AFLATOXIN B_1 RESIDUES IN LIVER OF JAPANESE QUAIL (*Coturnix japonica*) EXPOSED TO CONTAMINATED FEED AND EXOGENOUS CORTICOSTERONE*

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ABSTRACT. Magnoli A.P., Chiacchiera S.M., Rosa C.A. da R., Dalcero A.M. & Marin R.H. **Aflatoxin B_1 residues in liver of Japanese quail (*Coturnix japonica*) exposed to contaminated feed and exogenous corticosterone.** [Resíduos de aflatoxina B_1 em fígado de codornas japonesas (*Coturnix japonica*) expostas a alimentos contaminados e à corticosterona exógena]. *Revista Brasileira de Medicina Veterinária*, *35(4):378-384, 2013*. Departamento de Química, Universidad Nacional de Rio Cuarto, Rio Cuarto, Córdoba, Argentina. E-mail: schiacchiera@exa.unrc.edu.ar

The combined effect of aflatoxin B_1 (AFB_1) exposition and stress induced by corticosterone (CORT) upon the residual aflatoxin levels in livers of Japanese quail was examined. A total of 144 Japanese quail (*Coturnix japonica*) were divided into 6 treatments with 6 replicates per treatment, each containing 2 males and 2 females. The different treatments resulted from the combination of the presence or absence of CORT in drinking water (5 mg/L) with the presence or absence of AFB_1 supplementation (100 or 500 ng/g). Dietary treatments were offered from 5 to 11 weeks of age The different diets were: Treatment (T) 1: basal diet (B) AFB_1 (15 ng/g); T2: B plus AFB_1 (100 ng/g); T3: B plus AFB_1 (500 ng/g); T4: B plus CORT (5 mg/L); T5: B plus AFB_1 (100 ng/g) and CORT (5 mg/L); and T6: B plus AFB_1 (500 ng/g) and CORT (5 mg/L). The residual levels of AFB_1 were remarkably higher in livers of birds fed with AFB_1 in comparison with those belonging to the group that received a combination of AFB_1 and CORT ($P< 0.05$). A plausible explanation about the facts that could be responsible for observed reduction in the toxin carryover in liver was suggested. These results are very striking and may constitute the start point for a series of studies that aim to elucidate the precise influence of hypothalamic-pituitary-adrenal axis (HPA) response to stress in birds exposed to AFB_1.

KEY WORDS. Aflatoxicosis, corticosterone, stress response, Japanese quail, liver, Aflatoxin B_1 residues.

RESUMO. Estudou-se o efeito combinado da exposição à aflatoxina B1 (AFB_1) e o estresse induzido por corticosterona (CORT) sobre os níveis de residuais de AFB_1 em fígados de codornas japonesas. Um total de 144 codornas japonesas (*Coturnix japonica*) foram divididas em 6 tratamentos com

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6 repetições por tratamento, cada uma contendo 2 machos e 2 fêmeas. As dietas dos tratamentos foram oferecidas da 5ª até a 11ª semana de idade. Os diferentes tratamentos resultaram da combinação da presença ou ausência de CORT na água de beber (5 mg / L), com a presença ou ausência de suplementação AFB_1 (100 ou 500 ng /g) As dietas diferentes foram: Tratamento (T) 1: dieta basal (B) AFB_1 (15 ng/g); T2: B mais AFB_1 (100 ng / g); T3: B mais AFB_1 (500 ng / g); T4: B mais CORT (5 mg / L); T5: B mais AFB_1 (100 ng / g) e CORT (5 mg / L), e T6: B mais AFB_1 (500 ng / g) e CORT (5 mg / L). Os níveis residuais de AFB_1 foram significativamente mais elevados em fígados de aves alimentadas com AFB_1 em comparação com os que pertencem ao grupo que recebeu uma combinação de AFB_1 e CORT ($P< 0,05$). Sugere-se uma possível explicação para a redução dos níveis da toxina observada naqueles animais submetidos a dieta com CORT. Estes resultados constituem-se de um ponto de início para uma série de estudos que visam a elucidar a influência da resposta do eixo hipotalâmico-hipofisário-adrenais (HPA) ao stress em aves expostas a AFB_1.

