Changes in heterophil to lymphocyte ratios of heat-stressed chickens in response to dietary supplementation of several related stress agents

Veränderung des Verhältnisses von Heterophilen zu Lymphozyten bei Hühnern unter Hitzestress in Abhängigkeit von der Zugabe verschiedener Anti-Stress-Komponenten zum Futter

J. L. Campo and S. G. Dávila


Introduction

The heterophil to lymphocyte ratio has become widely accepted as a reliable and accurate physiological indicator of the stress response in chickens (GROSS and SIEGEL, 1983), because exposure to stressors causes it to increase progressively. GROSS and SIEGEL (1993) suggested that reference values for the heterophil to lymphocyte ratio of about 0.2, 0.5 and 0.8 are characteristic of low, optimal and high degrees of stress, respectively.

Several feed compounds are related to stress levels in animals and some may be used to alleviate stress; between them are: yeast, ascorbic acid, tocopherol, tryptophan, niacin, and lactic acid. Usually, poultry diets contain α-tocopherol, niacin and sometimes ascorbic acid and definitely tocopherol and niacin. The brewer’s yeast (Saccharomyces cerevisiae) is known to reduce stress in animals (PHILLIPS and VON TUNGELN, 1984), in broiler chickens, the use of yeast reduced significantly the effects of aflatoxicosis (STANLEY et al., 1993) and the frequency of Salmonella and Campylobacter colonization in birds subjected to transport stress (LINE et al., 1997). Furthermore, CHUKWU and STANLEY (1997) found that the performance of laying hens during severe high temperature could be maintained with dietary inclusion of yeast. On the other hand, avian species are not able to synthesise sufficient ascorbic acid (vitamin C) to replace the severe losses of this vitamin during stress (PARDUE and THAXTON, 1986). GROSS (1988) suggested that ascorbic acid may reduce the effect of Escherichia coli and AMAYE-ANIM et al. (2000) suggested that supplementation of ascorbic acid has beneficial effects on antibody response to infectious bursal disease. Furthermore, KUTLU and FORBES (1993) found that ascorbic acid ameliorated heat-induced deterioration in performance and metabolism of broilers. Similarly, dietary needs of α-tocopherol (vitamin E) increase during stress, and supplementation of vitamin E resulted in partially preventing a decrease in egg production (SCHIEDELER, 1996; BOLLENGIER-LEE et al., 1998, 1999) and alleviating heat stress in hens (WILLIAMS, 1997; LEWIS et al., 1999; SMITH, 2000). Furthermore, vitamin E supplementation enhances macrophage activity (KONJUFCA et al., 1999) and alleviated the impact of E. coli infection (YANG et al., 2000).

Increased dietary tryptophan markedly reduces aggression in broiler breeders (SHEA et al., 1990) and the incidence of pecking damage in growing bantams (SAVORY et al., 1990). Furthermore, MENCH (1991) showed that increasing the dietary tryptophan content to levels found to be effective in reducing aggressive behaviour reduced plasma corticosterone levels in feed-restricted broiler breeders, and NEWBERRY and BLAIR (1993) suggested that the addition of tryptophan to the diet might have a mild fear-reducing effect. Although SHEA-MOORE et al. (1996) showed that decreases in aggression in tryptophan-supplemented broiler breeders were not due to increases in blood niacin levels (this vitamin is synthesised from the tryptophan), elevations in niacin levels have been reported to control nervousness and hysteria in chickens (SCHUTZ, 1965; HANSEN, 1976).

Finally, high levels of lactic acid (metabolite from glycolysis) were reported in blood of broilers that suffered from sudden death syndrome (IMAEDA, 2000a). Lactic acid levels in blood are elevated when chickens are reared under high levels of stress caused by high stocking density, warm ambient temperature (IMAEDA, 2000b) and saturated dietary fat content (ROTTER et al., 1985). FRONING et al. (1978) and NGOKA and FRONING (1982) observed deterioration of breast meat colour of birds that were allowed to struggle before slaughter compared to anaesthetised birds. The darker breast meat colour is caused by depletion of muscle glycogen and less lactic acid build-up during post mortem glycolysis. On the contrary, supplementation of lactic acid in drinking water during pretransport may reduce Salmonella and Campylobacter contamination of broiler carcass at processing (BYRD et al., 2001).

