Effect of feed contamination with aflatoxin B_1 and administration of exogenous corticosterone on Japanese quail biochemical and immunological parameters

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ABSTRACT Stress is the loss of homeostasis by external forces or stressors. Manipulation, transport, contamination, and other procedures involved in production could be considered stressors. Contamination is a problem commonly faced by producers in the poultry industry. Aflatoxicosis is one of the most common infections resulting from feed contaminated with *Aspergillus flavus* and *Aspergillus parasiticus*. This study evaluated the potential effects of the combined administration of aflatoxin B_1 (AFB_1) and corticosterone on biochemical (concentration of globulins, proteins, and albumin) and immunological (inflammatory response and heterophil:lymphocyte ratio) parameters of Japanese quail. Potential sex effects on those parameters were also considered. The provision of corticosterone in drinking water is a method used for mimicking the effects of chronic stress in avian species. At 35 d of age, 24 mixed-sex groups of 4 animals (2 males and 2 females) were housed in cages and assigned to 1 of 4 treatments: plain drinking water and laying diet, corticosterone administration in drinking water, feed contamination with AFB_1 (100 μg/kg of feed), or corticosterone plus AFB_1 administration. There were 6 cages per treatment. No significant effect of sex in any of the parameters analyzed was detected. Hypoproteinemia, hypoalbuminemia, and hypoglobulinemia were observed in animals treated with corticosterone or contaminated feed. These responses were exacerbated when the factors were combined. The immunodepressive effect of corticosterone administration was confirmed, and a higher effect was noticed when combined with the aflatoxin contamination. Aflatoxin contamination affected birds' physiology similar to a chronic stressor stimulation because it elevates the heterophil:lymphocyte ratio. This study suggests that the effects of the AFB_1 contamination are further increased when overlapped with a chronic stressful stimulation and emphasizes the importance of controlling potential stressor combinations during animal rearing to preserve not only the animal's health status but also their welfare.

Key words: aflatoxin contamination, corticosterone, stress response, Japanese quail

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INTRODUCTION

The loss of homeostasis by external forces or stressors has been defined as the state of stress by Siegel (1995). The stress response has been well-characterized and it involves increased levels of glucocorticoids and catecholamines released into the blood stream as the main responses to stressors (Jones et al., 2000; Cheng and Muir, 2004; Mumma et al., 2006). The increase of these compounds is the result of the activation of 2 neuroendocrine axes, the sympathetic and the hypothalamic-pituitary-adrenal axes (De Kloet and Derijk, 2004; Kuenzel and Jurkevich, 2010). Avian production nowadays implies handling, vaccination, transport, contamination, and various other instances that lessen animals' welfare (Schulz et al., 2000; Marco et al., 2006; Dickens et al., 2009, 2010). These situations could lead to a state of stress in which animals invest resources and energy to re-establish the lost homeostasis. Studies of stress response physiology in poultry have emphasized that corticosterone (**CORT**) plays a multifunctional role through the alteration of neuroendocrine and im-

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mune components (Cheng and Muir, 2004). Negative consequences of stress involve, among others, diminished egg production, fertility, and hatchability (Marin et al., 2002b; Marin and Satterlee 2004; Leone and Estévez 2008); altered social interaction with conspecifics (Jones, 1996; Marin et al., 2001; Marin and Satterlee, 2003; Guzman and Marin, 2008); and a strong incapability to adequately use resources (Jones, 1996; Mormède et al., 2007). The immune system is one of the most affected systems during the stress response, especially if the stressor persists in time, inducing what is known as chronic stress (Glick, 1984; Dohms and Metz, 1991; Fair et al., 1999; Shini et al., 2008a,b; Bauer et al., 2009, Nazar and Marin, 2011). The consequences of a sustained stress response on the immune system have been widely reported. For example, Japanese quail react to a chronic restraint stressor with diminished cellular and humoral responses (Nazar and Marin, 2011). The provision of CORT in the drinking water has been used as a method for mimicking the effects of chronic stress in avian species (Shini and Kaiser, 2009). Leukocyte populations decrease after the administration of CORT in drinking water (Shini and Kaiser, 2009); inoculation of chickens with CORT using pharmacological doses results in a rapid lymphoid depletion in the thymus, bursa, and spleen (Dohms and Metz, 1991).