PALAVRAS-CHAVE. Corticosterona, codornas japonesas, fígado, aflatoxina B1.

INTRODUCTION

Aflatoxin B_1 (AFB_1), B_2, G_1 and G_2 are fungal metabolites, mainly produced by *Aspergillus flavus* and *A. parasiticus*, which have been frequently detected as natural contaminants of feeds (Shareef 2010). Aflatoxin B_1 was classified as carcinogen Group 1 by the International Agency for Research on Cancer (IARC 1993). Many countries have established tolerance AFB_1 levels in food in order to reduce the toxin exposure. For animal feed, the European Union established maximum limits of AFB_1 ranging from 5 to 20 μg/kg depending not only on the type of product but also of the animal to be fed (European Commission 2003). An action level of 100 μg/kg has been established by the Food and Drug Administration for aflatoxins in feeds intended to mature poultry (FDA 1994). In Latin American, Brazil has established a limit of 50 μg/kg for aflatoxins B_1, G_1, B_2 and G_2, while MERCOSUR, the Latin American common trade market, accept 20 μg/kg of aflatoxins B_1, G_1, B_2 and G_2 (FAO 2003).

The toxicity of AFB_1 in birds has been widely investigated, being the liver the toxin target organ. Biochemical, haematological, immunological, and pathological effects of AFB_1 have also been well-described (Mutlu et al. 2010, Magnoli et al. 2011a, Magnoli et al. 2011b, Yunus et al. 2011, Nazar et al. 2012, Magnoli et al. 2012). The dose-response relationship is markedly altered by intraspecific factors such as age, sex, and breed (CAST 2003). Liver is also the main site where AFB_1 is accumulated, metabolized and/or conjugated to nucleic acids and proteins. Aflatoxin B_1 is activated by cytochrome P450 enzymes (CYP), including CYP1A2, CYP3A4 and CYP2A6, and converted to epoxides (AFB_1-8,9-exo-epoxide, and AFB_1-8,9-endo-epoxide), aflatoxin M_1 (AFM_1), aflatoxin P_1 (AFP_1), aflatoxin Q_1 (AFQ_1), or its reduced form aflatoxicol (AFL) (Yunus et al. 2011). Recent studies have shown that CYP2A6 and to a lesser extent CYP1A1 are the enzymes responsible for bio-activation of AFB_1 into epoxide forms in the liver of chicken and quail (Díaz et al. 2010). Residues of AFB_1 and some of its metabolites have been detected in eggs, liver, gizzard and kidney, as a consequence of the direct intake of feed contaminated with aflatoxins. This fact represents a potential risk for the bird's health, with the economic losses associated to aflatoxicosis as well as the toxin carryover in the food chain (Oliveira et al. 2000, Bintvihok et al. 2002a, Rizzi et al. 2003, Mutlu et al. 2010).

For many years, researchers have evaluated the effects of environmental factors that may affect the behavioral, physiological and performance responses of birds (Mashaly et al. 2004, Shini et al. 2008). Several external factors are known to induce a state of stress response that has been well-characterized and involves increased levels of glucocorticoids and catecholamines released into the blood stream (Satterlee & Marin 2006, Odihambo Mumma et al. 2006). The increase of these compounds is the result of the activation of two neuroendocrine axis, the sympathetic and the hypothalamic-pituitary-adrenal axes (Kuenzel & Jurkevich 2010). These stress-induced physiological changes can seriously affect animal's growth, development and health during production (Lin 2006). The increase in circulating corticosterone induces in poultry a state of stress response, affecting metabolic (Shini & Kaiser 2009), physiological (Satterlee & Marin 2006) and immunological functions among others (Nazar et al. 2012).

