An unusually high production of hepatic aflatoxin B$_1$-dihydrodiol, the possible explanation for the high susceptibility of ducks to aflatoxin B$_1$

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A study was conducted to determine the enzymatic kinetic parameters $V_{\text{max}}$, $K_M$, and intrinsic clearance (CL$_{\text{int}}$) for the hepatic in vitro production of aflatoxin B$_1$-dihydrodiol (AFB$_1$-dhd) from aflatoxin B$_1$ (AFB$_1$) in four commercial poultry species, ranging in sensitivity to AFB$_1$ from highest (ducks) to lowest (chickens). Significant but small differences were seen for $V_{\text{max}}$ while large significant differences were observed for $K_M$. However, the largest inter-species differences were observed for the CL$_{\text{int}}$ parameter, with ducks being extraordinarily efficient in converting AFB$_1$ into AFB$_1$-dhd. Since AFB$_1$-dhd is considered the metabolite responsible for the acute toxic effects of AFB$_1$, the high hepatic production of AFB$_1$-dhd from AFB$_1$ in ducks is the possible biochemical explanation for the extraordinary high sensitivity of this poultry species to the adverse effects of AFB$_1$.

Since the discovery of aflatoxins after the turkey “X” disease outbreak that killed over 100,000 turkey poultlets in Britain in 1960, it has been known that there are extremely large differences in sensitivity to aflatoxin B$_1$ (AFB$_1$) among commercial poultry species. The sensitivity to the acute effects of AFB$_1$, expressed as LD$_{50}$ values, ranges from 0.4 mg/kg in day-old ducklings to 6.8 mg/kg in day-old chicks. The minimum dietary concentration of AFB$_1$ capable of affecting growth in ducks, turkeys, chickens and laying hens is about 50, 200, 500 and 5000 ng/kg when exposed to the toxic diets for 3 to 4 weeks. The 100-fold difference between ducks and laying hens reflects the extreme tolerance to aflatoxins in adult chickens and the large sensitivity in ducks. Chickens even grow better when there are aflatoxins in their diet, whereas ducks are so sensitive that they were used as a biological assay for testing feedstuffs, prior to the development of the modern analytical techniques for aflatoxins.

To become a toxic compound, AFB$_1$ requires biotransformation by cytochrome P450 enzymes (CYP). Several AFB$_1$ metabolites from mammalian and avian CYPs have been identified including aflatoxins M$_1$, B$_2$, P$_1$, and Q$_1$, and the electrophilic unstable AFB$_1$-exo-8,9-epoxide (AFBO). The epoxide can alkylate RNA in vitro as well as the N7 position of guanine residues in DNA, forming irreversible adducts; these adducts eventually cause the transversion G $\rightarrow$ T at codon 249 of the p53 tumor suppressor gene in human hepatocytes, leading to hepatic cancer. Chronic exposure to AFB$_1$ causes hepatocellular carcinoma not only in humans but also in such species as rats, primates and ducks. The AFB$_1$ metabolite responsible for the acute toxic effects of AFB$_1$ has not been clearly identified but one possible candidate is the AFB$_1$-exo-8,9-dihydrodiol (AFB$_1$-dhd) that results from the nucleophilic trapping process of the AFB$_1$-exo-8,9-epoxide by water. AFB$_1$-dhd has been shown to inhibit protein synthesis in vitro and its furanopen-rIng-opened oxyanionic metabolite (AFB$_1$-hydroxydialdehyde) can form lysine adducts in serum albumin in vivo. Further, an aldehyde reductase with activity toward the AFB$_1$ dialdehyde has been associated with decreased liver toxic effects in rats. Therefore, the dihydrodiol/dialdehyde forms, which occur in equilibrium at physiological pH, appear to be responsible for the cytotoxic acute effects of AFB$_1$ exposure. For more than a decade our research group has been looking for biochemical differences in the hepatic biotransformation of AFB$_1$ that could explain the in vivo differences in response to AFB$_1$, among the main poultry species. The present study shows for the first-time large differences in the enzymatic kinetic parameters for the hepatic production of AFB$_1$-dhd from AFB$_1$.}

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parameters of AFB1-dhd production in liver microsomes that could explain the different in vivo sensitivity to AFB1 of resistant (chickens and quail), sensitive (turkeys) and highly sensitive (ducks) poultry species.

Results
Due to the lack of a commercially available AFB1-dhd standard, a mass spectrometric analysis of the putative AFB1-dhd peak was conducted to determine its monoisotopic mass. The putative peak observed at 6.7 min Fig. 2a corresponded to a compound of 347 Da, which is consistent with the monoisotopic protonated mass of AFB1-dhd Fig. 2b.

