# THE ONTOGENY OF THE IMMUNE RESPONSE TO SHEEP ERYTHROCYTES AND RESISTANCE TO AFLATOXINS IN CHICKENS

by

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# INTRODUCTION

Organisms respond to toxins and infectious agents in several ways. The primary organ for detoxification of drugs and mycotoxins in chickens is the liver with variation in the degree of resistance of various target organs. The basis of immune response to natural antigens such as bacteria, viruses and fungi consists of a process of multiplication and differentiation of immunocytes induced by antigen stimulation. This process is subject to genetic control at the level of antigen recognition and antibody synthesis.

Historically, the main genetic approach used by man to reduce disease susceptibility in the fowl has been selection for resistance to specific pathogens. An approach for examining disease resistance involves genetic responses to a specific and/or general antigen. In the mouse, Biozzi and colleagues (1969, 1970) have developed, through genetic selection, high and low antibody responder lines to sheep red blood cells (SRBC). The differences observed between the genetic lines were greater for IgM and IgG classes than for the IgA class. They observed no differences between the lines for skin-graft rejection, although cytotoxic activity of serum antibodies against histocompatability antigens was greater in the high than low line (Biozzi _et_ _al_., 1971). In chickens, Siegel and Gross (1980) established through

genetic selection high and low antibody lines to SRBC antigen. Correlated responses included resistance to specific diseases and parasites (Gross *et al*., 1980) and response to stressors (Gross and Siegel, 1981) as well as various production and behavioral traits (Siegel *et al*., 1982; Cherry *et al*., 1984; Dunnington *et al*., 1984).

Early immune competence of chicks is complicated by maternal antibodies which provide protection against *Escherichia coli*, Marek's disease, and encephalomyelitis. For other diseases, early immune competence relates to cell-mediated or local immunity and macrophage activity. Although young chicks may fail to respond or respond weakly to immunizations to SRBC antigen (Skeeles *et al*., 1980), data on genetic variation for the ontogeny of antibody production are generally lacking. There is, however, genetic variation for the kinetics of responses to SRBC to antigen in mice (Biozzi *et al*., 1979) and chickens (Van der Zijpp, 1982), and lines known to differ in antibody production (Siegel and Gross, 1980) provide an opportunity for *in vivo* studies of the ontogeny and kinetics of responses to SRBC antigen.

Aflatoxicosis in poultry is an acute disease characterized by growth inhibition, inefficient feed conversion and increased mortality and condemnations. Effects of high doses of aflatoxin at any stage of purity

consists of acute hepatitis, hemorrhagic disease and death. Lowered productivity and carcinogenesis are associated with continued ingestion of low doses of aflatoxins. In addition, aflatoxin and other mycotoxins may contribute further to economic losses because they impair immunity. Although the mechanisms of reduced resistance and impaired immunogenesis is not fully understood, aflatoxins increase the susceptibility of vaccinated chickens to challenges with particular agents. The theoretical and practical implications of these findings make it desirable to investigate the effect of dietary aflatoxin on production of specific circulating antibodies without the complications of pathogenesis. This view is cogent because of differences among populations in responses to aflatoxins.

This dissertation consists of three phases involving chickens from lines selected for high and low antibody production to sheep erythrocytes. They involve comparisons among the parent populations and reciprocal $F_1$ generation crosses for (1) the ontogeny of production of antibody to sheep erythrocyte antigen, (2) the kinetics of response to sheep erythrocyte antigen, and (3) the response to feeding varying levels of aflatoxin $B_1$.