Contamination along the production course, as mentioned above, could also lead to stressful situations. These situations could imply behavioral responses, such as avoiding contaminated materials, in some cases, or lead to sickness or even death in other occasions in which the animals cannot overcome the negative effects of contamination. Birds' commercial feed contains several cereals and oil seed, such as wheat, corn, soybean, barley, oats, sorghum, and sunflower. These raw materials may be contaminated with toxigenic fungi (genus *Aspergillus*, particularly *A. flavus* and *A. parasiticus*; Cotty and Garcia, 2007) containing different mycotoxins; among them, aflatoxins (**AF**) are the prevalent toxic compounds of avian feed (CAST, 2003). Twenty AF have been identified, the major ones being B_1, B_2, G_1, and G_2; with aflatoxin B_1 (**AFB_1**) being the most common and toxic compound (Hussein and Brasel, 2001). The effects of AF are dose- and time-dependent, and 2 different forms of contamination with AF (aflatoxicosis) have been reported: acute and chronic (Osweiler, 1990). The liver is the main target organ of AF (Sawhney et al., 1973). Biochemical-hematological, immunological, and pathological effects of AF have also been well-described (Kiran et al., 1998; Qureshi et al., 1998; Oğuz et al., 2000b). The susceptibility to AF varies between avian species (Japanese quail appear to be more resistant to aflatoxicosis than Bobwhite quail; Ruff et al., 1992) and it is also markedly altered by intraspecific factors, such as age, sex, and breed (Pier, 1992; CAST, 2003).

During rearing, as said, birds are normally simultaneously exposed to different stressful situations to which they must adjust in order to cope with them. The effects induced would depend on the type and combination of the stressors, the duration of the exposure, and the susceptibility of each species to them. Moreover, stress response may also vary according to animal sex, with males being more susceptible to stressors than their female counterparts (Huff et al., 1999; Wideman and French, 2000; Marin et al., 2002a).

In a recent study, we found that a combination of chronic stress inducing manipulation with an AF contamination further reduced BW and feed conversion in Japanese quail (Magnoli et al., 2012). The present study focuses on whether the administration of feed contaminated with a low dose of AFB_1 (100 μg/kg), a provision of a low dose of CORT in the drinking water, or a combination of both treatments affects Japanese quail biochemical and immune parameters. To our best knowledge, this is the first study that evaluates the combined effects of an AF contamination concomitant with a chronic stress manipulation. The parameters measured were the concentration of proteins, albumin, globulins and the albumin:globulin ratio, heterophil:lymphocyte ratio, and a lymphoproliferative response. This design allowed us to answer 3 main questions: 1) can the supplementation of a low CORT dose in the drinking water change immune-related parameters in Japanese quail, 2) is the immune system of Japanese quail sensitive to a low dose of AFB_1, and if so, 3) is the combination of CORT and AFB_1 treatment able to increase the effects of each individual factor? Considering the sex differences in stress response mentioned above, we also examined whether males and females may differ in their response to AFB_1 contamination or CORT treatment.

