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The Effect of Lighting Program and Melatonin on the Alleviation of the Negative Impact of Heat Stress on the Immune Response in Broiler Chickens

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Abstract: Two experiments were conducted to study the effect of lighting program and melatonin addition to the diet on the immune responses of broiler chickens under chronic heat stress. In the first experiment, two groups of male broiler chickens received Continuous Light (CL) (23L: 1D) while another two groups received Intermittent Light (IL) (1L: 3D). From 4 to 6 wk of age, a group from each light program was exposed to 35°C versus 24°C for the other two groups. Heat stressed chickens under IL had significantly lower ($p<0.05$) body temperatures, pro-inflammatory cytokines levels and corticosterone concentrations in their plasma, compared to the heat stressed chickens under CL. Furthermore, in the heat stressed groups, the IL group had a significantly higher ($p<0.05$) cutaneous basophil hypersensitivity response and T-cell proliferation, compared to the CL group. In the second experiment, two groups of male broiler chickens were fed a diet containing melatonin (40 ppm), while the other two groups received a melatonin free diet (0 ppm), from 4 to 6 wk of age. Concurrently, a group from each melatonin treatment was exposed to 35°C versus 24°C in the other group. The heat stressed chickens receiving melatonin had significantly lower ($p<0.05$) body temperatures, pro-inflammatory cytokines IL-1 and IL-6 and corticosterone concentrations. Furthermore, in the heat stressed birds, the melatonin group had a higher but not significant cutaneous basophil hypersensitivity response and T-cell proliferation. The current study indicates that intermittent light and melatonin administration can be used to ameliorate immunosuppression associated with heat stress in broiler chickens.

Key words: Light, melatonin, heat stress, broilers, immunity

Introduction

The negative impact of high environmental temperature on the poultry industry has been well established. Exposure of broiler chickens to chronic heat stress leads to the deterioration of most production parameters (Bonnet *et al*., 1997; Cooper and Washburn, 1998). In addition exposure to high environmental temperatures will compromise most immune functions in chickens; decreasing total White Blood Cells (WBC) count in the circulation (Nathan *et al*., 1976), decreasing CD4+T and CD8+T-cells in the circulation (Trout and Mashaly, 1994), decreasing antibody titers to sheep red blood cells (Bartlett and Smith, 2003) and decreasing T and B-cells mitogenic proliferation (Atta *et al*., 1996). Furthermore, exposure to high environmental temperature will activate the hypothalamo-pituitary-adrenal-axis in chickens culminating in increased plasma corticosterone levels (Nathan *et al*., 1976). Glucocorticoids have a suppressive effect on many immune functions (Mc Ewen *et al*., 1997). Increasing the scotoperiod in poultry light programs was reported to have a positive immunomodulatory effect. Kliger *et al*. (2000) reported that using an intermittent instead of a constant light program can have an immune enhancing effect on broiler chickens. Moore and Siopes (2000) reported improved cellular and humoral immune responses for Japanese quail raised under decreasing photoperiods compared to quail raised under constant light condition. Chickens raised under intermittent light programs are likely to have higher serum melatonin levels than chickens raised under constant light programs. Melatonin is a hormone secreted in the dark from the pineal gland and sets the internal biological clock that governs different daily and seasonal cycles or rhythms in various physiological systems in birds (Pang *et al*., 1996). Kliger *et al*. (2000) reported that *in vitro* melatonin addition enhanced T and B cells mitogenic activity of broiler chickens raised under constant light conditions. Brennan *et al*. (2002) also reported enhanced mitogenic activity for T and B cells as well as increased total WBC counts following the subcutaneous injection of melatonin in chickens. Adding melatonin to the drinking water improved cellular and humoral immune responses of Japanese quail raised under constant light conditions (Moore and Siopes, 2000). Furthermore, melatonin addition to the diet was reported to ameliorate the negative effects of heat stress on performance parameters in Japanese quail (Sahin *et al*., 2003).

There is a wealth of literature on possible techniques that can be utilized to alleviate the adverse effects of heat stress in broiler chickens. However, little is known about the extent to which photoperiodic changes and melatonin can redress the negative effects of chronic heat stress on the immune response in broilers. Hence, this study was carried out to investigate whether applying an intermittent light program or melatonin addition to broilers' diets can ameliorate the negative effects of chronic heat stress on the immune response in broiler chickens.

Materials and Methods

Experimental protocol: This study was carried out at the Poultry Educational and Research Center (PERC) of The Pennsylvania State University. The study consisted of two experiments. In the first experiment, the effect of lighting program on the immune response of heat stressed broiler chickens was investigated. In the second experiment, the effect of melatonin addition to the diet on the immune response of heat stressed broiler chickens was investigated. Both Experiments were conducted using male broiler chickens and lasted for 6 wks.

Experiment. I

Experimental design: Three hundred and twenty one-day-old male broiler chicks (Cobb×Cobb) obtained from a local hatchery were used. The chicks were housed in battery cages with *ad libitum* access to water and feed that met NRC (1994) recommendations. Chicks were randomly assigned into four equal experimental groups to four environmental chambers located at the PERC. All groups received 24-hr lighting for the first three days. From the fourth day to the end of the experiment, the first and second group were exposed to continuous light (CL) (23Light:1Dark), whereas the third and fourth group were exposed to Intermittent Light (IL) (1Light:3Dark). A brooding temperature of 33°C was maintained for the first 3 days then it was decreased to 30°C for the rest of the first wk. The temperature was reduced by an increment of 2°C a wk until the third wk of age. From the beginning of the fourth week to the end of the experiment, the second and fourth groups were exposed to 35°C, whereas the first and third groups were exposed to 24°C. Relative humidity was set at 50% in the four chambers.