Previous studies have shown that biochemical and immunological parameters as well as the immunodepression were exacerbated with the combined administration of AFB_1 (500 ng/g of feed) and corticosterone (5 mg/L) in drinking water (Nazar et

al. 2012). Negative effects on performance indices (i.e., body weight, feed conversion and egg production) as well as on macroscopic and microscopic liver parameters were also observed (Magnoli et al. 2012). To the best of our knowledge there is no data available on the effect of CORT upon the carryover of AFB_1 in liver.

Therefore, taking into account that during avian breeding the stressors and aflatoxin exposure are frequently unavoidable, the aim of this study was to evaluate the effect of these factors, alone or in combination, upon residual levels of aflatoxin B_1 in liver. Japanese quail were chosen not only because of their importance for meat and egg production (Caron et al. 1990, Baumgartner 1994), but also because they are useful models for chickens and other commercially important poultry species (Minvielle 2009).

MATERIALS AND METHODS

Chemicals

Standards of purchased AFB_1 (Sigma, Aldrich Inc., St Louis MO) were assayed by HPLC. Their purities were confirmed as being higher than 99%. Demineralized water (HPLC grade) was obtained using Labconco equipment (model 90901-01). Corticosterone or **11β,21-Dihydroxy-4-pregnene-3,20-dione,** (CORT) (Sigma, Aldrich Inc., St Louis MO) dose was selected according to previous experiments, showing that a dose of 5 mg/L of CORT in drinking water was able to elevate CORT in blood at similar levels to the ones obtained in a stressful manipulation (Hull et al. 2007, Wall & Cockrem 2010).

Aflatoxin 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 mould aqueous suspension containing 10^6 spores/mL. Cultures were allowed to grow for 7 d at 25 ºC in darkness. On the seventh day, Erlenmeyer flasks were autoclaved; and culture material was dried for 48 h at 40ºC in a forced-air oven and ground to a fine powder. The AFB_1 levels in rice powder were measured by HPLC (Trucksess et al. 1994, AOAC 2000). The milled substrate was added to the basal diet to provide the working levels of AFB_1 contamination.

Experimental design and husbandry

One hundred and sixty five Japanese quail (*Coturnix japonica*) hatchlings were randomly housed in 3 white wooden boxes (55 quail each) measuring 90 x 90 x 60 cm (length x width x height), and remained in the same boxes until 4 weeks old. Each box had two 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 the passage of excreta and a lid prevented the birds from escaping. Brooding temperature was 37.5 °C during the first wk of life, with a weekly decline of 3.0 °C until room temperature (24 to 27 °C) was achieved. At 4 wk of age, birds were randomly selected by similar body weight (BW), sexed by plumage coloration, peak trimmed and wing banded for further identification. One hundred and forty four birds were housed in mixed groups of four animals (two males and two females) in 36 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 Rios, 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. Temperature was kept between 22 ± 2 ºC during the experimental period. The AFB_1 contend in basal diet was 15 ng/g.

After 5 wk of age, quail were weighed and randomly assigned to 1 of 6 dietary treatment groups (see below). The different treatments resulted from the combination of the presence or absence of CORT in drinking water with the presence or absence of AFB_1 supplementation in diets. During the treatment (lasting 6 wk), birds were monitored daily for signs of morbidity and mortality. To facilitate application of CORT treatment, water was provided through a manual bottle-pipe drinker. The AFB_1 contamination in feed was obtained by AFB_1 supplementation of basal diet to the required toxin levels according to the treatment.

The experimental diets for each one of the 6 treatments were as follows: Treatment 1 (T1): basal diet (AFB_1 15 ng/g) (B); Treatment 2 (T2): B plus AFB_1 (100 ng/g); Treatment 3 (T3): B plus AFB_1 (500 ng/g); Treatment 4 (T4): B plus CORT (5 mg/L); Treatment 5 (T5): B plus AFB_1 (100 ng/g) and CORT (5 mg/L); Treatment 6 (T6): B plus AFB_1 (500 ng/g) and CORT (5 mg/L).