MATERIALS AND METHODS

Husbandry and Experimental Design

In total, 108 Japanese quail (*Coturnix coturnix japonica*) hatchlings were randomly housed in 2 white wooden boxes (54 quail each) measuring 90 × 90 × 60 cm (length × width × height), and they remained in the same boxes until 4 wk of age. Each box had 2 feeders covering the front part and 16 automatic nipple drinkers (8 on each side). A wire-mesh floor (1-cm grid) was raised 5 cm to allow for the passage of excreta, and a lid prevented the birds from escaping. The brooding temperature was 37.5°C during the first week of life, with a weekly decline of 3.0°C until room temperature (24–27°C) was achieved. At 4 wk of age, birds were randomly selected by similar BW, sexed by plumage coloration, had their beak trimmed, and were wing-banded for further identification. Ninety-six birds were housed in mixed groups of 4 animals (2 males and 2 females) in 24 cages measuring 50.8 × 15.2 × 26.7 cm (length × width × height). Birds were allowed 6 d to adapt to the cages, and during this period, were fed with a laying diet and water ad libitum. The laying diet (Marcelo E. Hoffman e Hijos S.A., Entre Ríos, Argentina) contained

corn meal, soybean meal, wheat shorts, sunflower meal, limestone, sodium chloride, dicalcium phosphate, vitamins, and minerals, with 21.5% CP and 2,750 kcal of ME/kg. Quail were subjected to a daily cycle of 16 h of light and 8 h of dark during the study, with the light (300–320 lx) setting on at 0600 h. The temperature was kept between 22 ± 2°C during the experimental period and water was continued ad libitum.

At 5 wk of age, all of the quail from each cage were randomly assigned to 1 of 4 treatment groups (6 cages/treatment) and the experiment was conducted for 46 more days (11 wk total). The experimental treatments were as follows: treatment 1: laying diet; treatment 2: laying diet + AFB_1 (100 μg/kg); treatment 3: laying diet + CORT (5 mg/L); and treatment 4: laying diet + AFB_1 (100 μg/kg) + CORT (5 mg/L).

To ensure the follow-up of the CORT treatment, water was provided through a manual and controlled bottle-drinker system. The CORT dose was selected according to previous studies that showed that a concentration of 5 mg/L in drinking water is able to elevate CORT blood levels similar to a stressful manipulation (Hull et al., 2007; Wall and Cockrem, 2010)

AF Production and Diet Preparation

Aflatoxins were produced via fermentation of rice by *Aspergillus parasiticus* NRRL 2999 (USDA, Agricultural Research Service, Peoria, IL). The sterile substrate, placed in Erlenmeyer flasks, was inoculated with 2 mL of the mold aqueous suspension containing 10^6 spores/mL. Cultures were allowed to grow for 7 d at 25°C in darkness. On d 7, Erlenmeyer flasks were autoclaved; culture material was dried for 48 h at 40°C in a forced-air oven and ground to a fine powder. The AFB_1 levels in rice powder were measured by thin-layer chromatography and high-performance liquid chromatography as described previously (Trucksess et al., 1994; AOAC, 1995). The milled substrate was added to the laying diet to provide the level of 100 μg of AFB_1/kg of feed. The concentrations of AFB_1 in each diet were confirmed by high-performance liquid chromatography-tandem mass spectrometry, following procedures previously proposed by Sulyok et al. (2007). The natural level of AFB_1 in the laying diet was 15 μg/kg of feed and in the contaminated diets was 100 μg/kg of feed. The dose selected for AFB_1 is a low dose that was proven to induce aflatoxicosis in Japanese quail, also affecting performance during the laying period (Oliveira et al., 2002).

Chemicals

Standards of purchased AFB_1 (Sigma, Aldrich Inc., St. Louis, MO) were assayed by high-performance liquid chromatography. Purities were confirmed as being greater than 99%. For corticosterone (Sigma Aldrich Inc.), 5 mg was dissolved in 1 mL of ethanol solution per 1 L of drinking water. The CORT solution used was selected because it was previously shown as being a low dose capable of elevating plasma CORT concentrations within a physiological range in Japanese quail (Wall and Cockrem, 2010).