Only a few studies have examined the effects of these compounds on the heterophil to lymphocyte ratio. The effect of yeast on the heterophil to lymphocyte ratio has been reported by GUO and LIU (1997), who indicated that the addition of yeast reduced significantly the heterophil to lymphocyte ratio in broilers under heat stress. A strong relationship between ascorbic acid and heterophil to lymphocyte ratio has been documented; ascorbic acid reduced significantly the heterophil to lymphocyte ratio in birds under heat, cold, sound, fasting, or management stress (GROSS, 1992; MCKEE and HARRISON, 1995; ZULKIFLI et al., 2000). Vitamin E supplementation improved the het-
erophil to lymphocyte ratio suggesting improved phagocyte ability of the immune system (Boa-Ampomsem et al., 2000). Blair et al. (1993) showed that the heterophil to lymphocyte ratio was not affected by dietary tryptophan in broilers under light stress. Although the relationship between lactic acid and heterophil to lymphocyte ratio has not been reported up to now, feed withdrawal has been reported to cause a decreased lactic acid concentration (Corrier et al., 1999) and an increased heterophil to lymphocyte ratio (Maxwell et al., 1992; Zulkipli et al., 1995; Alodan and Mashaly, 1999).

The objectives of this study were to evaluate the effects of several additives related to stress levels: supplemental yeast, ascorbic acid, tocopherol, tryptophan, niacin, or lactic acid in diets, on the heterophil to lymphocyte ratio in chickens under heat stress, one of the most important stressors in poultry production.

Materials and methods

The data were obtained from hens of a White Leghorn population, originating from crossing three strains selected for egg number and egg weight (Campos and Jurado, 1982). The six different experiments were: 1) brewer's yeast supplemented at 0.5%; 2) L-ascorbic acid supplemented at 1000 ppm; 3) feed was supplemented with 250 ppm of α-tocopherol; 4) tryptophan supplemented at 0.5%; 5) niacin supplemented at 250 ppm; and 6) feed was supplemented with 2% lactic acid. Products were bought from Sigma Chemical Co.

Two treatment groups were used in each experiment. Group 1 consisted of twenty 40-wk-old hens subjected to 37.5 °C heat stress, for 3 days before measurement of heterophil to lymphocyte ratio. Group 2 consisted of 20 additional hens subjected to heat stress (37.5 °C for 3 days) with the addition of a dietary agent for 3 days. In Experiment 1, there was an additional group of 20 hens without heat stress and Group 1 to 3 had an additional older age group of 72-wk-old hens. Hens in each treatment were kept in separated floor pens. They were fed standard layer diet (containing 16% CP, 2,700 kcal ME/kg, 3.5 Ca, 0.5 P, 12 ppm ascorbic acid, 12 ppm tocopherol and 18 ppm niacin); feed and water were provided ad libitum. The room temperature of the hens prior to heat stress was about 25 °C.

To obtain the heterophil to lymphocyte ratio, hens were carried to a separate room, and collection of blood was made immediately. Two drops of blood were taken from a small puncture in the comb of each bird, one drop being smeared on each of two glass slides. The smears were stained using May-Grünwald and Giemsa stains (McAuliffe et al., 1982). The smears were made immediately. Two drops of blood were taken from a small puncture in the comb of each bird, one drop being smeared on each of two glass slides. The smears were stained using May-Grünwald and Giemsa stains (LuCAS et al., 1993; Zulkipli et al., 1995; Alodan and Mashaly, 1999).