The enzymatic kinetic parameters for AFB1-dhd production by the four poultry species investigated are presented in Fig. 3. Chicken and quail enzymes did not saturate even at the highest AFB1 concentration evaluated (256 μM) Fig. 3a; however, turkey and duck enzymes seemed to become completely saturated with only 56 μM AFB1. The average values for the V_max were the highest in Rhode Island Red chickens (11.2 ± 1.48 nmol of dhd-AFB1/mg protein/minute) and quail (9.57 ± 3.06 nmol of dhd-AFB1/mg protein/minute), while no differences (P > 0.05) were observed among Ross chickens, turkeys, and ducks (5.75 ± 1.95, 5.84 ± 2.07 and 5.55 ± 1.33 nmol of dhd-AFB1/mg protein/minute, respectively) Fig. 3b. Rhode Island Red chicks had a higher V_max value compared with Ross chickens. Regarding differences by sex, only quail and turkeys showed significant differences between males and females. The average values for K_m showed large (P < 0.05) differences, with ducks presenting the lowest K_m value by far (3.84 ± 1.01 μM of AFB1), followed by turkeys (49.33 ± 7.66 μM of AFB1), quail (77.79 ± 22.14 μM of AFB1) and the chicken breeds Rhode Island Red and Ross (112.5 ± 33.4 and 131.8 ± 26.2 μM of AFB1, respectively) Fig. 3c. No differences between males and females were found in any species for this enzyme kinetic parameter. Furthermore, no differences between the chicken breeds were found either. Regarding the CL_int parameter, very large differences among the species evaluated were observed, with ducks being extraordinarily efficient in converting AFB1 into AFB1-dhd compared to the other poultry species investigated Fig. 3d. CL_int values for ducks, turkey, quail and Rhode Island Red and Ross chickens were 1.64 ± 1.00, 0.12 ± 0.04, 0.14 ± 0.08, 0.11 ± 0.02 and 0.05 ± 0.02 mL/mg protein/minute, respectively. No differences between males and females were observed.

Discussion
Since the discovery of aflatoxins in the early 1960’s it was observed that different animal species exhibit very different adverse effects upon exposure to the toxins. For example, ducklings, pigs and dogs die acutely at dietary concentrations that are well tolerated by humans, chickens and rats22–24. In some animal models, these differences in the ability to trap AFB1 with glutathione (GSH) ultimately determine the degree of AFB1-induced liver damage: while rats develop hepatocellular carcinoma upon chronic exposure to AFB1, mice are resistant. The reason for this differential response lies in the constitutive expression of high levels of an Alpha-class glutathione transferase (GST) that catalyzes the trapping of AFBO in the mouse that is only expressed at low levels in the rat25. Among poultry species exposed chronically to AFB1, the only one that develops liver cancer is the duck26; however, due to the short life-span of commercial poultry, it is actually the acute effects the ones that are more important. For more than a decade our research group has been searching for a biochemical explanation for the differences in susceptibility to AFB1 among the main poultry species. We have found that AFB1 is essentially bio- transformed into aflatoxical and AFB1-dhd by chicken, quail, turkey and duck liver microsomes and that at least four CYPs can bioactivate AFB1 into the epoxide in ducks, whereas CYP2A6 is the main cytochrome responsible for this reaction in chickens, quail and turkeys8,18–21. However, none of these findings could explain the extraordinarily high sensitivity of the duck compared to other poultry. In the present study we investigated the in vitro kinetic constants V_max and K_m, as well as their ratio, also known as intrinsic clearance. Measurement of CL_int has been used to predict the hepatic extraction of a compound27, and it is considered to be a measure of the total amount of enzyme that is coupled to the substrate and engaged in the conversion of the substrate into the product24, in other words it is a means to express enzyme efficiency28. Maximal velocity did not differ significantly between duck and turkey (sensitive species) or Ross chickens (highly resistant species); however, large significant differences in K_m were seen among the poultry species studied. Duck presented the lowest value: almost 13 times lower that turkey, 20 times lower than quail and 30 times lower than the chicken breeds. The calculation of the CL_int values revealed that duck liver microsomes clear AFB1 as AFB1-dhd at rates between 15 and 33 times higher than chickens. These values are due the low duck K_m values for AFB1-dhd production, which means that duck CYPs require very low concentrations of AFB1 to reach maximal velocity. More tolerant or resistant species require higher amounts of AFB1 to reach V_max making their
CYP enzymes a low performance biotransformation system. Based on these results we propose an order of AFB1 clearance as AFB1-dhd in the poultry species studied as follows: duck > quail > turkey > chicken (Ross), with values of 1.64, 0.14, 0.12 and 0.05 mL/mg/minute, respectively. In regard to differences between males and females we confirmed previous results obtained in our laboratory, where no significant differences were found by sex.