Blood Sampling and Variables Measured

At 77 d of age, 2 birds from each cage (1 male and 1 female) randomly selected the previous day (see below) were bled and killed by cervical dislocation. Blood samples with anticoagulant addition were collected, used for preparing smears (see below), and then centrifuged at 2,500 × *g* for 15 min. The serum obtained was stored at −20°C for further analysis of biochemical parameters.

Lymphoproliferative Response to Phytohemagglutinin-P. To determine cell-mediated immunity, the responses to phytohemagglutinin-p (**PHA-P**) injection [a lectin from *Phaseolus vulgaris* (Sigma Chemical, St. Louis, MO)] was measured in the wing web of each bird following a test described elsewhere (Stadecker et al., 1977; Smits and Williams, 1999; Nazar and Marin, 2011). Briefly, 2 birds from each cage were randomly selected and at 76 d of age, a 0.1-mL intradermal injection of a solution of PHA-P in PBS (1,000 μg/mL) was given in the wing web, at 2 mm from the brachial vein. The dermal swelling response was measured as the percentage increase in wing-web thickness at the injection site 24 h post-PHA-P injection. The swelling was calculated using the following formula: percentage of inflammation = (previous 24 h inflammation/post 24 h inflammation) × 100. Measurements were recorded to the nearest 0.01 mm using a mechanical micrometer. Following Smits and Williams, (1999) and previous work in our laboratory (Nazar and Marin, 2011), only one wing was injected, without a control vehicle injection in the other wing because the inflammation resulting from the injection of PBS alone disappears gradually 24 h later, thus the inflammation measured is a consequence of the migration and recruitment processes due to the PHA-P injection.

Heterophil:Lymphocyte Ratio. Leukocyte counts were obtained by analyzing blood smears stained with May-Grünwald-Giemsa. Smears were done immediately after blood collection on d 77. Differential counts of 100 white cells per blood smear were made (Fair et al., 1999). The heterophil:lymphocyte ratio was calculated using the following formula: heterophil:lymphocyte ratio = number of heterophils/number of lymphocytes (Gross and Siegel, 1983).

Biochemical Parameters. Biochemical determinations as total protein, albumin (**ALB**), and globulin (**GLOB**) concentrations and ALB:GLOB ratio for each bird were measured in the serum obtained after centrifugation (Oğuz et al., 2000a; Rosa et al., 2001). These concentrations were determined with a clinical chemistry analyzer (Commercial kit 2000, Wiener Lab, Rosario, Argentina) calorimetric method for determination of total protein, albumin, and serum transaminase) according to the manufacturer's recommended procedure.

Statistical Analysis

Analyses were performed using a 3-way ANOVA (InfoStat, 2004) that examined the main effects of CORT treatment (non-CORT and CORT administrated), AFB_1 contamination (non-AFB_1 and AFB_1 contaminated), sex (male and female), and their interactions. Assumptions of the ANOVA were verified. Heterophyl:lymphocyte ratio data were subjected to a square-root transformation before analysis to fit the ANOVA assumptions. Transformations were not required for inflammatory response and biochemical parameters. Post hoc treatment group comparisons were conducted using the Fisher least significant difference test. A P-value of <0.05 was considered to represent significant differences.

RESULTS

Analysis of variance revealed a significant main effect of the CORT treatment ($F_{1,40} = 15.75$; $P < 0.001$) and AFB_1 contamination ($F_{1,40} = 4.64$; $P = 0.03$). No effects of sex were detected for this variable ($F_{1,40} = 0.28$; $P = 0.59$). The post-hoc test was performed combining male and female data and showed that the inflammatory response was the highest in the animals that were neither treated with CORT nor contaminated with AFB_1 in their diet (Figure 1). Quail that were contaminated with AFB_1 but not treated with CORT showed a significant fall in their inflammatory response compared with that of the untreated group. The group of animals that were contaminated with AFB_1 and that received exogenous CORT was the treatment combination that showed the lowest inflammatory response. Animals that received CORT but that were not contaminated with AFB_1 showed an intermediate inflammation response but not statistically different from the CORT and AFB_1 treated group.

