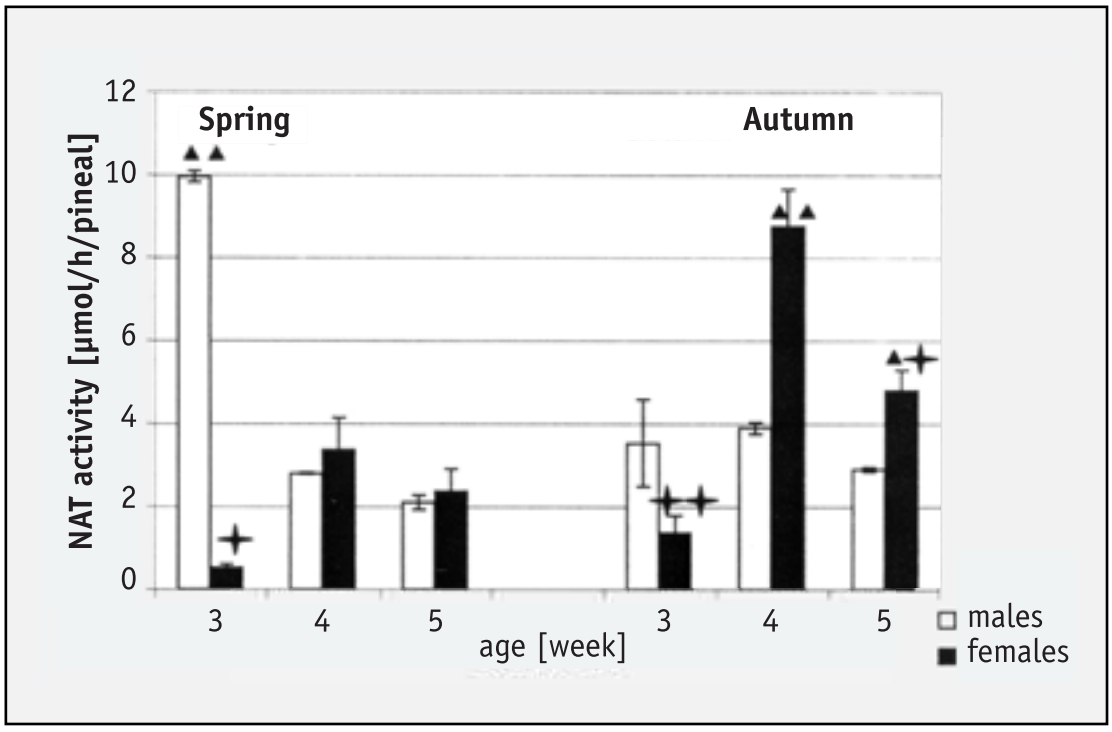


Fig. 1. Effect of sex, season and age on the nocturnal NAT activity in the chicken pineal gland (n = 8 birds per group in 2-week-olds and n = 5 per group in 3- and 4-week-olds)
▲▲ -p< 0.01 vs. another sex in the same age group
✦ -p< 0.05 vs. 4-week-old group in the respective season
✦✦-p< 0.01 vs. 4-week-old group in the respective season