Experiment II

Experimental design: One hundred and sixty one-day old male broiler chicks (Cobb×Cobb) were obtained from a local hatchery. The chicks were housed in battery cages with *ad libitum* access to water and feed that met NRC (1994) recommendations. Chicks were randomly assigned into four equal experimental groups to two environmental chambers located at the PERC. All groups received 24h of light for the first 3 days, then were exposed to 23L:1D from the fourth day until the end of the experiment. The brooding temperature was 33°C for the first three days and then it was decreased to 30°C for the rest of the first wk. The temperature was reduced by an increment of 2°C a wk until the third wk of age. From the beginning of the fourth wk to the end of the experiment, the third and fourth groups were exposed to 35°C, whereas the first and second group were exposed to 24°C. Furthermore, from the beginning of the fourth wk to the end of the experiment, the second and fourth groups received 40 ppm melatonin in the feed, whereas the first and third groups received no melatonin (0 ppm). Relative humidity was set at 50% in the two chambers.

Measurements

Body temperature: Body temperature for birds in the different groups in both experiments were measured using a thermocouple rectal thermometer with a 3-cm insertion probe at 6 wk of age. Time of measurement was set to be in the middle of a light cycle for chickens exposed to intermittent light in the first experiment.

Blood collection: At 6 wk of age, blood samples from chickens in the different groups in both experiments were obtained and the plasma was divided into multiple aliquots and stored in-20°C until assayed. Chickens from all groups in the first experiment were bled twice. The first bleeding was set to be in the middle of a dark cycle and the plasma samples were used for hormonal assays. The second bleeding was set to be in the middle of a light cycle and the plasma samples were used for both cytokines and hormonal assays. The time elapsing between the first and second bleeding was >12 h. However, chickens in the second experiment were bled once in light conditions. For the T-cell proliferation assay, five mL of blood were collected from different chickens from all the groups in the two experiments. Blood was collected in the middle of a light cycle from chickens' of the first experiment.

Hormonal assay: Corticosterone and melatonin were measured using radioimmunoassay (RIA) kits (Gehad *et al.*, 2002).

Cytokine assays

IL-1 like activity in the plasma: IL-1-like activity in the plasma was measured using the thymocyte comitogensis assay as previously described by Korver and Klasing (1997) and Gehad *et al.* (2002). This assay is based on measuring the ability of IL-1 to enhance thymocytes proliferation. Briefly, thymocytes, at a concentration of 40×10^6/ml live cells, were prepared using thymuses of 6 wk-old broiler chickens from a different hatch. Plasma samples were diluted 1:1 using 24% polyethylene glycol (PEG) to yield a total volume of

600 µL per tube. One hundred µL of diluted plasma samples were added to each well of a 96-well round bottom plate, then the samples were serially diluted until the concentration of PEG was 1.5% in the sample using RPMI 1640 medium plus 5% Fetal Bovine Serum (FBS). Fifty µL of PHA-P (20 mg/ml) and another 50 µL of the thymocyte suspension were added to the wells. Control wells received 100 µL of RPMI 1640 5% FBS, 1% L-glutamine and 3% PEG instead of diluted plasma. The cells were incubated for 48 h at 41°C in 5%CO_2, then 50 µl of ^{3}H-thymidine was added to all the wells to give an activity of 1 µci/well. Cells were incubated for an additional 18 h, harvested onto glass fiber filters and ^{3}H-thymidine uptake was measured as counts per min. (cpm) using a scintillation counter.

Interleukin-1 activity was reported as a stimulation index, which is the ratio of the cpm of the thymocytes incubated with the plasma 3% PEG (IL-1 source) to the thymocytes incubated in the medium 3% PEG (no IL-1).

TNF-like activity in the plasma: This assay is based on the ability of TNF to induce the spontaneous lysis of murine L929 fibroblasts (Flick and Gifford, 1984; Gehad *et al.*, 2002). Briefly, L929 murine fibroblast cells were plated at a concentration of 4×10^4 cells in 100 µL RPMI 1640 5% FBS and L-glutamine in 96-well plates and allowed to adhere overnight at 37°C in 5%CO_2. The next day, 100 µL of 1:2 RPMI diluted plasma and another 100 µL of actinomycin D (2 mg/ml in RPMI 1640 5% FBS) were added to each well. Control wells received 100 µL of RPMI with 5% FBS instead of diluted plasma plus 100 µL of actinomycin D. Maximum cytolysis wells received 100 µL of 0.5% Triton-100 plus 100 ml of actinomycin D. The cells were then incubated for 18 h at 37°C in 5%CO_2, after which the supernatant was removed and replaced with 100 µL of MTT dye (1 mg/ml in sterile PBS). The cells were incubated for another hour, then 100 µL of extraction buffer (sodium dodecyl sulphate and dimethylformamide) was added to the cells and incubated for another 20 h. Plates were then read at 595 nm wave length. TNF activity in plasma was expressed as percent cytotoxicity against L929 using the following equation described by Zhang *et al.* (1995).