# LITERATURE REVIEW

## Humoral Immune Responsiveness and Disease Resistance

Research on the inheritance of disease resistance in livestock and poultry have focused on susceptibility to specific infectious agents (Hutt, 1958; Gavora and Spencer, 1978). Recently, however, there have been attempts to examine resistance by studying "disease packages", such as genetic variation for response to specific complex antigens (see reviews by Gavora, 1982; Gavora and Spencer, 1983; van der Zijpp, 1983a). Although differences among breeds and strains of chickens for response to sheep erythrocytes are documented (van der Zijpp, 1978; Lamont and Smyth, 1984) and genetic variation for responses to sheep erythrocytes has been shown in several species (Biozzi *et al*., 1968; 1979; 1981; Siegel and Gross, 1980; Ibanez *et al*., 1980; Mouton *et al*., 1981; van der Zijpp, 1983a), external factors such as sex (van der Zijpp and Leenstra, 1980; Siegel and Gross, 1980), maternal antibodies (van der Zijpp, 1978), environmental temperatures (Subba Rao and Glick, 1977), nutrition (Cheville, 1979), social interactions (Siegel and Latimer, 1975) and route of administration (Gross, 1979) also influence the antibody response to SRBC.

van der Zijpp (1978) showed that a low dose (.0005 ml) of SRBC failed to evoke a measurable antibody response, and a very high dose (6 ml) produced the maximal response. The

antibody titers reflected the antigen dosage at all stages of the immune reaction and genetic differences were detected at all dosages tested. In a similar study, Gross (1979) observed that differences among stocks were greater at lower than higher doses of SRBC. Biozzi et al. (1972) observed that to reach an optimal response, smaller amounts of antigen were required by low than high responder mice. These mice also showed a decreased susceptibility to antigen stimulation as shown by high threshold dose of sheep erythrocyte antigen. A similar pattern was observed for immune responsiveness to pneumococcal polysaccharide in high and low responder mice (Howard et al., 1972). Whereas high responders required a smaller dose of pneumococcal polysaccharide to elicit an immune response, they needed a larger dose to be rendered tolerant than low responders. Biozzi et al. (1972) concluded from these results that both immune responsiveness and tolerance were subject to the same type of genetic control.

van der Zijpp (1983b) reported that the primary response to SRBC in chickens consisted almost completly of 2-ME sensitive antibodies (IgM) with the peak observed on day 7 post-injection. The secondary response had a maximum of total antibody on day 5 post-injection which coincided with the peak of 2-ME resistant (IgG) antibodies.

Seto (1981) postulated that thymus-derived cells

limited the onset of immunocompetence in neonatal chicks. This view was consistent with those of Skeeles et al. (1980) who noted that immune responsiveness developed with age in chickens and complement activity, while low in the embryo, increased with age. Gross et al. (1980) compared chickens from high and low SRBC antibody (HA and LA) lines and observed that the HA birds produced more antibody to Newcastle Disease virus than LA birds. In mice, differences in antibody production between lines were due to a faster rate of multiplication and differentiation of B-lymphocytes as well as a stronger catabolic activity of macrophages in the low than in the high line (Biozzi et al., 1979). An inverse relationship, therefore, exists between antibody synthesis and bacterial capacity. They pointed out that quantitative antibody production was subject to polygenic control. Differences also exist between inbred lines of chickens in humoral response to various antigens with the capability for formation of antibodies inherited as a dominant trait (Balcarova et al., 1973). Recently, Lamont and Smyth (1984) immunized three lines of chickens at different ages with SRBC and Brucella abortus, and observed that differences between lines at early ages did not necessarily persist at older ages. McCorkle and Glick (1980) found that antibody production to SRBC but not Brucella abortus declined with age in chickens.

SRBC and most viruses belong to the group of T-dependent antigens (Burns, 1975). Thus, it is not surprising that vaccination against viral diseases can significantly increase antibody production to SRBC chickens (van der Zijpp, 1982), or that inactivated infectious bursal disease vaccine injected subcutaneously can stimulate agglutinin production against horse red blood cells and SRBC (Sharma and Tuomi, 1973; Burns, 1975).

Leduc *et al*. (1955) stated that there was a marked difference between the initial and secondary antibody responses; the former were characterized by the development of very few antibody-containing cells, while in the latter there were hundreds in a similar area. They postulated that the morphology of the cells involved in both responses were identical. Ispen (1959) reported that the secondary antigen dose functioned essentially as a stimulus to the mature cells for specific antibody production. The low dependency of response on secondary doses indicated that only a minority of the functions of the secondary dose was to stimulate new cells into maturation.