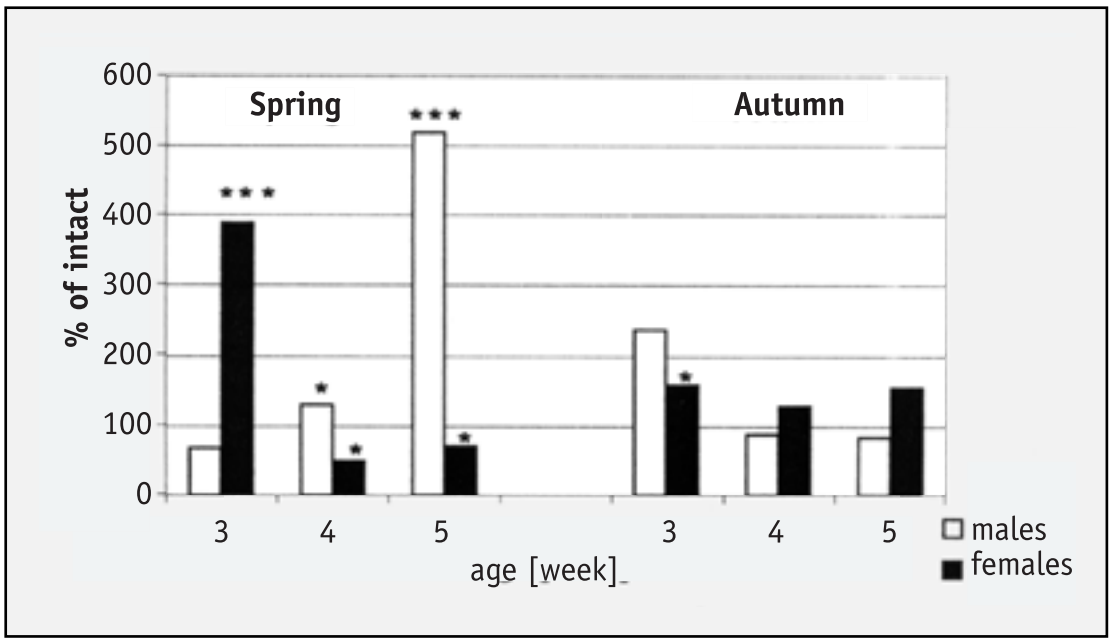
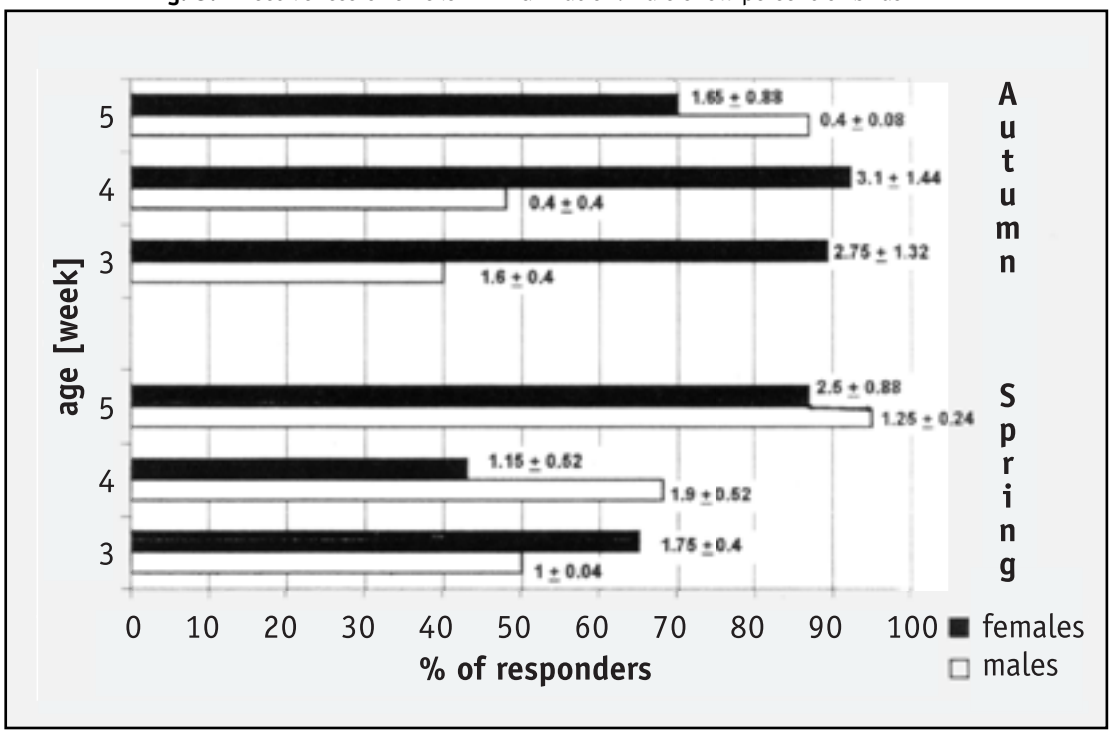