Percentage Cytotoxicity = (A-B)/(A-C)×100, where:
A = Optical density of control wells
B = Optical density of sample wells
C = Optical density of wells receiving 0.5% Triton-100

IL-6 bioassay: The IL-6 levels of plasma samples were measured using an IL-6 dependent murine hybridoma 7TD1 cells (Shuster *et al.*, 1993). Plasma samples were diluted 1:10 with RPMI 1640 medium. One hundred uL of diluted plasma were added to 7TD1 cells (1×10^6 cells/100 uL/well), whereas control wells received 100 µL of RPMI 1640 5% FBS and L-glutamine. The culture plates were incubated for 44h at 37°C in 5% CO_2. Following incubation, 25 uL of MTT dye (4mg/ml in PBS), was added to all wells and the plates were incubated for an additional 4h. Following MTT incubation, 100 µL of medium were removed and another 100 uL of 2-propanol/0.4N HCl were mixed into each well. The absorbance was determined at a wave length of at 550 nm, using an automated plate reader and software. The concentrations of IL-6 in plasma samples were calculated from a standard curve generated in the same assay using recombinant human IL-6.

Cell mediated immune responses

Peripheral blood T-cells proliferation response to Concanavalin-A mitogen: Briefly, 100 µL whole blood was diluted thirty times with RPMI 1640 medium 10% FBS. One hundred µL of diluted blood was plated in triplicate wells in 96-well round-bottom plates. Fifty µL of Concanavalin-A (Con-A) (50 µg/ml) was added to selected wells, while control wells received 50 µL of RPMI 1640 10% FBS. The cultures were incubated for 48 h at 42°C in 5% CO_2. Following incubation, 50 µL of ^{3}H-thymidine (2 µci/well) was added to each well. Eighteen hours later, the cultures were harvested onto glass fiber filter paper. ^{3}H-thymidine uptake was measured as counts per min. (cpm) using a scintillation counter to determine T-cell proliferation. The net cpm was obtained by subtracting the mean cpm of the control wells from the mean cpm of its corresponding mitogen wells.

The Cutaneous Basophil Hypersensitivity response (CBH): At 6 wk of age, five birds from each experimental group in the two experiments were injected with 100 µg/100 µL Phytohemagglutinin-P (PHA-P) intradermally into the wattle (Brake *et al.*, 1988). The wattle of the other side received Phosphate Buffer Saline (PBS) to serve as a control. Wattle thickness was measured before and 24 h post PHA-P and PBS injections using a digital micrometer. The CBH response was expressed in cm as the difference between the wattle thickness before and after injection with PHA-P.

Statistical analysis: The general linear models procedures (SAS Institute, 1996) was used to analyze data with two-way analysis of variance with environmental temperature and light program as the main effects in the first experiment and environmental temperature and dose of melatonin as the main effects in the second experiment. Means were separated for significance using Duncan's multiple-range test with significance set at $p<0.05$.

Results

Body temperature: Chronic Heat Stress (CHS) from 4-6 wk of age significantly increased broiler chickens body temperatures regardless of the light program applied or

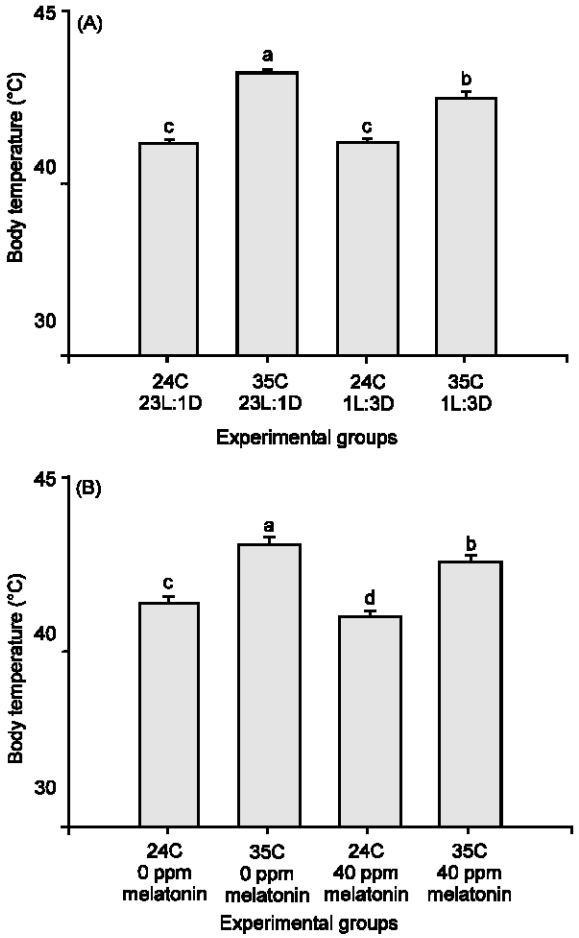

Fig. 1: The effect of light program (A) and melatonin addition to the diet (B) on body temperature of 6 wk old broiler male chickens exposed to chronic heat stress from 4-6 wk. Bars are means+SE. Bars with common letters are not significantly different ($p>0.05$) (n = 8)

melatonin addition compared to chickens housed under control temperatures (Fig. 1 a and b). Chickens raised under the Intermittent Light program (IL) and exposed to CHS had significantly lower body temperatures than chickens raised under the Constant Light program (CL) and exposed to CHS (Fig. 1a), but still significantly higher than those chickens in control temperatures regardless of the light program (Fig. 1a). Melatonin addition was able to significantly decrease body temperature in chickens exposed to CHS compared to chickens exposed to CHS with no melatonin added (Fig. 1b). Moreover, melatonin addition significantly lowered body temperature of chickens in control temperatures when compared to chickens under control temperature with no melatonin added (Fig. 1b).