## Aflatoxin and the Avian Humoral Immune System

Research directed toward the practical objective of controlling aflatoxin contamination of animal diets and meat has provided an opportunity for scientists from diverse disciplines to integrate views on susceptibility of poultry

to such toxins. Aflatoxins are toxic metabolites produced by the fungus *Aspergillus flavus*. Aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$ were identified with aflatoxin $B_1$ being the most toxic strain. Genetic variation also exists in the response of individuals to aflatoxins. This variation is documented in several avian species (e.g., Brown and Abrams, 1965; Carnaghan *et al*., 1967; Gumbmann *et al*., 1970; Smith and Hamilton, 1970; Marks and Wyatt, 1979; Bryden *et al*., 1980).

The ability of aflatoxin to depress protein formation has attracted the interest of several researchers (e.g., Brown and Abrams, 1965; Carnaghan *et al*., 1966; Cysewski *et al*., 1968; Sisk *et al*., 1968) to its effects on immunogenesis. The problem is of both theoretical and practical importance because of the (1) natural occurrence of the toxin in feeds, (2) susceptibility of chickens, turkeys, ducks, pigs and calves to the toxin, and (3) frequent use of immunization techniques to protect animals from infectious agents.

Pier and Heddleston (1970) reported that aflatoxin (250-500 ppb) consumed during or after the period of immunization against *Pasteurella multocida* interfered with the development or manifestation of acquired resistance in turkey poults and chicks. Aflatoxin consumed immediately before immunization did not impair the development of such immune responses. Yet, in mice there was a marked

depression in antibody formation when aflatoxin was given before or during the vaccination period and a moderate depression when aflatoxin was given after vaccination (Galikeev et al., 1968). It was postulated by Pier and Heddleston (1970) that in order to affect acquired resistance, aflatoxin had to be present either when the body was exposed to the antigen or while it was producing an immune response. They observed that aflatoxin was capable of exerting a reversible effect on the ability of turkey poults to develop or exhibit immunity, and proposed that if aflatoxin was being consumed at the time of exposure to the antigen or later, when the immunity was challenged, the resistance was diminished. Edds et al. (1973), Hamilton and Harris (1971) and Smith and Hamilton (1970) observed that low concentrations of aflatoxin in poultry feed reduced resistance to infection with Pasteurella multocida, Salmonella spp, Marek's disease virus, Eimeria tenella, and Candida albicans. Pier et al. (1971) and Richard et al. (1973) did not observe reduced resistance to Newcastle disease virus or Aspergillus fumigatus infections but aflatoxin reduced interferon levels.

Thaxton et al. (1974) investigated the effect of aflatoxin on the immunity of chickens in a system uncomplicated by pathogenesis. They found that dietary aflatoxin caused a dose-related suppression of

hemagglutinins against SRBC antigen and a regression of the thymus and Bursa of Fabricius. Dietary aflatoxin causes a dose related impairment of the reticuloendothelial system of chickens (Michael et al., 1973). This system is responsible for the removal of foreign particulate matter from the circulation and for the protection of the tissue from invasion by pathogenic organisms.

The effects of aflatoxin in antibody formation may be caused by defective interaction with the antigen or by a depression of a humoral substance other than antibody which had a relatively short half-life in the body (Pier, 1973). It is likely that affected humoral substances are of relatively short half-life ($<$ 3 weeks) in the circulating blood and located in one or more of the serum fractions which are notably affected by aflatoxin, i.e., $\alpha$- or $\beta$-globulins or albumin. The formation of hemagglutinating antibody in response to Newcastle Disease Lasota vaccine was impaired when chicks consumed 460 or 640 mg aflatoxin per kg of feed for a period of 20 days (Chenchev et al., 1978). The $\lambda$-globulin was higher in test animals while $\alpha$-1, $\alpha$-2, $\beta$-1 and $\lambda$-2 globulins in the blood were higher in untreated controls. Dzuik et al. (1978) incorporated 5000 ppb of aflatoxin to the diet fed poults from 49 to 70 days of age. When the poults were challenged with an avirulent *Pasteurella multocida*, it was noted that aflatoxin did not

affect the development of immunity. Also, Campbell *et al.* (1983) did not observe alterations of antibody production to either SRBC or *Brucella abortus* antigen in chicks fed diets containing aflatoxin-ochratoxin combinations.