Fig. 2. Effect of immunization with SRBC on the nocturnal NAT activity in the chicken pineal gland (n = 8 birds per group in 2-week-olds and n = 5 per group in 3- and 4-week-olds). Results are expressed as a percent of activity found in nonimmunized birds in the same experimental group.
* - p< 0.05 intact vs. immunized group
*** - p< 0.001 intact vs. immunized group

Fig. 3. Effectiveness of chicken immunization. Bars show percent of birds



developing immune response to SRBC and numbers beyond respective bars show the serum anti-SRBC agglutinin titer expressed as the $\log_2$ of the last agglutinating serum dilution. (n = 8 birds per group in 2-week-olds and n = 5 per group in 3- and 4-week-olds).

ished significantly. In younger males the effect of immunization was significant (an increase) only in 4-week-olds. In autumn, immunization evoked an increase in NAT activity in 3-week-old birds, but it was significant only in females.

Effectiveness of immunization

Fig. 3. Primary antibody response to the same dose of antigen (SRBC) was not developed in all birds examined. The percent of responders varied, according to sex, season and age. In males in both seasons the percent of responders increased gradually in the function of age (from 40% in 3-week-olds to 87% in 5-week-olds in autumn, and from 50% to 95% in spring, respectively) and generally was higher in the spring. In females there was not so clear regularity—the number of responders was higher in autumn than in the spring (70–93% vs. 42–86%, respectively), but the dependence on the age was less pronounced. The lowest number of female responders was in spring in the 4-week-old group, whereas in autumn—in the oldest birds examined. Anti-SRBC agglutinin titer in serum was relatively low and the differences between groups were statistically nonsignificant. Only a tendency of the level to be higher in females (1.1–3.1) than in males (0.4–1.9) was seen, and the dependence on the season varied in both sexes. In females it was higher in autumn (1.65–3.1) than in spring (1.1–2.5), but in males the effect of season was opposite (1.0–1.9 in spring vs. 0.4–1.55 in autumn). The influence of age was not clear.

Discussion

Pineal NAT activity, a key enzyme in MEL synthesis pathway, remains under adrenergic control. In birds, however, in contrast with mammals, noradrenaline (NA) released from postsynaptic sympathetic terminals during the day acts via α2-adrenergic receptors and strongly inhibits adenylate cyclase (AC) activity and cAMP level, lead to the decrease in NAT activity [20, 21]. Thus, it is admissible that the changes observed by us in the pineal NAT activity might be, at least partly, caused by the inversely correlated changes in the level of NA. On the other hand, all agents stimulating pineal AC activity and cAMP level may cause an increase in NAT activity and MEL production [22, 23].

In experiments presented herein, NAT activity measured in chickens of both sexes was comparable with the results obtained previously by other authors [24, 25], and it was influenced by all factors examined: sex, season and age. In the youngest birds examined (3-week-olds), regardless of the season, males exhibited significantly higher NAT activity than females and this result is in agreement with the reports on the sexual dimorphism in NAT activity in the Syrian hamster Harderian gland [26] and in optic lobes of the giant freshwater prawn [27]. On the other hand, a sharp increase in NAT activity was observed in female chickens between 3rd and 4th week of life, regardless of the season. It could be attributed to the important decrease in the plasma progesterone concentration in the female chicken at that age, described by Vanmontfort et al. [28]. Actually, it was demonstrated in both mature and peripubertal female rats that estrogens exerted a suppressive effect on pineal AC activity [29], and a similar effect of the elevated progesterone level on MEL synthesis was described [30]. In the male chickens at the same age a progressive increase in testosterone concentration occurs [28], and it seems to be necessary to maintain the amplitude of the nocturnal melatonin peak [30]. To verify above mentioned suggestion the measurement of the developmental changes in the sex hormones concentration in the same strain of chickens are in progress in our laboratory.