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Results

The results of the Experiment 1, indicating yeast effects on heterophil to lymphocyte ratio, are shown in Table 1. Age and treatment by age interaction were not significant for either analysed measurement (heterophil to lymphocyte ratio, and its numerator or denominator), and they were pooled with the residual to give a one-way model of the treatment effect. No differences were found between treatments (heat stress, heat stress + yeast and without heat stress). The heterophil to lymphocyte ratios presented in Table 1 differ from those calculated with the presented means for heterophils and lymphocytes (0.58, 0.53, and 0.58 in heat stress, heat stress + yeast, and without heat stress groups, respectively) due to the effect of variance and covariance on the value of the ratio: x/y = mₓ/mᵧ + 2mₓmᵧSₓSᵧ/ mₓ²mᵧ² = mₓmᵧ/SₓSᵧ, being mₓ and mᵧ the mean values of the numerator and denominator, Sₓ the covariance between them, and SₓSᵧ the variance of the denominator. This applies for all the other experiments.

Addition of ascorbic acid to the diet (Experiment 2) did not change significantly measurements for heterophil to lymphocyte ratio (Table 1). In hens under heat stress + ascorbic acid, there was a significant (P < 0.05) increase in lymphocyte number, but heterophil number was not significantly different between treatments.

Hens under heat stress showed significantly (P < 0.05) higher heterophil to lymphocyte ratio than heat-stressed hens with tocopherol (Experiment 3; Table 1). In hens with tocopherol added to the diet, there was a significant (P < 0.05) increase in lymphocyte number, whereas heterophil number did not differ significantly between treatments.

There was no significant difference between heat-stressed hens in the two groups of dietary tryptophan (Experiment 4) in terms of heterophil to lymphocyte ratio (Table 1). Difference between treatments was significant (P < 0.05) for lymphocyte number, with the hens under heat stress + tryptophan addition showing higher lymphocyte numbers. No difference was found between treatments for number of heterophils.

The results of Experiment 5, demonstrating a dietary niacin effect on heterophil to lymphocyte ratio, are summarised in Table 1. Heat-stressed hens without niacin added to the diet manifested a significantly (P < 0.05) higher heterophil to lymphocyte ratio than those with niacin. In hens with supplemental niacin, there was a significant (P < 0.05) increase in lymphocyte number, though no response was observed for heterophil number.

Finally, the results of Experiment 6 showed the effect of lactic acid supplementation on the heterophil to lymphocyte ratio of hens (Table 1). Heat-stressed hens with dietary lactic acid had the opposite result compared to the rest of the other experiments, with a significantly (P < 0.05) higher heterophil to lymphocyte ratio than hens under heat stress without supplemental lactic acid. In the former
### Table 1. Mean heterophil/lymphocyte ratio, number of heterophils and number of lymphocytes in each experiment (n = 20)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Heterophil/lymphocyte</th>
<th>Heterophil number</th>
<th>Lymphocyte number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Heat stress</td>
<td>0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Heat stress+yeast</td>
<td>0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Without heat stress</td>
<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.05</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Heat stress</td>
<td>0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Heat stress+ascorbic acid</td>
<td>0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>SEM</td>
<td>0.07</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Heat stress</td>
<td>0.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Heat stress+tocopherol</td>
<td>0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>SEM</td>
<td>0.07</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Heat stress</td>
<td>0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Heat stress+tryptophan</td>
<td>0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>SEM</td>
<td>0.08</td>
<td>2</td>
<td>3</td>
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<tr>
<td>5</td>
<td>Heat stress</td>
<td>0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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<td></td>
<td>Heat stress+nacin</td>
<td>0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>SEM</td>
<td>0.07</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Heat stress</td>
<td>0.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Heat stress+lactic acid</td>
<td>1.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.20</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Means within the same experiment and column with no common superscript differ (P < 0.05)

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A group of hens, there was a significant (P < 0.05) increase in heterophil number, but lymphocyte number was similar in both groups.

**Discussion**

The results show that the addition of tocopherol, niacin, or lactic acid to the diet of chickens under heat stress involves adjustments in the heterophil to lymphocyte ratio. This ratio was significantly lower compared with that of the heat-stressed hens when tocopherol or niacin was incorporated in feed, suggesting that they are effective to alleviate heat stress levels. On the contrary, the addition of lactic acid produced a significant increase in the heterophil to lymphocyte ratio, suggesting that this compound reinforces the stress induced by high temperature. The significant decreases on the heterophil to lymphocyte ratio observed for the tocopherol and niacin treatments were due to increases in its denominator (lymphophilia), whereas the significant increase observed for the lactic acid was due to a significant heterophilia.