Chicks fed diets containing 2500 ppb of aflatoxin from 14 to 28 days of age or from hatching to 28 days of age were deficient in cell-mediated immunity (Giambrone *et al.*, 1978). At the same time, a decrease in concentrations of serum IgG and IgA but not IgM occurred. Edds *et al.* (1973) reported that aflatoxicosis increased susceptibility of turkeys to *candidiasis*, *Pasteurella* and *Salmonella* infections, coccidiosis and Marek's disease. They also observed that prior exposure to aflatoxin $B_1$ increased susceptibility and mortality from cecal coccidiosis but did not interfere with the protection afforded by a coccidiostat. Likewise, chicks vaccinated against Marek's disease and fed aflatoxin $B_1$ seemed more resistant against challenge exposure with Marek's virus than did non-vaccinated control chicks. Boonchuvit and Hamilton (1975) observed mortality in chickens fed aflatoxin and infected with *Salmonella washington*, *S. thompson*, *S. derby* and *S. typhimurium var. copenhagen*, and they suggested that aflatoxin impaired components of the immune system other than those forming humoral antibodies.

Hamilton and Harris (1971) observed that aflatoxin made

chickens more sensitive to stressors. The reaction of chickens to heat was influenced by the intensity of the stressor and by picking an appropriate temperature, mean survival time could be altered to show no effect or reduced disease resistance. For example, dietary aflatoxin had no effect on the mean survival time of chickens exposed to a temperature of 40°C and 45% relative humidity, while it significantly decreased survival time at 37°C and 45% humidity (Wyatt et al., 1977) and increased resistance at 43°C (Hamilton and Harris, 1971).

Aflatoxin and Protein Synthesis

Cysewski et al. (1968) analyzed serum proteins obtained from pigs before exposure to aflatoxin and 24 hours after intoxication. They observed a decrease in total serum proteins and all components (α-, β-, λ-globulins and albumin with the greatest percentage decrease in the β-globulins). Sisk et al. (1968) proposed that the inhibition of mitosis and protein synthesis in pigs suffering from acute aflatoxicosis resulted in enlarged hyperchromic centrilobular hepatic cells with karyomegaly and deranged morphological features. Brown and Abrams (1965) hypothesized that the suppression of protein synthesis, particularly albumin in chickens and ducklings suffering from aflatoxicosis, was due to a lowered rate of ATP synthesis, a consequence of mitochondrial injury. They

suggested that the decrease of serum protein concentrations associated with aflatoxicosis would be expected to adversely influence pathogenesis and morbidity. An increase in the activity of certain plasma enzymes, notably those of lactic dehydrogenase, aldolase and glutamic-oxalacetic or glutamic-pyruvic transaminases was also observed. The increase in the activity of plasma enzymes was correlated with hepatic lesions.

Biochemical and pathological aspects of groundnut poisoning in chickens were examined by Carnaghan et al. (1966). They found in chicks which consumed rations that contained aflatoxin, only minor differences in DNA values and consistently smaller RNA content in the livers. The aflatoxin $B_1$ content of the commercial ration fed to the chicks was approximately 10,000 ppb. It has been speculated (Osborne and Hamilton, 1970), that during aflatoxicosis, there is a marked decrease in the synthesis of pancreatic hydrolytic enzymes involved in the digestion of starch, protein, lipids, and nucleic acids with those responsible for starch and lipid digestion being the most severely affected. Clifford and Rees (1966) observed that aflatoxin binds with DNA shortly after consumption and effected an inhibition on the formation of DNA-dependent RNA.