The dependence of the mammalian immune system activity on the season is the well known phenomenon [for review see 5, 32], and it seems to reflect the physiological coping with the stressful external conditions. Winter suppression of immune function is observed in wild-living animals whereas in short-day laboratory conditions an enhancement of immune parameters seems to be the rule. The difference in reaction of the immune system of wild-living rodents vs. laboratory animals was considered in terms of the relative superiority of immunosuppressing stressful conditions in the former case vs. an immunoenhancing increase of melatonin synthesis in the latter [32]. Recently, some data indicating the existence of the interdependence between lighting conditions and immunity in avian species has been published as well. In particular, juvenile Japanese quails of both sexes in L:D conditions (both in L:D=8:16 and 16:8) exhibited the cellular and humoral immune response significantly greater than those in LL (continuous light) [33]. Moreover, in photostimulated male European starlings (housed for several weeks in L:D=18:6) immune functions measured as ConA-stimulated splenocyte proliferation were compromised compared with photorefractory ones [34]. This last result was interpreted in terms of the direct effect of reproductive state on immune function. On the other hand, a sexual dimorphism of immune system function, with a more efficient one in females, has been already well accepted [35]. In experiments presented here, chickens hatched in different seasons (late spring, i.e. long days and late autumn, i.e. short days), and

housed from hatching under artificial photoperiod L:D=12:12 exhibited the well pronounced sex- and season-dependent differences in the immune response against the same T-dependent non-pathogenic antigen, SRBC. In females, the efficiency of immunization, measured by both percent of responders and a serum antibody titer, was higher in winter than in spring (70–93% and 1.65–3.1 *vs.* 42–86% and 1.1–2.5, respectively). In males, the response evoked by the same immunization was weaker, and the influence of the season was not so clear, but generally immunization was more efficient in the spring (50–95% and 1.0–1.8 *vs.* 40–83% and 0.4–1.6, respectively). Moreover, in males the number of responders increased with age from 3- to 5-week olds, indicating a progressive maturation of the chicken immune system. Similar regularity was not seen in females. Results obtained herein support the existence of the sexual dimorphism in chicken immune system and their dependence on the season, even in artificial intermediate L:D=12:12 lighting conditions. An explanation of the last result is difficult at present, especially in comparison with the results obtained recently by Demas and Nelson [36], that immune function is enhanced in short days in both female and male deer mice and this effect does not appear to be due to reduction in circulating steroids.

Above mentioned differences in the chicken immune response are reflected by the effect of immunization on the pineal NAT activity. Actually, immunization itself influenced nocturnal synthesis of MEL, but there was no simple correlation between the efficiency of immunization and NAT activity. Introduction of a particulate, thus thymo-dependent, antigen into the vertebrate's body (immunization, infection) is followed by a sequence of processes, leading to the cytokine secretion and antibody synthesis. The humoral factors secreted by activated immune cells are numerous, and their messages are understood not only for the immune cells but also for the other parts of the body. There are some indications that they could be transmitted also into the pineal gland. Namely, it was demonstrated by Mucha et al. [6] that the treatment of the male rats with IL-1 caused a dose-dependent decrease in the serum MEL content and this effect was reversed by an injection of the antibody against IL-1 receptors (IL-1R). These results indirectly suggest that rat pinealoctyes may express the membrane-bound IL-1 receptors which are, on the other hand, linked with protein G leading to the increased levels of intracellular cAMP [37]. It is admissible that cytokine receptors could be expressed on the chicken pinealocytes as well as making them sensitive to the message(s) derived from the immune system. Therefore, the results presented here, indicating that immunization may cause an increase, a decrease or no effect of the nocturnal NAT activity, suggest that the immune cell-derived signal(s), transduced into the chicken pineal gland may be modified by several factors (e.g. gonadal steroids), other than simple binding of IL-1 to its receptors on the pinealocyte membrane.

In conclusion, we wish to emphasize the existence of the direct effect of immunization on the biosynthetic activity of the chicken pineal gland, and, on the other hand, the dependence of the immune system as well as the pineal gland activity of the young, sexually immature chicken, on sex, age and season. The last observation seems to be particularly important because the chickens examined were hatched at the different seasons (spring *vs.* autumn), and immediately thereafter they were kept in the same, controlled laboratory conditions. The results obtained suggest that in chickens information on the external lighting conditions may be transmitted, probably *via* maternal hormones deposed in the egg, to the developing embryo, and hatched bird, influencing thereby some seasonally changing physiological functions, including the pineal gland and immune system activity.