Pro-inflammatory cytokines: There were no significant differences in the levels of pro-inflammatory cytokines (IL1, IL-6 and TNF) in the plasma of control temperature chickens regardless of light program or melatonin addition (Fig. 2 a, b, c, d, e and f). Exposure of chickens to CHS under CL significantly increased the plasma levels of the pro-inflammatory cytokines (IL-1, IL-6 and TNF) compared to the other three groups (Fig. 2 a, b and c). However, chickens under IL and exposed to CHS had significantly lower plasma levels of pro-inflammatory cytokines than chickens under CL and exposed to CHS (Fig. 2 a, b and c).

Melatonin addition to the diet significantly decreased the levels of the pro-inflammatory cytokines; IL-1 and IL-6 in the plasma of CHS chickens compared to their levels in CHS chickens with no melatonin added to the diet (Fig. 2 d and e). Tumor necrosis factor levels were slightly lower in CHS chickens with melatonin added to their diets but not significant from CHS chickens with no melatonin added to their diets (Fig. 2f).

Cell-mediated immune responses: Exposure to CHS significantly inhibited *in vitro* T-cell proliferation in response to Con-A mitogen compared to chickens in control temperatures regardless of light program or melatonin addition (Fig. 3 a and b). Chickens exposed to CHS under IL exhibited significantly higher T-cell proliferation than chickens exposed to CHS under CL, however, they were still significantly lower than chickens in control temperatures under either CL or IL (Fig. 3a). Furthermore, chickens under IL in control temperatures exhibited significantly higher T-proliferation than chickens under CL in control temperatures (Fig. 3a).

Melatonin added to the diet significantly increased the proliferation of T-cells in chickens raised in control temperatures (Fig. 3b) On the other hand, in CHS chickens where T-cell proliferation was inhibited, melatonin addition to the diet did not result in a significant increase of T-proliferation (Fig. 3b).

The cutaneous basophil hypersensitivity response as measured by increases in wattle thickness 24h following PHA-P injection was also used to test cellular immune responses. Chickens exposed to CHS under CL had a significantly lower CBH response, whereas in chickens exposed to CHS under IL this response was restored to the levels of control temperature chickens (Fig. 4a). Melatonin supplementation was able to significantly increase the CBH response in control temperature chickens compared to the other groups (Fig. 4b). Exposure to CHS significantly decreased the CBH response in chickens that did not receive melatonin. However, melatonin addition restored the response to the level observed in control temperature chickens.

Hormones

Corticosterone: In the middle of the dark cycle within the same light program, chickens exposed to CHS had significantly higher levels of corticosterone in their plasma than chickens in control temperatures (Fig. 5a).