Gregory et al. (1983) reported that aflatoxin residue levels of 10 to 1190 ppb of liver muscle tissue cleared when

the feeding of dietary aflatoxin was discontinued. Wong and Hsieh (1980) noted that species susceptibility to the acute and carcinogenic effects of aflatoxin $B_1$ is closely related to the rate and extent of tissue penetration, distribution, metabolism, and elimination. Recently, Dalvi and McGowan (1984) reported that either activated charcoal (.1% in the feed) or reduced glutathione (.05%) or phenobarbital (.05%) given intermittently in drinking water improved feed consumption with < 10% and < 28% reversal, respectively, and prevented the inhibitory effect of aflatoxin $B_1$ on microsomal cytochrome P-450 in birds receiving 10,000 ppb of aflatoxin $B_1$.

With a spiraling increase in the poultry population, the economic implications of aflatoxin infections have become ever more important. Concentrated efforts should be made toward research directed to finding out the subclinical ramifications when avian aflatoxicosis interacts with common poultry diseases, e.g., coccidiosis, *Escherichia coli*, adenovirus or mycoplasma infections in the chicken.

# EXPERIMENT I

## RESPONSES TO AFLATOXIN $B_1$ BY LINES OF CHICKENS SELECTED FOR ANTIBODY PRODUCTION TO SHEEP ERYTHROCYTES

# Introduction

Aflatoxins are toxic metabolites produced by the fungus *Aspergillus flavus* (Newberne *et al*., 1964). Aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ have been identified with $B_1$ being the most toxic (Wogan, 1965). Species susceptibility to acute and carcinogenic effects of aflatoxin $B_1$ is closely related to its rate of metabolism and elimination (Wong and Hsieh, 1980). In poultry, aflatoxicosis is characterized by growth retardation, inefficient feed conversion, and increased condemnations (Smith and Hamilton, 1970). Consumption of high levels of aflatoxin can cause acute hepatitis, hemorrhagic disease, and death.

Genetic differences exist for response of broilers (Brown and Abrams, 1965; Carnaghan *et al*., 1967; Gumbmann *et al*., 1970; Smith and Hamilton, 1970; Bryden *et al*., 1980) and quail (Gumbmann *et al*., 1970; Marks and Wyatt, 1979) to aflatoxin. Pier and Heddleston (1970) postulated that in order to affect acquired resistance to *Pasteurella multocida* in turkey poults and New Hampshire chicks, aflatoxin had to be present when the body was exposed to the antigen or while it was producing immune response. In chickens, Thaxton *et al*. (1974) found that dietary aflatoxin caused a dose-related suppression of hemagglutinins against sheep erythrocytes and a dose-related suppression of the thymus and bursa of Fabricius.

Lacking is information concerning the effect of aflatoxin on chickens known to differ in their response to sheep erythrocytes. The experiment reported here was designed to measure the effects of feeding different levels of aflatoxin $B_1$ fed to such populations.

## Materials and Methods

Lines of White Leghorn chickens selectively bred for either high (H) or low (L) antibody response 5 days after intravenous inoculation of 0.1 ml of 0.25% suspension of sheep red blood cells, SRBC, (Siegel and Gross, 1980) were used in this study. Progeny from the parental lines and reciprocal crosses between them were obtained by random matings among $S_9$ generation chickens. Symbols denoting the four mating combinations are HH, HL, LH, and LL with the line of the sire denoted first and the dam second.

Parental populations were age contemporaries and all of the eggs used to produce chicks were incubated on the same day. On the 22nd day of incubation, chicks were removed from the hatcher, wing-banded, vent-sexed, vaccinated against Marek's disease, and placed in electrically heated, wire-floored starter batteries. Within each sex, 100 chicks from each of the 4 populations were randomly allotted to 5 dietary aflatoxin treatments.

Five levels of aflatoxin $B_1$ were incorporated into a soy-corn diet containing 20% protein and 2684 kcal/kg. The dietary formulation (Siegel, 1962) was the same as that under which the selected lines were developed. By analysis, the aflatoxin $B_1$ in the 5 dietary treatments was 0, 966, 1830, 2890 and 5697 ppb. A very low level of aflatoxin $G_1$ (91 ppb) was detected in the basal diet. All chicks were

fed the basal diet until 2 days of age when they were placed on their assigned diets.