REFERENCES

1 Collin JP, Voisin P, Falcon J, Faure J, Brisson P, Defaye JR. Pineal transducers in the course of evolution: molecular organization, rhythmic metabolic activity and role. Arch Histol Cytol 1989; Suppl **52**:441–49.
2 Skwarlo-Sonta K. Functional connection between the pineal gland and immune system. Acta Neurobiol Exp 1996; **56**: 341–57.
3 Liebmann PM, Wolfler A, Felsner P, Hofer D, Schauenstein K. Melatonin and the immune system. Int Arch Allergy Immunol 1997; **112**:203–11.
4 Maestroni GJM. The immunoendocrine role of melatonin. J Pineal Res 1993; **14**:1–10.
5 Nelson RJ, Demas GE, Klein SL, Kriegsfeld LJ. The influence of season, photoperiod, and pineal melatonin on immune function. J Pineal Res 1995; **19**:149–65.
6 Mucha S, Zylinska K, Zerek-Melen G, Swietoslawski J, Stepien H. Effects of interleukin-1 on in vivo melatonin secretion by the pineal gland in rats. Adv Pineal Res 1994; **7**:177–81.
7 Withyachumnarnkul B, Nonaka KO, Attia AM, Reiter RJ. Changes in indole metabolism in organ cultured rat pineal glands induced by interferon-γ. J Pineal Res 1990; **8**:313–22.
8 Withyachumnarnkul B, Reiter RJ, Lerchl A, Nonaka KO, Stokkan KA. Evidence that interferon-γ alters pineal metabolism both indirectly via sympathetic nerves and directly on the