At the end of the experiment, two birds from each replicate (one male and one female) were randomly selected and killed for liver removal and determination of aflatoxin B_1 residues.

Determination of aflatoxin B_1 in feed

The concentration of AFB_1 in each diet was estimated by HPLC-MS/MS following procedures proposed by Sulyok et al. (2007). A Waters 2695 Alliance HPLC instrument (Waters Corporation) equipped with a Waters Alliance 2685 pump and a Waters Alliance 2695 autosampler, interfaced with a Quattro Ultima Platinum tandem quadrupole mass spectrometer with electrospray ionization source was used. An XBridge C18 column (3.5µm, 2.1 × 150 mm) with a guard column was utilized. The experimental conditions for the determination were published elsewhere (Nazar et al. 2012). The assayed levels were 15 ng/g of AFB_1 for the basal diet and 100 and 500 ng/g for each supplementation level.

Determination of aflatoxin B_1 residue in liver tissue

Liver samples of birds from each group were pooled and stored at 20 ºC for later analysis. The AFB_1 in the tissues was extracted, purified using an immunoaffinity column and estimated by a HPLC-fluorescent detection method with pre-column derivatization according to a methodology adapted from the literature (Tavčar-Kalcher et al. 2007, Hussain et al. 2010). Twenty-five grams of each defrosted sample were homogenized and blended with 2.5 mL of a 20% aqueous citric

acid solution and diatomaceous earth (5 g). The mixture was extracted with 50 mL of dichloromethane by shaking for 30 min. The filtered extract was dried by addition of Na_2SO_4 and filtered again, and an aliquot (10 mL) was evaporated to dryness. The residue was reconstituted with 10 mL of acetonitrile: H_2O (75:25, V/V) and extracted with 5 mL of hexane. Afterward, 5 mL of the aqueous phase were taken and evaporated to dryness. The concentrate was reconstituted with 50 mL methanol: water (80:20, V/V). An aliquot of 40 mL was applied onto the immunoaffinity column (AFLAPREP® R-Biopharm) without preconditioning. The column was washed with 10 mL of phosphate buffered saline. Aflatoxin B_1 was eluted from the column with 2 mL of methanol and evaporated to dryness. The residue was resuspended with 200 µL of mobile phase and derivatized with 700 µL trifluoroacetic acid: acetic acid: water (20:10:70) (AOAC 2000). An aliquot of 50 µL of the derivatized extract was injected into HPLC (Water 2475) with reverse phase column (Luna 5 µm C18 (2) 100A, 150 x 4,6mm, Phenomenex Inc.) equipped with a guard column and a Waters 2475 Multi-Wavelength Fluorescence Detector, with excitation at 360 nm and emission at 440 nm. The mobile phase of the chromatographic procedure was a mixture of methanol (solvent A), acetonitrile (solvent B) and water. The pump program started with acetonitrile-methanol-water (1:1:4) during 7 min, followed by 5 min of washing (2:2:1) and 6 min of stabilization at initial conditions before next injection. The flow rate was 1.5 mL/min. Figures 1a and 1b show the chromatograms obtained from liver samples of T1 (Basal diet), and T6 (Basal diet plus 500 ng/g AFB_1 and 5 mg/L corticosterone), respectively. The detection (LOD) and quantification (LOQ) limits of the analytical method were 0.001 and 0.004 ng/g, respectively. Results were not corrected by recovery.