The effects of CORT treatment and AFB_1 contamination on the quail heterophil:lymphocyte ratio are given in Figure 2. The ANOVA revealed a significant main effect of the CORT treatment ($F_{1,40} = 4.73$; $P = 0.03$) and AFB_1 contamination ($F_{1,40} = 4.48$; $P = 0.04$). No significant differences ($F_{1,40} = 0.42$; $P = 0.52$) between males and females were detected. Thus, post-hoc analysis was also performed combining male and female data. The test revealed that the control quail (not CORT treated and not AFB_1 contaminated) and the CORT treated and reared with AFB_1 contaminated feed showed respectively the lowest and the highest H:L ratio. The non-CORT-treated birds reared with AFB_1 and the CORT treated with noncontaminated feed showed similar and intermediate values (Figure 2).

The statistical analysis on the effects of CORT and AFB_1 contamination showed a similar response pattern in the 3 biochemical parameters evaluated. The ANOVA revealed significant interactions among CORT and AFB_1 contamination for total protein concentration ($F_{1,40} = 28.01$; $P < 0.0001$), ALB ($F_{1,40} = 31.07$; $P < 0.0001$), and GLOB ($F_{1,40} = 8.52$; $P < 0.01$). No significant effect was detected on the ALB:GLOB ratio (data not shown), and there was no significant effect of sex on total protein concentration ($F_{1,40} = 0.02$; $P = 0.87$), ALB ($F_{1,40} = 0.12$; $P = 0.73$), and GLOB ($F_{1,40} = 0.32$; $P = 0.57$) (Figures 3, 4, and 5, respectively). The group of animals not CORT-treated and not AFB_1-contaminated showed the highest values of the biochemical parameters measured, and the CORT-

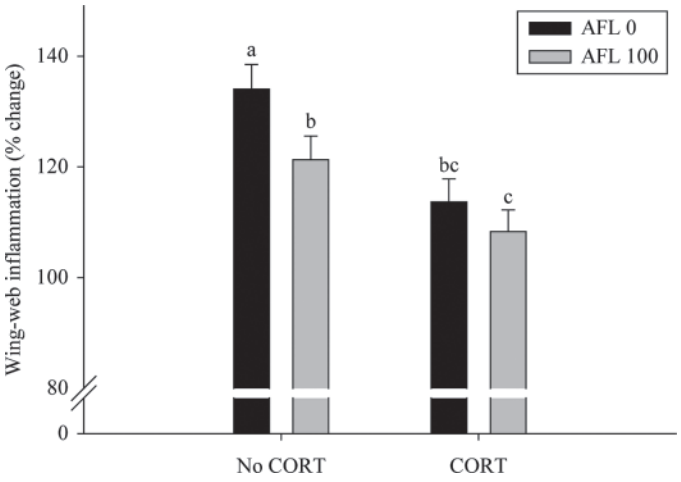

Figure 1. Percentage of change in the wing-web thickness 24 h postinjection of phytohemagglutinin-p in Japanese quail submitted to the administration of corticosterone (CORT) in drinking water and feed contaminated with aflatoxin (AFB_1). [a–c]Different letters indicate significant ($P < 0.05$; Fisher least significant difference test) differences between groups. Bars represent treatment means and lines represent the SE (number of birds/group = 12). No effect of sex was detected, thus, data from female and male were pooled to improve visualization. AFL 0 = 0 μg of AFB_1/kg of feed; and AFL 100 = 100 μg of AFB_1/kg of feed.