There was no significant effect of dietary yeast supplementation on stressfulness of hens as estimated by heterophil to lymphocyte ratio. This result is in disagreement with those indicating that the use of *S. cerevisiae* significantly reduced the effects of stress in general (Phillips and Von Tungen, 1984; Stanley et al., 1993; Line et al., 1997) and heat stress in particular (Chukwu and Stanley, 1997). It also disagrees with the significantly decrease reported by Guo and Liu (1997) on the heterophil to lymphocyte ratio in broilers under heat stress and supplemental yeast in the feed.

Though the heterophil to lymphocyte ratio was numerically low in the heat stress group with ascorbic acid compared to the control group, no significant effects of dietary ascorbic acid supplementation on heterophil to lymphocyte ratio was observed, although it produced significant lymphophilia. The results found in the literature reported in general a significant decrease for the heterophil to lymphocyte ratio (Gross, 1992; Mckee and Harrison, 1995; Zulkifli et al., 2000). However, Campo and Carnicer (1994) did not find a significant effect of ascorbic acid on fearfulness of hens as estimated by tonic immobility duration (a measure of psychological stress in chickens), in hens that had not been deliberately stressed in any way.

The significant reduction in stress levels, as indicated by the lower heterophil to lymphocyte ratio, observed in the hens supplemented with tocopherol is in agreement with positive effects by other authors that reported decreased adverse effects of heat stress in tocopherol supplemented birds (Schedeler, 1996; Bollegren-Lee et al., 1998, 1999; Williams, 1997; Lewis et al., 1999; Smith, 2000). Although Boa-Ampson et al. (2000) reported a statistical significant increase in heterophil to lymphocyte ratios produced by the addition of high doses of tocopherol, this change seems to have little practical significance (0.44 vs. 0.36).

There was no significant effect of tryptophan supplementation on heterophil to lymphocyte ratio, although this amino acid produced a tendency to decrease the heterophil to lymphocyte ratio and significantly increase the number of lymphocytes. This result agrees with that of Blair et al. (1993), who also reported no significant effect of dietary tryptophan addition (0.2%) in the heterophil to lymphocyte ratio of light-stressed broilers. In contrast, Mench (1991) found that dietary tryptophan (1.5%) reduced plasma corticosterone levels in feed-restricted broiler breeders, and Newberry and Blair (1993) showed that the addition of tryptophan to the diet (0.4%) might had a significant effect on tonic immobility duration. The significant decrease for the heterophil to lymphocyte ratio observed in heat stressed hens with supplemental niacin was in agreement with the results of Schutz (1965) and Hansen (1976), who reported that high levels of niacin (200 ppm) prevented symptoms of nervousness and hysteria in chickens.

The heterophil to lymphocyte ratio increased significantly in hens exposed to heat and lactic acid supplemen-
tation, due to significant heterofilia. This fact is consistent with those reported by Fröning et al. (1978), Ngoka and Fröning (1982), and Imaeda (2000a, b), who indicated that lactic acid was related to stress situations, but disagrees with the indirect negative relationship between heterophili to lymphocyte ratio and lactic acid observed in fasting stressed birds by different authors (Maxwell et al., 1992; Zulkipli et al., 1995; Aloodan and Mashaly, 1999; Corrier et al., 1999).

In conclusion, the results of this manuscript suggest that tocopherol and niacin are effective to alleviate heat stress levels, whereas lactic acid reinforces the stress induced by the high temperature.