In summary, the present findings not only provide a biochemical explanation for the large differences in susceptibility to AFB1 between chickens and ducks, but also provide strong evidence that AFB1-dhd is the metabolite...
responsible for the acute toxicity of AFB1. We hypothesize that the large production of AFB1-dhd by the duck liver is the cause of the mortality and liver lesions observed with dietary concentrations that do not affect other poultry. Further, the large production of AFB1-dhd, which is in turn produced by the AFB1-exo-8,9-epoxide, might be related to the fact that ducks are the only poultry species that develop hepatic cancer after AFB1 exposure.

**Methods**

**Reagents.** AFB$_{180}$, glucose 6-phosphate sodium salt, glucose 6-phosphate dehydrogenase, nicotinamide dinucleotide phosphate (NADP$^+$), ethylenediaminetetraacetic acid (EDTA), bicinchoninic acid solution (sodium carbonate, sodium tartrate, sodium bicarbonate and sodium hydroxide 0.1 N pH 11.25), copper sulphate pentahydrate, formic acid, dimethylsulfoxide (DMSO), sucrose, glycerol, and bovine serum albumin were from Sigma-Aldrich (St. Louis, MO, USA). Aflatoxin B$_1$ was from Fermentek Ltd. (Jerusalem, Israel). Sodium chloride and magnesium chloride pentahydrate were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Sodium phosphate monobasic and sodium phosphate dibasic anhydrous were from Merck (Darmstadt, Germany). Methanol, acetonitrile and water were all HPLC grade.

**Microsomal fraction processing.** Liver fractions were obtained from 12 healthy birds (6 males and 6 females) from each of the following species and age: seven-week old Ross and Rhode Island Red chickens (*Gallus gallus ssp. domesticus*), eight-week old turkeys (*Meleagris gallopavo*), eight-week old quails (*Coturnix coturnix japonica*) and nine-week old Pekin ducks (*Anas platyrhynchos ssp. domesticus*). The birds were sacrificed by cervical dislocation, and their livers extracted immediately, washed with cold PBS buffer (50 mM phosphates, pH 7.4, NaCl 150 mM), cut into small pieces and stored at $-70^\circ$C until processing. The experiment was conducted following the welfare guidelines of the Poultry Research Facility and was approved by the Bioethics Committee, Faculty of Veterinary Medicine and Zootecnics, National University of Colombia, Bogotá D.C., Colombia (approval document CB-FMVZ-UN-033-18). Frozen liver samples were allowed to thaw, and 2.5 g were minced and homogenized for 1 minute with a tissue homogenizer (Cat X120, Cat Scientific Inc., Paso Robles, CA, USA) with 10 mL of extraction buffer (phosphates 50 mM pH 7.4, EDTA 1 mM, sucrose 250 mM). The homogenates were then centrifuged at 12,000 $\times g$ for 30 minutes at 4 $^\circ$C (IEC CL31R Multispeed Centrifuge, Thermo Scientific, Waltham, MA, USA). After this first centrifugation, the supernatants (approximately 10 mL) were transferred into ultracentrifuge tubes kept at 4 $^\circ$C and centrifuged for 90 minutes at 100,000 $\times g$ (Sorval WX Ultra 100 Centrifuge, Thermo Scientific, Waltham, MA, USA). The resulting pellets (corresponding to the microsomal fraction) were resuspended in 3 mL of storage buffer (phosphates 50 mM pH 7.4, EDTA 1 mM, sucrose 250 mM, 20% glycerol), fractioned in microcentrifuge tubes and stored at $-70^\circ$C. An aliquot of each sample was taken to determine its protein content by using the bicinchoninic acid protein quantification method according to Redinbaugh and Turley.

**Microsomal incubations.** Incubations were carried out in 1.5 mL microcentrifuge tubes kept at 39 $^\circ$C (the normal average avian body temperature) containing 5 mM glucose 6-phosphate, 0.5 U.L. of glucose 6-phosphate dehydrogenase, 0.5 mM NADP$^+$, 1 $\mu$L of AFB1 in DMSO at concentrations ranging from 1.23 to 256 $\mu$M, and 5 $\mu$g of microsomal protein. All volumes were completed with incubation buffer (phosphates 50 mM pH 7.4, MgCl$_2$ 5 mM, EDTA 0.5 mM), and the reaction stopped after 10 minutes with 250 $\mu$L of ice-cold acetonitrile. The stopped incubations were centrifuged at 15,000 $\times g$ for 10 minutes and 2 $\mu$L of a 1:10 dilution in mobile phase were analyzed by High Performance Liquid Chromatography (HPLC) as described below.