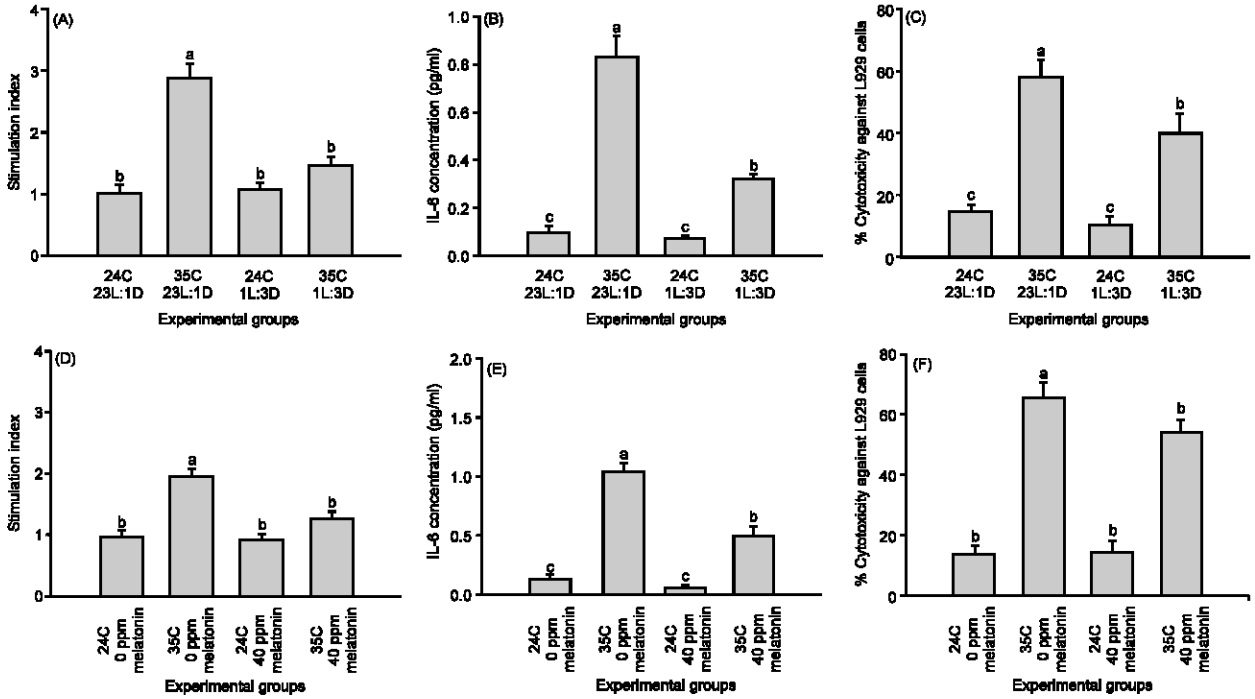

Fig. 2: The effect of light program (A, B, C) and melatonin addition to the diet (D, E, F) on the plasma levels of pro-inflammatory cytokines of 6 wk old broiler male chickens exposed to chronic heat stress from 4-6 wk. Bars are means+SE. Bars with common letters are not significantly different (p>0.05) (n = 5)

Table 1: The Effect of Melatonin on plasma melatonin levels (ng/ml) of 6 wk old broiler male chickens exposed to chronic heat stress from 4-6 wk

0ppm		40ppm	
24°C	35°C	24°C	35°C
7±2.95^{c}	14±3.56^{c}	4077±370^{a}	591±55.93^{b}

Values are means±SE. Values with no common letters are significantly different (p<0.05) n = 5

In the middle of the dark period, chickens exposed to CHS under IL had lower levels of corticosterone in their plasma than chickens exposed to CHS under CL but not statistically significant. In the middle of the light cycle, there were no significant differences in plasma corticosterone levels among groups, except for the group exposed to CHS under CL that had significantly higher corticosterone levels than all other groups.

Exposure to CHS significantly increased corticosterone levels in the plasma regardless of melatonin addition (Fig. 5b). Melatonin addition to the diet significantly decreased corticosterone levels in CHS chickens compared to CHS with no melatonin added to the diet.

Melatonin: In the middle of the dark cycle within the same light program, chickens exposed to CHS had significantly higher levels of melatonin in their plasma than chickens in control temperatures (Fig. 6). In the middle of the light cycle, there were no significant differences in plasma melatonin levels among groups except for the group exposed to control temperatures under CL that had significantly lower melatonin levels than all other groups. Melatonin addition to the diet resulted in high levels of melatonin in the plasma (Table 1). Chickens exposed to CHS with melatonin added to their diets had significantly lower levels of melatonin in their plasma than chickens in control temperatures with melatonin added to their diets.

Discussion

Exposure of broiler chickens to chronic heat stress negatively affected their immune response. In accordance with an earlier report by Cooper and Washburn (1998), exposure to CHS significantly increased chickens' body temperatures. Body temperature is not an immunological parameter *per se*, however it is a good indicator when testing for methods that can effectively ameliorate the negative impact of heat stress. Broiler chickens exposed to CHS under IL had significantly lower body temperatures than chickens exposed to CHS under CL. Intermittent light can be influencing body temperature directly by reducing energy expended on muscular effort (MacLeod *et al.*, 1988) or indirectly through enhancing melatonin secretion (Rozenboim *et al.*, 1998). Since the duration of melatonin release is proportional to the length of the dark phase (Illnerova *et al.*, 1984), it was reasonable to

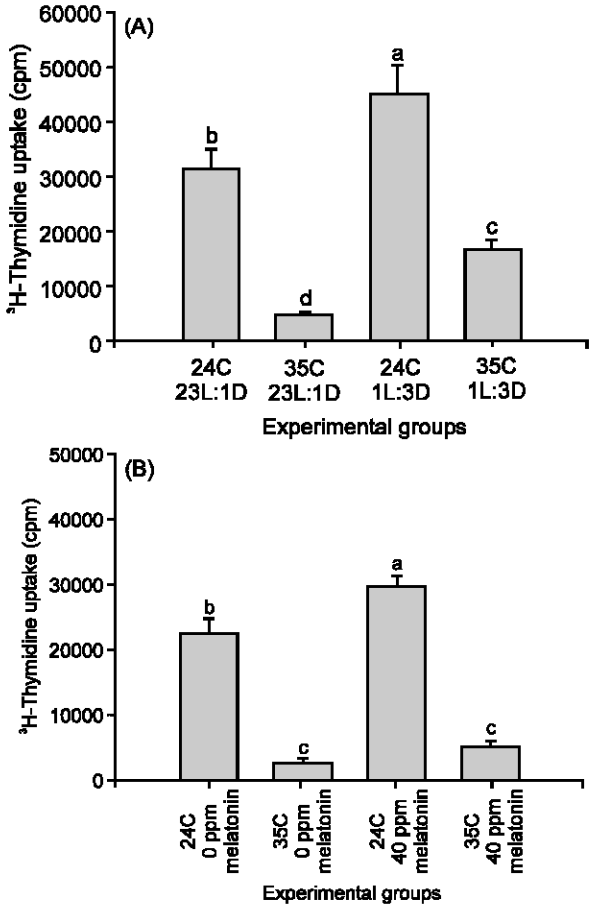

Fig. 3: The effect of light program (A) and melatonin addition to the diet (B) on the proliferation of peripheral blood T-cells in response to Con-A mitogen of 6 wk old broiler male chickens exposed to chronic heat stress from 4-6 wk. Bars are means+SE. Bars with common letters are not significantly different (p>0.05) (n = 8)