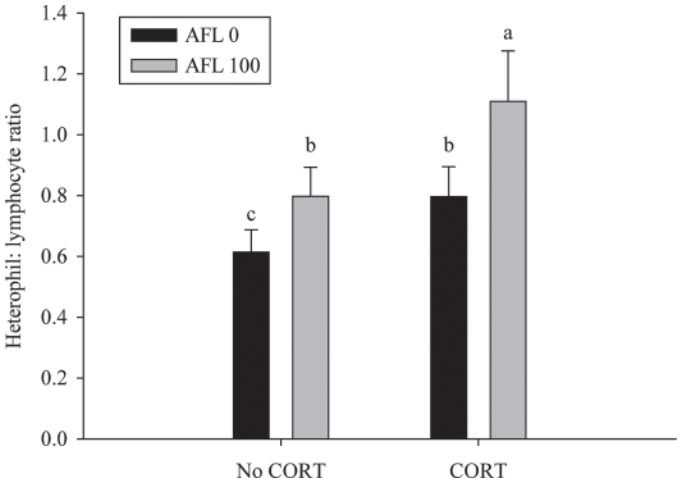

Figure 2. Heterophil:lymphocyte ratio of Japanese quail submitted to the administration of corticosterone (CORT) in drinking water and feed contaminated with aflatoxin (AFB_1). [a–c]Different letters indicate significant ($P < 0.05$; Fisher least significant difference test) differences between groups. Bars represent treatment means and lines represent the SE (number of birds/group = 12). No effect of sex was detected, thus, data from female and male were pooled to improve visualization. AFL 0 = 0 μg of AFB_1/kg of feed; and AFL 100 = 100 μg of AFB_1/kg of feed.

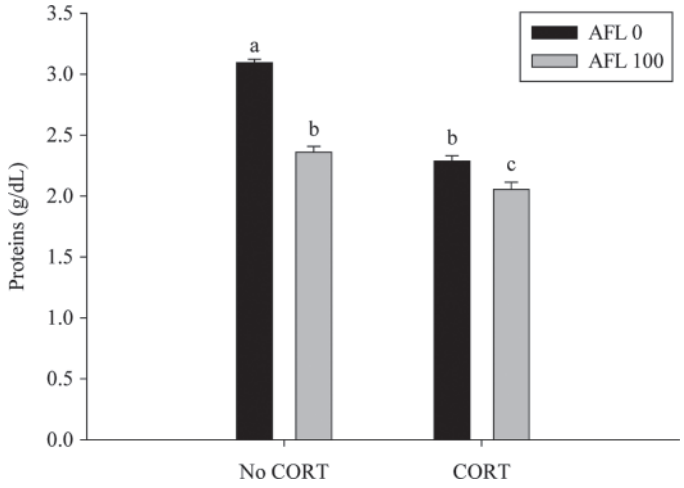

Figure 3. Protein concentration in plasma of Japanese quail submitted to the administration of corticosterone (CORT) in drinking water and feed contaminated with aflatoxin (AFB_1). [a–c]Different letters indicate significant ($P < 0.05$; Fisher least significant difference test) differences between groups. Bars represent treatment means and lines represent the SE (number of birds/group = 12). No effect of sex was detected, thus, data from female and male were pooled to improve visualization. AFL 0 = 0 μg of AFB_1/kg of feed; and AFL 100 = 100 μg of AFB_1/kg of feed.