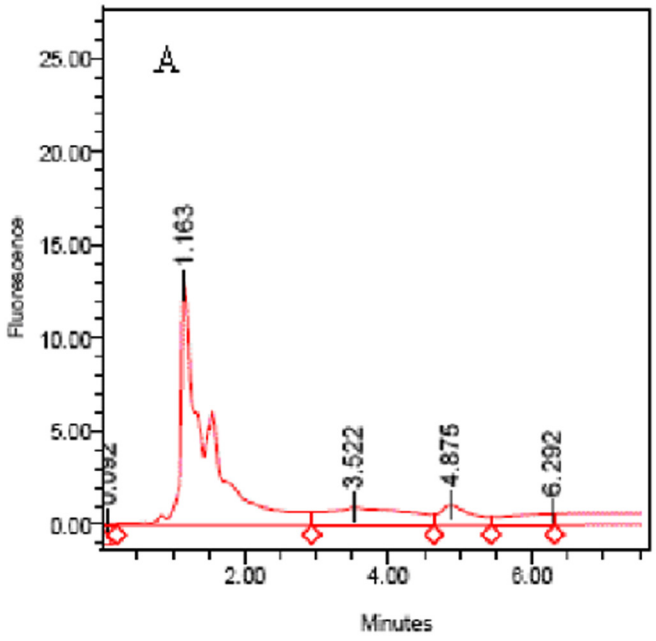

Figure 1a. Chromatogram of AFB_1 residues in liver samples of Japanese quail, corresponding to Treatment 1 (Basal diet).

Assay of spiking and recovery of AFB_1 in livers

In order to determine the efficacy of the analytical method, duplicated toxin free livers samples were fortified at three spiking levels to reach concentrations of 50, 100 and 250 ng/g of AFB_1. Recovery values were in the range 70–80%. Intraday and interday relative standard deviations were 4.6 and 14.7%, respectively.

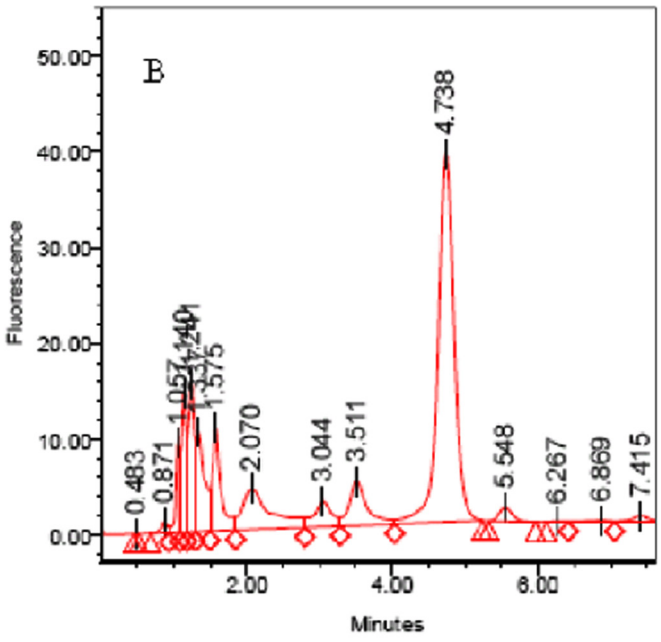

Figure 1b. Chromatogram of AFB_1 residues in liver samples of Japanese quail, corresponding to Treatment 6 (Basal diet plus AFB_1 (500 µg/kg) and Corticosterone (5 mg/L).

Data analysis

Data analyses were performed by analysis of variance. The Fisher's least significant difference test (LSD) was used to determine the significant differences ($P<0.05$) among means of AFB_1 residues in liver samples.