Summary

The effects of several dietary additives that are related to stress levels on the heterophil to lymphocyte ratio (an indicator of stress) were studied in heat-stressed chickens from a White Leghorn population. There were six experiments: 1) brewer’s yeast at 0.5% in the diet; 2) ascorbic acid at 1000 ppm; 3) tocopherol at 250 ppm; 4) tryptophan at 0.5%; 5) niacin at 250 ppm; 6) lactic acid at 2%. Treatments were begun 3 days before measurements of heterophili to lymphocyte ratio. Two treatment groups were used in each experiment (heat stress versus heat stress + dietary additive). In Experiment 1 there was an additional group of hens without heat stress. Addition of yeast, ascorbic acid or tryptophan did not change significantly measurements for heterophili to lymphocyte ratio. Addition of tocopherol or niacin decreased significantly the heterophili to lymphocyte ratio of the heat-stressed hens (0.43 ± 0.07 vs. 0.65 ± 0.07 and 0.45 ± 0.07 vs. 0.66 ± 0.07, respectively; P < 0.05). Hens of the ascorbic acid, tocopherol, tryptophan and niacin groups had significant lymphophili (P < 0.05). Heat-stressed hens with lactic acid added to the diet had significantly higher heterophili to lymphocyte ratio than hens without supplemental lactic acid (1.19 ± 0.20 vs. 0.62 ± 0.20; P < 0.05). The significant increase observed for the lactic acid group was due to a significant heterofilia (P < 0.05). The results suggest that tocopherol and niacin are effective to alleviate heat stress levels, whereas lactic acid reinforces the stress induced by the high temperature.

Keywords

Layers, nutrition, heat stress, ascorbic acid, niacin, tocopherol, tryptophan, yeast, heterophils, lymphocytes

Zusammenfassung

Veränderung des Verhältnisses von Heterophilen zu Lymphozyten bei Hühnern unter Hitzestress in Abhängigkeit von der Zugabe verschiedener Anti-Stress-Komponenten zum Futter

Die Veränderung des Verhältnisses von Heterophilen zu Lymphozyten als Indikatoren für Stress wurde bei Hühnern der Rasse Weiße Leghorn unter Hitzestress in Abhängigkeit von der Zugabe verschiedener Anti-Stress-Komponenten zum Futter untersucht. Es wurden sechs Versuche durchgeführt, bei denen folgende Komponenten den Rationen zugefügt wurden: 1) 0.5% Brauerhefe; 2) 1000 mg/kg Ascorbinsäure; 3) 250 mg/kg α-Tocopherol; 4) 0.5% Tryptophan; 5) 250 mg/kg Niacin; 6) 2% Milchsäure. Die jeweilige Fütterung wurde 3 Tage vor der Messung des Heterophilen-Lymphozyten-Verhältnisses begonnen. In jedem Versuch wurden zwei Behandlungsgruppen (Hitzestress gegen Hitzestress + Anti-Stress-Komponente) miteinander verglichen. In Versuch 1 wurde zusätzlich eine Behandlung ohne Hitzestress einbezogen. Die Zugabe von Hefe, Ascorbinsäure oder Tryptophan hatte keinen signifikanten Einfluss auf das Verhältnis von Heterophilen zu Lymphozyten. Demgegenüber reduzierte die Zugabe von α-Tocopherol oder Niacin das Heterophilen-Lymphozyten-Verhältnis der Hennen um signifikant (0.43 ± 0.07 vs. 0.65 ± 0.07 bzw. 0.45 ± 0.07 zu 0.66 ± 0.07; P < 0.05). Die Hennen der Behandlungen Ascorbinsäure, α-Tocopherol, Tryptophan oder Niacin wiesen eine signifikante Zunahme an Lymphozyten auf (P < 0.05). Hennen unter Hitzestress wiesen bei Zugabe von Milchsäure ein signifikant höheres Heterophilen-Lymphozyten-Verhältnis auf als Hennen ohne Milchsäure-Zusatz (1.19 ± 0.20 zu 0.62 ± 0.20; P < 0.05). Die signifikante Zunahme des Verhältnisses wurde in der Behandlung Milchsäure durch eine signifikant signifikante Zunahme der Heterophilen verursacht (P < 0.05). Die Ergebnisse deuten darauf hin, dass α-Tocopherol und Niacin effektiv Hitzestress kompensieren können, während Milchsäure die negativen Effekte hoher Temperaturen verstärkt.

Stichworte

Legehennen, Fütterung, Hitzestress, Ascorbinsäure, Niacin, Tocopherol, Tryptophan, Hefe, Heterophile, Lymphozyten

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