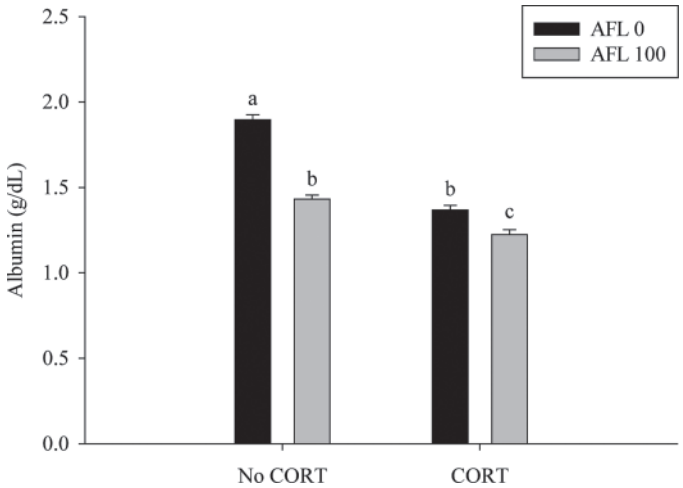

Figure 4. Albumin concentration in plasma of Japanese quail submitted to the administration of corticosterone (CORT) in drinking water and feed contaminated with aflatoxin (AFB_1). [a–c]Different letters indicate significant ($P < 0.05$; Fisher least significant difference test) differences between groups. Bars represent treatment means and lines represent the SE (number of birds/group = 12). No effect of sex was detected, thus, data from female and male were pooled to improve visualization. AFL 0 = 0 μg of AFB_1/kg of feed; and AFL 100 = 100 μg of AFB_1/kg of feed.

treated and AFB_1-contaminated animals showed the lowest values. Birds that were not CORT-treated and AFB_1-contaminated and those birds that were CORT-treated but not AFB_1-contaminated showed intermediate but significantly different values compared with those of the extreme groups mentioned above (see Figures 3, 4, and 5).

DISCUSSION

The present study evaluates the potential effects of the combination of the administration of AFB_1 and CORT on biochemical and immunological parameters of Japanese quail. Mycotoxicosis is a current problem faced by poultry farmers (Madheswaran et al., 2004; Ortatatli et al., 2004), and AF is one of the most frequently encountered mycotoxins. Previous studies inform about the negative consequences of AF administration on birds (Arafa et al., 1981). Aflatoxicosis has been experimentally induced several times in chicken (Oğuz et al., 2000a,b) and in quail (Bintvihok et al., 1993; Oliveira et al., 2002, 2007). Hypoproteinemia, hypoalbuminemia, and hypoglobulinemia were previously observed in AF toxin-treated quail groups (Madheswaran et al., 2004). This phenomenon is confirmed by the results of the present study. Moreover, our data also show that a chronic stressor (induced by CORT in drinking water) and AFB_1 contamination combined their negative effects on the biochemical parameters evaluated. Thus, for example, quail in the treatment combining the effects of the administration of CORT and AFB_1 showed the highest hypoproteinemia, hypoalbuminemia, and hypoglobulinemia. The fall in these biochemical variables has been previously attributed to the inactivation of biosynthetic enzymes and the impairment of protein synthesis by AF (Madheswaran et al., 2004). Based on our results, we could propose that the inactivation suggested is reinforced by the effect of the chronic stressor used (CORT), leading to an exacerbated phenomenon in the animals submitted to the combination of these 2 factors. It is important to mention that Japanese quail submitted to the same experi-

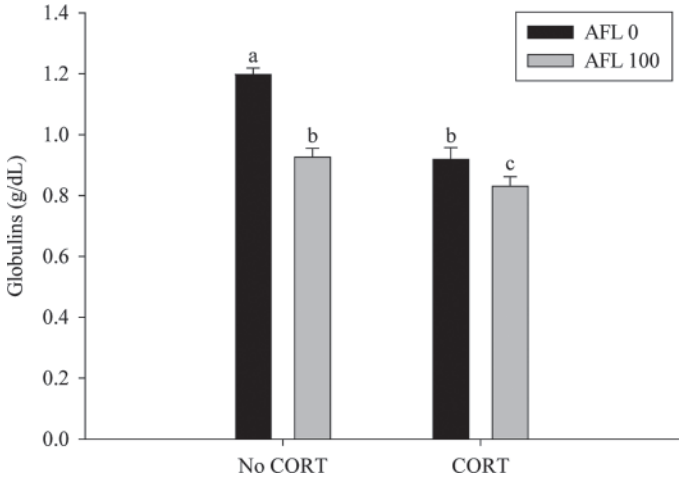

Figure 5. Globulin concentration in plasma of Japanese quail submitted to the administration of corticosterone (CORT) in drinking water and feed contaminated with aflatoxin (AFB_1). [a,b]Different letters indicate significant ($P < 0.05$; Fisher least significant difference test) differences between groups. Bars represent treatment means and lines represent the SE (number of birds/group = 12). No effect of sex was detected, thus, data from female and male were pooled to improve visualization. AFL 0 = 0 μg of AFB_1/kg of feed; and AFL 100 = 100 μg of AFB_1/kg of feed.