RESULTS

Aflatoxin B_1 residues levels in liver of Japanese quail fed different experimental diets from 35 to 77 days of age are shown in Table 1. All AFB_1 residue levels in liver were statistically different between treatments ($P<0.05$). The highest AFB_1 mean level, (0.299 ± 0.005 ng/g) was detected in the livers of

Table 1. Levels of Aflatoxin B_1 residues in liver samples of Japanese quail after 6 weeks of treatment

Treatments[a]	AFB_1 in diet ng x g	CORT mg/L	AFB_1 in liver ng × g	Ratio[b]
T1	15±1	-	0.055±0.005^{bc}	272
T2	100±7	-	0.078±0.005^{c}	1,282
T3	500±25	-	0.299±0.005^{e}	1,672
T4	15±1	5	0.016±0.003^{a}	937
T5	100±7	5	0.039±0.002^{ab}	2,564
T6	500±25	5	0.245±0.076^{d}	2,040

[a]Values are presented as Mean ± SE. Each treatment group consisted of six replicates (12 birds per treatment). Values in column with no common superscripts are significantly different (P < 0.05) according to LSD Fisher's tests.

[b]Ratio: AFB_1 levels in the feed versus AFB_1 residual levels in the liver.

birds feed with 500 ng of the toxin /g feed (T3). A residual mean value almost four times lower (0.078 ± 0.005 ng/g) was observed in treatment T2, i.e. birds feed with 100 ng/g of AFB_1. On the other hand, livers from control birds (T1), which were fed with naturally contaminated feeds (15 ng/g of AFB_1), showed a residual toxin mean value of 0.055 ± 0.005 ng/g. As can be observed in Table 1, the ratio of AFB_1 in the feed to the residual level AFB_1 in the liver in absence of corticosterone was 272 in quail fed the basal diet (15ng/g), while ratios of 1,282 and 1,672 were obtained for quail fed, with 100 and 500 ng/g of AFB_1, respectively. On the other hand, the ratio of AFB_1 in the feed to the residual levels AFB_1 in the liver in presence of corticosterone was 937 in quail fed the basal diet (15ng/g), while ratio of 2,564 and 2,040 were obtained for quail fed with 100 and 500 ng/g of AFB_1 respectively.

In the case of birds under conditions that mimic physiological stress (administered with corticosterone in water), significantly ($P<0.05$) lower AFB_1 residual values in liver were obtained. Compared with T1, the livers of birds received CORT alone (T4) showed 3.5 times lower residual toxin levels. As the toxin intake level increased, the effect became weaker. Thus, a two times decrease in the toxin residual mean value was observed in T5 group while 1.2 times reduction was observed in T6 group when compared with their group counterparts receiving 100 and 500 ng of AFB_1/g of feed (i.e., T2 and T3, respectively).

DISCUSSION

A critical aspect of animal production is to determine whether or not an aflatoxicosis is affecting the animals and if so, what are the levels of contamination that are present at the end of the production chain. Residues of AFB_1 and their metabolites have been previously found in eggs and poultry tissues after the consumption of diets contaminated with aflatoxins (Pandey & Chauhan 2007). This data is important both from the animal health and the consumer's point of view. Although much literature has been published about the adverse effects of AFB_1 or CORT on productive, biochemical and immunological parameters in birds (Yunus et al., 2011; Shini et al., 2008), little is known about the combined effects of these stressors during animal rearing (Caron et al., 1990; Baumgartner 1994). The present study evaluates the effects of CORT on the residue levels of AFB_1 in Japanese quail liver under induced aflatoxicosis. Aflatoxin B_1 levels of 0.055, 0.078 and 0.299 ng/g were found in livers of Japanese quail fed 15, 100 and 500 ng of AFB_1/g of diets, respectively. Eshak et al. (2010) reported a much higher AFB_1 level (8.64 ng/g) in livers of Japanese quail feed during 35d with a concentration of 500 ng/kg. In the present work, the experiment began with 35d of age birds which could explain the lower observed carryover in liver. Bintvihok et al. (2002b) also reported a quite high AFB_1 mean value (7.83 ng/g) in livers of birds after a shorter exposition time (21d) of contamination but in quail fed with a six times greater AFB_1 dose (3,000 ng/kg). Previous studies showed similar residual mean level of AFB_1 in livers (0.40 ng/g) of broilers fed diets containing 50 µg/kg of AFB_1 over a period of 28d (Magnoli et al. 2011b). Zaghini et al. (2005) found residues of AFB_1 in livers of laying hens (4.13 ng/g) given 2,500 ng of AFB_1/kg of feed during 28d. Chen et al. (1984) reported levels of 3 ng of AFB_1/g of liver in chickens fed with 2,060 ng of AFB_1/kg of diet during a 35d period. Bintvihok & Kositcharoenkul (2006) reported AFB_1 residual levels of 0.10 and 0.32 ng/g in livers of broilers fed with 50 and 100 µg/kg of AFB_1 during 42d, respectively. Factors such as animal strain, duration of exposure, nutrition, health, age, sex and individual variations might be responsible for the observed differences on the quantity of residues in liver samples (Wolzak et al. 1985).