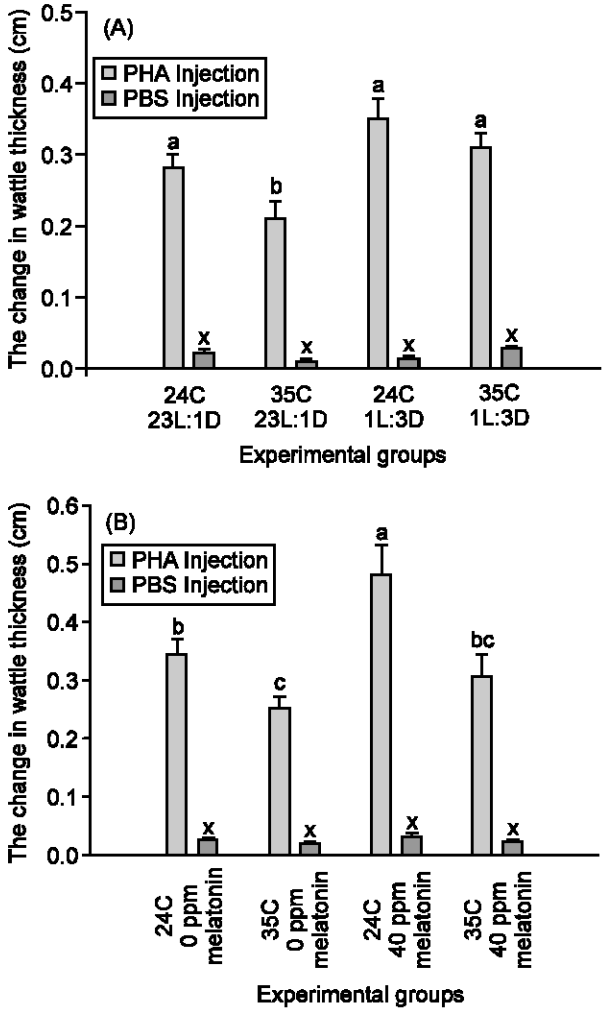

Fig. 4: The effect of light program (A) and melatonin addition to the diet (B) on the cutaneous basophilic hypersensitivity response to PHA-P of 6 wk old broiler male chickens exposed to chronic heat stress from 4-6 wk. Bars are means+SE. Bars with common letters within PHA or PBS treatments are not significantly different (p>0.05) (n = 5)

hypothesize that IL would raise plasma melatonin levels in chickens under IL and thereby induce hypothermia as reported by Rozenboim *et al.* (1998). However, plasma melatonin levels measured at 6 wk were not significantly different between CHS chickens in either light program, although numerically higher in CHS chickens under IL. Interestingly, melatonin levels in the dark were significantly higher in CHS chickens under CL than chickens exposed to control temperatures under CL.

Increased melatonin secretion can be a defense mechanism against heat stress and other types of stress and chickens exposed to CHS may tend to increase their melatonin secretion during dark hours. Maximal melatonin secretion in the rat occurred when temperature increased from 36°C to 40°C and 43.3°C (Zatz *et al.*, 1994). Plasma melatonin has also been reported to increase in rats exposed to cold restraint stress and was suggested to be involved in physiological adaptation to the given stress (Oxenkrug and McIntyre, 1985). Melatonin addition was able to significantly decrease body temperature in chickens exposed to CHS compared to chickens exposed to CHS with no melatonin added. Rozenboim *et al.* (1998), were able to induce hypothermia in layers with a single melatonin injection in a dose dependent manner. Injection of melatonin 30 minutes prior to heat exposure protected against heat stress induced hyperthermia, whereas melatonin injection 30 minutes past heat exposure failed to do so. In our study, melatonin was added to the diet at the time of the onset and throughout the heat treatment and chickens feeding on melatonin added diets acquired exceptionally high levels of