mental conditions that showed diminished biochemical parameters herein also showed histological and macroscopic indicators of aflatoxicosis in their livers and a significant reduction in BW and feed conversion ratio (Magnoli et al., unpublished data).

To our best knowledge, no previous studies inform about the effect of AF contamination on immune parameters of Japanese quail. According to our results, the administration of AFB_1 in the diet had a depressing effect on cell-mediated immunity. Our data also confirms that the previously demonstrated immunodepressing effect of CORT administration via drinking water in chickens (Shini and Kaiser, 2009) also occurs in Japanese quail, when referring to the cellular component of immunity. Collectively, the data could imply that the recruitment and migration mechanisms involved in cellular immunity are affected by both AFB_1 and CORT. Communication between cells of the immune system and innate responses frequently require different proteins in blood (Chamanza et al., 1999; Bals, 2000). The diminished level of proteins in the contaminated animals could also be a plausible explanation to the cellular immune depression observed. Although no direct study of humoral response was performed herein, it is worth mentioning that the fall in globulins is an indicator of diminished production of the family of proteins to which antibodies belong.

The elevation of the heterophil:lymphocyte ratio is a hematological indicator that animals are coping with chronic stress (Gross and Siegel, 1983). Induction of the hypothalamic-pituitary-adrenal axis has been shown not only to reduce the lymphocyte number but also to increase the number of neutrophils (equivalent to avian heterophils; Dhabhar et al., 1995, 1996). Glucocorticoids (predominantly CORT) can induce apoptosis in immature T and B cells (reviewed by Lechner et al., 2001). Coherently, those animals supplemented with CORT had elevated heterophil:lymphocyte ratios. Interestingly, those animals supplemented with AFB_1 also had an elevated ratio. These data could support the contention that the effects of AF administration on an organism are comparable or similar to those of a chronic stressor and, therefore, the elevation in the heterophil:lymphocyte ratio observed after providing the birds with AFB_1-contaminated feed. Although no significant interaction was detected between factors, birds supplemented with both AFB_1 and CORT were the animals with the highest observed ratio.

Considering the overall results, we suggest that consuming feed contaminated with AFB_1 could be considered a stressor for animals. The detriment in the cellular component of the immune response due to the contamination is a new aspect of this toxin consumption. This phenomenon could have 2 possible explanations: first, it could imply that the functioning of the immune response is affected by the contamination per se; or, second, that immune response is indirectly affected by the resources reallocation aimed to deal with contamination and its negative consequences. In the second context, immune response turns out to be another energy-demanding task (Klasing, 1998, 2004) for a challenged organism already coping with stress and contamination.

This study suggests that the effects of the AFB_1 contamination appear to be further increased when overlapped with a chronic stressful stimulation. This stimulation could be the result of various, and at the same time, unavoidable procedures in the commercial production of animals nowadays (Schulz et al., 2000; Marco et al., 2006; Dickens et al., 2009, 2010). Because neither sex effects nor interaction between sex, AFB_1, and CORT treatments were detected, our results also show that the detrimental consequences observed for the AFB_1 and CORT exposures affect male and female quail in a similar manner. To conclude, this study suggests that the effects of the AFB_1 contamination on biochemical and immunological parameters are further increased when overlapped with a chronic stressful stimulation, and therefore, it emphasizes the importance of controlling potential stressor combinations during animal rearing to preserve not only the animal's health status but also their welfare.

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