pinealocytes. Int J Biochem 1991; **23**:1397–401.
9 Zylinska K, Komorowski J, Robak T, Mucha S, Stepien H. Effect of granulocyte-macrophage colony stimulating factor on melatonin secretion in rats. In vivo and in vitro studies. J Neuroimmunol 1995; **56**:1187–90.
10 Skwarlo-Sonta K. Reciprocal interdependence between pineal gland and avian immune system. Neuroendocrinol Lett 1999; **20**:151–56.
11 Jankovic B, Kniezevic Z, Koji L, Nikoli V. Pineal gland and immune system. Immune functions in the chick embryo pinealectomized at 96 hours of incubation. Ann NY Acad Sci 1994; **719**:398–409.
12 Rosolowska-Huszcz D, Thaela MJ, Jagura M, Stepien D, Skwarlo-Sonta K. Pineal influence on the diurnal rhythm of nonspecific immunity indices in chickens. J Pineal Res 1991; **10**:190–95.
13 Besedovsky H, Sorkin E. Changes in blood hormone levels during the immune response. Proc Soc Exp Biol Med 1975; **150**:466–70.
14 Besedovsky H, Sorkin E, Felix D, Haas H. Hypothalamic changes during the immune response. Eur J Immunol 1977; **7**:323–25.
15 Reiter RJ. Neuroendocrinology of melatonin. In: Miles A, Philbrick DRS & Thompson C, editors. Melatonin-Clinical Perspectives. Oxford: Oxford University Press; 1988. p.1–42.
16 Skwarlo-Sonta K, Thaela MJ, Midura M, Lech B, Gluchowska B, Drela N, et al. Exogenous melatonin modifies the circadian rhythm but does not increase the level of some immune parameters in the chicken. J Pineal Res 1992; **12**:27–34.
17 Graetzer MA, Wolfe HR, Aspinal RL, Mayer RK. Effect of thymectomy annd bursectomy on precipitin and natural hemagglutinin production in the chicken. J Immunol 1963; **90**:878–87.
18 Deguchi T, Axelrod J. Sensitive assay for serotonin N-acetyltransferase activity in rat pineal gland. Analyt Biochem 1972; **50**:174–79.
19 Nowak JZ, Zurawska E, Zawilska J. Melatonin and its generating system: circadian rhythm, effect of environmental lighting, and interaction with dopamine. Neurochem Int 1989; **14**:397–406.
20 Voisin P, Collin JP. Regulation of chicken pineal arylalkylamine-N-acetyltransferase by postsynaptic α2-adrenergic receptors. Life Sci 1986; **39**:2025–32.
21 Zawilska JB, Rosiak J, Trzepizur K, Nowak JZ. The effects of near-ultraviolet light on serotonin N-acetyltransferase activity in the chick pineal gland. J Pineal Res 1999; **26**:122–27.
22 Takahashi JS, Murakami N, Nikaido SS, Pratt BL, Robertson LM. The avian pineal, a vertebrate model system of the circadian oscillator: cellular regulation of circadian rhythms by light, second messengers and macromolecular synthesis. Recent Prog Horm Res 1989; **45**:279–352.
23 Mackova M, Lamosova D, Zeman M. Regulation of rhythmic melatonin production in pineal cells of chick embryo by cyclic AMP. Cell Mol Life Sci 1998; **54**:461–66.
24 Binkley S, Mosher K, White BH. Photoperiod modifies daily maps of light and dark sensitivity for N-acetyltransferase activity in pineal glands of 3-week-old *Gallus domesticus*. J Comp Physiol B 1989; **159**:37–42.
25 Doi O, Koyama W, Nakamura T, Tanabe Y. Photoperiodic regulation of serotonin N-acetyltransferase activity in the pineal gland of the chicken. Comp Biochem Physiol A 1983; **74**:195–8.
26 Menendez-Pelaez A, Reiter RJ, Guerrero JM, Puig-Domingo M, Howes KA. Sexual dimorphism in N-acetyltransferase activity, hydroxyindole-O-methyltransferase activity and melatonin content in the Harderian gland of Syrian hamster: Changes following gonadectomy. Proc Soc Exp Biol Med 1988; **187**:287–91.
27 Withyachumnarnkul B, Ajpru S, Rachawong S, Pongsa-Asawapaiboon S, Sumritdthong A. Sexual dimorphism in N-acetyltransferase and melatonin levels in the giant freshwater prawn *Macrobrachium rosenbergii* de Man. J Pineal Res 1999; **26**:174–77.
28 Vanmontfort D, Berghman LR, Rombauts L, Verhoeven G, Decuypere E. Developmental changes in immunoreactive inhibin and FSH in plasma of chickens from hatch to sexual maturity. Poultry Sci 1995; **36**:779–90.
29 Hayashi K, Okatani Y. Mechanisms underlying the effects of estrogen on nocturnal melatonin synthesis in peripubertal female rats: Relation to norepinephrine and adenylate cyclase. J Pineal Res 1999; **26**:1178–83.
30 Alonso-Solis R, Abreu P, Lopez-Coviella I, Hernandez G, Fajardo N, Hernandez-Diaz F, et al. Gonadal steroid modulation of neuroendocrine transduction: a transynaptic view. Cell Mol Neurobiol 1996; **16**:357–82.
31 Nowak JZ, Zawilska JB. Melatonin and its physiological and therapeutic properties. Pharm World Sci 1998; **20**:18–27.
32 Nelson RJ, Demas GE. Seasonal changes in immune function. Q Rev Biol 1996; **71**:511–48.
33 Moore CB, Siopes TD. Effects of lighting conditions and melatonin supplementation on the cellular and humoral immune responses in Japanese quail *Coturnix coturnix japonica*. Gen Comp Endocrinol 2000; **119**:95–104.
34 Bentley GE, Demas GE, Nelson RJ, Ball GF. Melatonin, immunity and cost of reproductive state in male European starlings. Proc R Soc Lond B 1998; **265**:1191–5.
35 Grossman CJ, Roselle GA, Lendenhall CL. Sex steroid regulation of autoimmunity. J Steroid Biochem Molec Biol 1991; **40**:649–59.
36 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**:419–26.
37 Munoz E, Zubiaga AM, Sims JE, Huber BT. IL-1 Signal transduction pathways. I. Two functional IL-1 receptors are expressed in T cells. J Immunol 1991; **146**:136-43.