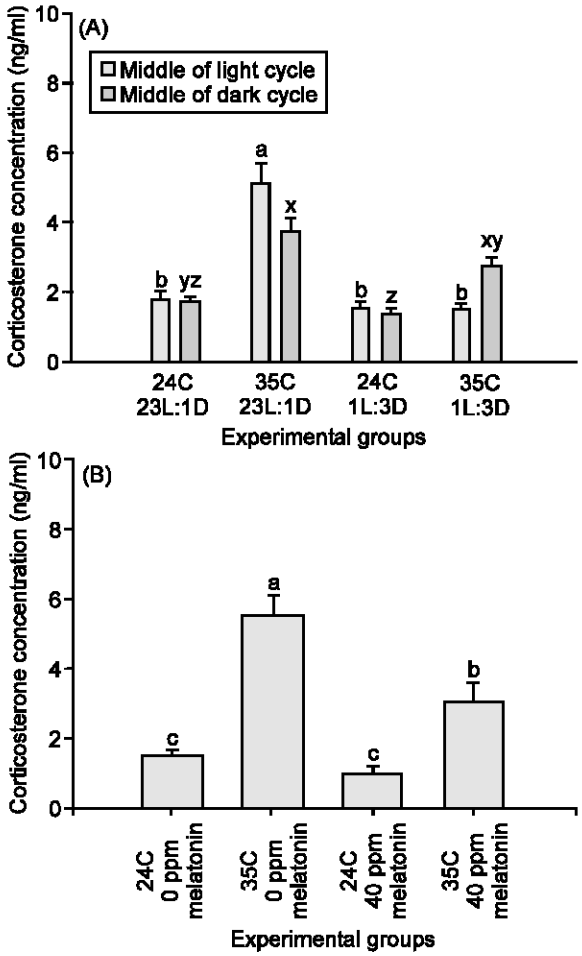

Fig. 5: The effect of light program (A) and melatonin addition to the diet (B) on the plasma corticosterone levels of 6 wk old broiler male chickens exposed to chronic heat stress from 4-6 wk. Bars are means+SE. Bars with common letters inside the same light/dark cycle are not significantly different (p>0.05) (n = 5)

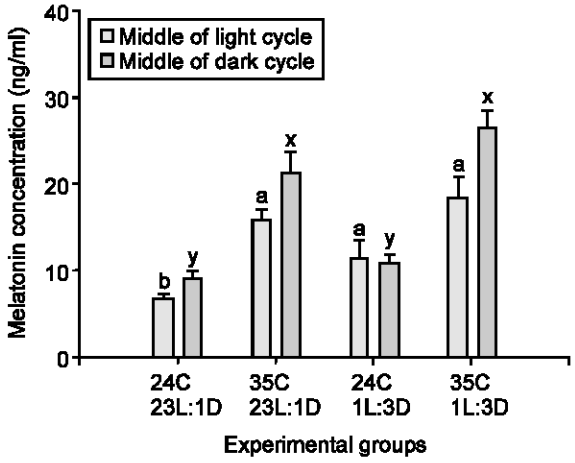

Fig. 6: The effect of light program on the plasma melatonin levels of 6 wk old broiler male chickens exposed to chronic heat stress from 4-6 wk. Bars are means+SE. Bars with common letters inside the same light/dark cycle are not significantly different (p>0.05) (n = 5)

melatonin in their plasma. Furthermore, melatonin addition significantly lowered body temperature of chickens in control temperatures when compared to chickens under control temperature with no melatonin added suggesting that the hypothermic effect of melatonin can occur without prior exposure to hyperthermia. In humans exposed to heat stress exogenous administration of melatonin during day time lowered the temperature threshold for cutaneous vasodilation and sweating allowing for better thermoregulation (Aoki *et al*., 2006). Melatonin can be inducing similar mechanisms in chickens by changing the temperature thresholds for nonevaporative skin heat loss and/or respiratory evaporation. Exposure to CHS under CL significantly increased the plasma levels of the pro-inflammatory cytokines (IL-1, IL-6 and TNF). Chronic heat stress can compromise gut integrity to release bacterial endotoxin in the circulation stimulating the pro-inflammatory cytokine network, alternatively or simultaneously CHS can cause tissue necrosis which would also activate the pro-inflammatory cytokine network (DuBose *et al*., 2002). Chickens exposed to CHS under IL had significantly lower levels of pro-inflammatory cytokinesin their plasma. Bilbo *et al*. (2002) reported that hamsters raised under short day conditions showed attenuated responses to endotoxin injection such as anorexia, fever and pro-inflammatory cytokine secretion over hamsters raised under long day conditions and suggested that increased melatonin secretion can be playing a role in this effect. Chickens raised under IL are exposed to longer dark hours and will experience longer durations of melatonin secretion than chickens under CL even if their plasma melatonin levels are similar. Melatonin was previously shown to attenuate the production of pro-inflammatory cytokines following endotoxin exposure in the rat (Nava *et al*., 1997). Exposure to CHS led to the elevation of TNF and IL-6. Tumor necrosis factor is capable of eliciting the metabolic manifestation of cachexia, which include weight loss, anorexia and skeletal muscle catabolism to yield amino acids for gluconeogenesis and Acute Phase Protein (APP) synthesis (Tracey *et al*., 1990), whereas IL-6 is the main cytokine responsible for regulation APP synthesis by the liver (Gauldie and Bauman, 1991). Chickens under IL are limited in their access to feed only during light hours. Under these conditions of chronic stress and feed restriction it is metabolically advantageous for those chickens to limit their inflammatory response to avoid going into a cachetic