**Chromatographic conditions (HPLC).** The production of AFB$_1$-dhd in each incubation was quantitated in a Shimadzu Prominence system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a DGU-20A3R degassing unit, two LC-20AD pumps, a SIL-20AC published autosampler, a CTO-20A column oven, an SPD-20AV UV-Vis detector, an RF-20A RF fluorescence detector, and a CBM-20A bus module, all controlled by “LC Solutions” software. The chromatography was carried out on an Alltech Altima HP C18, 150 mm × 3.0 mm (Alltech Associates Inc., Deerfield, IL, USA) kept at 40 $^\circ$C. The mobile phase was a linear gradient of solvent A (water – 0.1% formic acid) and B (acetonitrile: methanol, 1:1–0.1% formic acid), as follows: 0 min: 25% B, 1 min: 25% B, 10 min: 60% B, 10.01 min: 25% B, and 17 min: 25% B. The flow rate was 0.4 mL/min and the fluorescence detector was set at excitation and emission wavelengths of 365 nm and 425 nm, respectively. The in-vial concentration of AFB$_2$-dhd was quantitated using an external standard of AFB$_2$ since these two compounds share identical spectral properties. Further, the monoisotopic protonated mass of AFB1-dhd was determined by HPLC-MS by means of a 3200 QTrap mass spectrometer (Applied Biosystems, Toronto, Canada) using a thermospray ionization probe in positive mode and the following settings: probe voltage: 4,800 V, declustering potential: 140 V, entrance potential: 10 V, curtain gas value: 30, collision energy: 81 V and collision cell exit potential: 5 V.

**Statistical analysis.** The enzymatic parameters $K_M$ and $V_{max}$ were determined by non-linear regression using the Marquardt method adjusting the data to the Michaelis-Menten enzyme kinetics using the equation: $v = \frac{V_{max}[S]}{K_M + [S]}$, where $v$ is the enzyme reaction velocity, $[S]$ represents substrate concentration, $V_{max}$ represents maximal velocity and $K_M$ represents the Michaelis-Menten constant. Intrinsic clearance (CL$_{int}$) was calculated as the ratio $V_{max}/K_M$. Inter-species differences in enzymatic kinetic parameters were determined by using the Kruskal-Wallis test, while nonparametric multiple comparisons were made by using the Dwass-Steel-Critchlow-Fligner method. All analyses were performed using the Statistical Analysis System software.