The observed variations in data emphasize the influence of the toxin level in the diet upon the residual toxin in liver. The higher ratio of toxin in the feed to the residual level in the liver observed when the toxin level in the diet was increased could be due to an adaptive change of the overall metabolizing capacity of living organisms as observed for certain xenobiotics (Kliewer et al. 1999). On the other hand Bintvihok & Kositcharoenkul (2006) found a ratio of feed to the liver residual levels of only 383 in 20d old Japanese quail, but for a 7d induced acute aflatoxicosis. Differences in the exposition level could be the major reason of the observed differences between studies **(**Oliveira et al. 2000, Rizzi et al. 2003).

After exposure to AFB_1 and corticosterone, negative exacerbated effects were observed on: a) performance indices (i.e., body weight, feed conversion and egg production); b) biochemical and immunological parameters and c) macroscopic and microscopic quail liver parameters (Nazar et al. 2012, Magnoli et al. 2012). On the contrary, a gen-

eral decrease in the toxin carryover was observed in the presence of CORT. It is known that psychological, chemical or physical stress leads to dramatic changes of physiology in various regions of the body including the liver. Although the underlying hepatic mechanisms are still poorly understood, Ha et al. (2003) have shown that stress induces alterations in liver gene expression of mice. Particularly, an important group of genes involved in lipid metabolism and detoxification was found over expressed due to stress. These facts could be responsible for the observed reduction in AFB_1 carryover in liver, although further studies should be conducted in order to explain the underpinning mechanism of the observed effects. As mention above a lower amount of toxin in liver might be accompany of a greater amount of metabolic derivatives in liver, tissues and/or eggs. This is particularly important because among the possible derivatives, the 8,9-epoxides that are even more toxics than the precursor itself, are consider the main responsible of the toxin effects **(**CAST 2003). On the other hand, AFM_1 or AFL, reported in eggs by Oliveira et al. (2003), could be another metabolic way of AFB_1 biotransformation in liver. Aflatoxin M_1 itself has been classified as group II by the IARC (1993), and the natural mixture of aflatoxins (AFB_1, AFG_1 and AFM_1) as carcinogen group I (IARC 1993). Therefore, it have to be stressed that the present results are very striking and may constitute the start point for a series of future studies to elucidate the precise influence of HPA axis response to stress in birds exposed to AFB_1 as well as residual toxic metabolites in quail eggs and tissues.

In conclusions, stress induced changes in the liver detoxification mechanism could be responsible for a reduction in AFB_1 carryover in liver during aflatoxicosis. Further studies should perform in order to clearly elucidate the underlying physiological mechanism.

CONCLUSIONS

The residual levels of AFB_1 were remarkably higher in the livers of birds fed with AFB_1 alone compared to the livers of birds fed with AFB_1 in combination with CORT. An adaptive change of the overall metabolizing capacity of birds to increasing AFB_1 exposure might be operating. Stress induced changes in the liver detoxification mechanism could be responsible for the observed reduction in AFB_1 carryover in liver. Further studies should perform in order to clearly elucidate the underlying physiological mechanism.

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