state and increase their chances of survival. Melatonin addition to the diet was able to significantly decrease the pro-inflammatory cytokines; IL-1 and IL-6 levels in the plasma of CHS chickens compared to their levels in CHS chickens with no melatonin added to the diet. Melatonin was previously shown to reduce the levels of IL-1, TNF and IL-6 in rats' sera following endotoxin injection (Nava *et al*., 1997). The molecular mechanisms underlying this anti-inflammatory effect for melatonin are still being discovered. However, Li *et al.* (2005) reported that the nuclear translocation of the nuclear factor-kappa β, a transcription factor that regulates the transcription of a wide number of genes involved in immune and inflammatory responses was inhibited by melatonin. It is noteworthy to point out that reducing the release of pro-inflammatory cytokines by either IL or melatonin could be one of the mechanisms used to reduce the rise in body temperature following the exposure to CHS in this study. Pro-inflammatory cytokines such as IL-1, TNF and IL-6 are important mediators of fever (Engel *et al*., 1994). Exposure to CHS resulted in a significant inhibition of the *in vitro* T-cell proliferation in response to Con-A mitogen compared to chickens in control temperatures. Atta *et al*. (1996) indicated that exposure to heat stress significantly inhibits the *in vitro* mitogenic proliferation of chicken T-cells. Heat stress induces lipid peroxidation of cellular membranes (Freeman and Crapo, 1982) and oxidative stress due to hyperthermia inhibited the *in vitro* proliferation of T-cells (Pahlavani and Harris, 1998). Heat stress will also raise plasma corticosterone levels in chickens (Nathan *et al*., 1976). *In vitro* corticosterone inhibited chicken T-cells mitogenic proliferation (Trout and Mashaly, 1995). Chickens exposed to CHS under IL exhibited significantly higher T-cell proliferation than chickens exposed to CHS under CL. Furthermore, chickens under IL in control temperature exhibited the highest T-proliferation. Previously we have reported that broiler chickens raised under IL in normal temperatures exhibited significantly higher T and B-cell proliferation compared to broiler chickens raised under CL (Kliger *et al*., 2000). In addition, when melatonin was added simultaneously with the mitogen *in vitro*, it enhanced the proliferation of T-cells from chickens raised under CL (Kliger *et al*., 2000). In the current study, the enhancing effect of IL on T-cell proliferation can be due to different reasons. In CHS chickens under IL, corticosterone levels in the middle of the light period were significantly lower than CHS chickens under CL. Corticosterone inhibits the *in vitro* mitogenic proliferation of T-cells in chickens (Trout and Mashaly, 1995). The enhancing effect of IL on T-cell proliferation can be also due to melatonin. Although, there were no differences in melatonin levels between CHS chickens under either CL or IL, melatonin levels were numerically higher in IL chickens exposed to CHS. The number of dark hours in the IL schedule is higher than CL which entails that the duration of exposure to melatonin for birds under IL is longer. Melatonin is a powerful anti-oxidant that can protect against lipid peroxidation of cellular membranes (Hardeland, 2005). Melatonin can also increase lymphocyte proliferation through the stimulating the production of IL-2 and IFN-gamma by T helper cells and monocytes (Garcia-Maurino *et al*., 1997). Melatonin added to the diet significantly increased the mitogenic proliferation of T-cells in chickens raised in control temperatures. This is in agreement with previous findings that reported that melatonin addition *in vitro* (Kliger *et al*., 2000) or *in vivo* (Moore and Siopes, 2002) will enhance cell mediated immune responses in poultry. However, in CHS chickens where T-cell proliferation was inhibited, melatonin addition to the diet did not result in a significant increase of T-proliferation. Chronic melatonin treatment has been shown to attenuate adrenocortical secretory responses to acute and chronic stress (Konakchieva *et al*., 1997). However, in the current study melatonin added to CHS chickens' diets was unable to decrease corticosterone levels to levels in control temperature chickens. Corticosterone can inhibit T-cell proliferation in chickens (Trout and Mashaly, 1995). Furthermore, glucocorticoids will induce the apoptosis of T-cells (Kirsch *et al*., 1999). The cutaneous basophil hypersensitivity response following PHA-P injection is often reported as a good measure for cellular immunity (Brake *et al*., 1988; Moore and Siopes, 2002). In the current study, chickens exposed to CHS under CL exhibited a significantly lower CBH response, whereas in chickens exposed to CHS under IL this response was restored to control temperatures level. Moore and Siopes (2000) also found that quail raised under a short light schedule exhibited a significantly higher CBH response to PHA-P than quail raised under near constant light programs. Even though not statistically significant, chickens raised under IL in control temperatures had the highest CBH response further indicating that cellular immune responses in chickens can be enhanced by applying an IL program. Melatonin addition was able to significantly increase the CBH response in control temperatures chickens compared to the other groups. Moore and Siopes (2002) also reported that melatonin addition in the drinking water to quail significantly increased their CBH response compared to quail that did not receive melatonin. Exposure to CHS significantly decreased the CBH response in chickens that did not receive melatonin. However, melatonin addition to the diet of CHS chickens restored their CBH response to the level observed in control temperature chickens even though their plasma corticosterone levels were significantly higher than control temperatures chickens. Earlier Brake *et al.*

(1988) have reported that cortisol can enhance the CBH response to PHA-P. However, this report is in contrast to other studies that indicate an immunosuppressive action for glucocorticoids (Trout and Mashaly, 1995; Mc Ewen *et al*., 1997).

In conclusion, the results from the present study indicate that, applying an intermittent light program or melatonin addition to the diet can alleviate some of the immunosuppression associated with the exposure to chronic heat stress in broiler chickens. Whether the effects of intermittent light on the immune response seen here can be attributed solely to melatonin or melatonin and/or other factors will need further studying. Since neither intermittent light nor melatonin addition adversely affected any of the production parameters, in fact some were improved especially in heat stressed chickens (data not shown) both of them can be valid measures to protect against immunosuppression associated with heat stress. However, intermittent light schedules can be even more attracting considering they will not only enhance immune functions but also reduce the overall production costs by decreasing funds allocated to illumination.

To our knowledge this is the first report that either intermittent light or melatonin were used to alleviate the immunosuppression usually associated with chronic heat stress in broiler chickens.

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