**Data Availability**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
References
1. van der Zuiden, A. S. M. et al. Aspergillus flavus and turkey X disease: Isolation of crystalline form of a toxin responsible for turkey X disease. Nat. 195, 1060–1062, https://doi.org/10.1038/1951060a0 (1962).
2. Blount, W. P. Turkey "X" disease. Turkeys 9, 52–77 (1961).
3. Nesbitt, B. F., O’Kelly, J., Sargeant, K. & Sheridan, A. Aspergillus flavus and turkey X disease: Toxic metabolites of aspergillus flavus. Nat. 195, 1062–1063, https://doi.org/10.1038/1951062a0 (1962).
4. Smith, J. W. & Hamilton, P. B. Aflatoxicosis in the broiler chicken. Poult. Sci. 49, 207–215, https://doi.org/10.3382/ps.049207 (1970).
5. Diaz, G. J. Toxicología de las micotoxinas y sus efectos en avicultura comercial. In press (Editorial Acribia, 2019).
6. Diaz, G. J., Calabrese, E. & Blaini, R. Aflatoxicosis in chickens (gallus gallus): An example of hormesis? Poult. Sci. 87, 727–732, https://doi.org/10.3382/ps.2007-00403 (2008).
7. Armbrrecht, B. H. & Fitzhugh, O. G. Mycotoxins II: The biological assay of aflatoxin in Peking white ducklings. Toxicol. Appl. Pharmacol. 6, 421–426, https://doi.org/10.1016/0041-008X(62)90007-7 (1962).
8. Diaz, G. J. & Murcia, H. W. Biotransformation of aflatoxin B1 and its relationship with the differential toxicological response to aflatoxin in commercial poultry species. In Aflatoxins: Biochemistry and Molecular Biology (Ed. Guevara-Gonzalez, R. G.) 3–20, https://www.intechopen.com/books/aflatoxins-biochemistry-and-molecular-biology/biotransformation-of-aflatoxin-b1-and-its-relationship-with-the-differential-toxicological-response (Intech Publishing, 2011).
9. Swenson, D. H., Miller, J. A. & Miller, E. C. 2,3-dihydro-2,3-dihydroxy-aflatoxin B1: An acid hydrolysis product of an RNA-aflatoxin B1 adduct formed by hamster and rat liver microsomes in vitro. Biochem. Biophys. Res. Commun. 53, 1260–1267, https://doi.org/10.1016/S0006-291X(73)90601-3 (1973).
10. Guengerich, F. P. et al. Activation and detoxication of aflatoxin B1, Mutat. Res. 402, 121–128, https://doi.org/10.1016/S0027-5107(97)00289-3 (1998).
11. Aguilar, F., Hussain, S. P. & Cerutti, P. Aflatoxin B1, induces de transversion of G → T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. Proc. Natl. Acad. Sci. USA 90, 8586–8590, https://doi.org/10.1073/pnas.90.18.8586 (1993).
12. Johnson, W. W., Harris, T. M. & Guenguerich, F. P. Kinetics and mechanism of hydrolysis of aflatoxin B1 exo-8,9-epoxide and rearrangement of the dihydrodiol. J. Am. Chem. Soc. 118, 8213–8220, https://doi.org/10.1021/ja960525k (1996).
13. Josephy, P. D., Guengerich, F. P. & Miners, J. O. "Phase I" and "Phase II" drug metabolism: Terminology that we should phase out. Br. Poult. Sci. 47, 734–741, https://doi.org/10.1080/00071660601084390 (2006).
14. Swenson, D. H., Miller, J. A. & Miller, E. C. 2,3-dihydro-2,3-dihydroxy-aflatoxin B1: The formation of 2,3-dihydroxy-2,3-dihydro-aflatoxin B1 by duck liver microsomes isolated from certain avian and mammalian species and the possible role of this metabolite in the acute toxicity of aflatoxin B1, Toxicol. Appl. Pharmacol. 58, 431–437, https://doi.org/10.1016/0041-008X(81)90095-8 (1981).
15. Sabbioni, G., Skipper, P. L., Büchi, G. & Tannenbaum, S. R. Isolation and characterization of the major serum albumin adduct formed by hamster and rat liver microsomes in vitro. Biochem. Biophys. Res. Commun. 53, 1260–1267, https://doi.org/10.1016/S0006-291X(73)90601-3 (1973).
16. Redinbaugh, M. G. & Turley, R. B. Adaptation of the bicinchoninic acid protein assay for the use with microtiter plates and sucrose gradient fractions. Avian Pathol. 39, 279–285, https://doi.org/10.1080/03079457.2010.495109 (2010c).
17. Hayes, J. D., Judah, D. J. & Neal, G. E. Resistance to aflatoxin B1 is associated with the expression of a novel aldo-keto reductase formed by aflatoxin B1 in vivo rats. Cancer Res. 8, 819–824, https://doi.org/10.1093/carcin/8.6.819 (1987).
18. Swenson, D. H., Miller, J. A. & Miller, E. C. 2,3-dihydro-2,3-dihydroxy-aflatoxin B1 by duck liver microsomes in vitro. Biochem. Biophys. Res. Commun. 53, 1260–1267, https://doi.org/10.1016/S0006-291X(73)90601-3 (1973).
19. Hayes's Principles and Methods of Biochemistry (5th edn., John Wiley & Sons, 2001).
20. Northrop, D. B. On the meaning of K/V and K/V in enzyme kinetics. J. Chem. Educ. 75, 1153–1157, https://doi.org/10.1021/ed075p1153 (1998).
21. Guengerich, F. P. Analysis and characterization of enzymes and nucleic acids relevant to toxicology. In Haye's Principles and Methods of Toxicology Sixth Edition (eds Hayes, A. W. & Kruger, C. L.) 1939, ISBN-13: 978-1842145364 (CRC Press, 2014).
22. Redinbaugh, M. G. & Turley, R. B. Adaptation of the bischconinic acid protein assay for use with microtiter plates and sucrose gradient fractions. Analytical Biochemistry 153, 267–271, https://doi.org/10.1016/0003-2697(86)90091-6 (1986).
23. SAS Institute Inc. Base SAS 9.4 procedures guide: Statistical procedures, second edition. www.support.sas.com/bookstore (2015).

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G.J.D. designed the experiments, secured the necessary funding to perform them, analyzed the data, wrote some sections of the article and revised it thoroughly. H.W.M. designed and conducted the experiments, analyzed the data, wrote some sections of the article and revised all references.

Additional Information
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