Feed and water were provided *ad libitum* and lighting was continuous. There was an individual feeder and waterer for each pen to minimize contacts between chicks on different dietary treatments.

Traits measured

Individual body weights were obtained at 2, 14, 28 and 42 days of age. Feed consumption was measured on a pen basis and feed conversion (feed/weight gain) was calculated from 2 through 42 days of age.

Blood samples were collected at 18 days of age from the brachial vein of a random sample of 10 cockerels from each population-aflatoxin level subclass. The number of heterophils and lymphocytes from each blood sample was determined by examining a blood smear prepared by centrifugation with a Larc Spinner. Smears were stained with May-Grunewald-Giemsa stain (Gross and Siegel, 1983) and the heterophil/lymphocyte ratio for each bird was based on counts of a minimum of 50 cells (heterophils and lymphocytes) on each slide.

Ten chicks randomly selected at 28 days of age from each pen were immunized by injecting intravenously 0.1 ml of 0.25% SRBC antigen. Blood samples were obtained from the brachial vein 5 and 13 days post-immunization, plasma

titrated individually by the microtiter procedure of Wegmann and Smithies (1966) and antibody was expressed as $\log_2$ of the reciprocal of the greatest dilution in which agglutination occurred.

At 41 and 42 days of age (half of the pens each day), core and foot pad temperatures were obtained from 9 randomly selected birds in each pen using the protocol described by Dunnington and Siegel (1984). At 42 days of age, 9 birds from each pen were randomly selected, sacrificed by cervical dislocation, and their liver, bursa of Fabricius, spleen and thymus removed and weighed to the nearest 0.1 g. Also, liver samples from 3 control and from 4 birds of the 5697 ppb aflatoxin level treatment were assayed for microsomal and soluble enzyme activity. Specific assays were for cytochrome P-450/448 quantity, O-demethylase, glucuronyl transferase and GSH-S-transferase using procedures described by Ehrich and Larsen (1983).

Statistical analyses

Prior to analysis, body weights were transformed to natural logarithms and relative weights of the organs (liver, bursa of Fabricius, spleen and thymus) and heterophil/lymphocyte ratios were transformed to arc sines. When significant differences were observed, means were separated by Duncan's multiple range test.

The following statistical model was used:

$$Y_{ijkl} = \mu + L_i + T_j + S_k + (LT)_{ij} + (LS)_{ik} + (TS)_{jk} + (LTS)_{ijk} + e_{ijkl}$$

where $i = 1, 2 \ldots 4$ populations, and $j = 1, 2 \ldots 5$ aflatoxin levels, $k = 1, 2$ sexes of chicks, $l = 1 \ldots n$ individuals per subclass.

Since heterophil, lymphocyte, and heterophil/lymphocyte ratios were measured in males only, the variable sex was deleted from the statistical model. Feed consumption was obtained on a pen basis, and the measurement unit was the mean per bird per pen with values converted to natural logarithms prior to analysis. For temperatures, the variable day was included in the analysis. Comparisons between the 0 and 5697 ppb level of aflatoxin for hepatic toxin metabolism were by t-test.

## Results and Discussion

### Body weight and feed conversion

Since no interactions were observed among main variables for body weight at any age, results are presented for each main effect at each age. Significant differences were found among stocks for body weights at 14, 28, and 42 days of age with LL birds being significantly heavier than HH birds (Figure 1). This result was consistent with previous observations (Siegel and Gross, 1980). HL birds were consistently heaviest with LH birds intermediate to the parental lines. There was no difference among populations for feed consumption (Table 1).

Dietary aflatoxin significantly reduced body weight by 14 days of age (Figure 2) with the extent of depression dose related and cumulative over ages. Birds fed the 966 ppb level were 1 to 4% lower in weight than controls with the difference being significant at 14 days but not at older ages. At 1830 ppb, there was a significant depression of 5, 10 and 12% at 14, 28 and 42 days, respectively. The 2890 ppb and 5697 ppb of aflatoxin significantly depressed weight by 10 and 19% at 14 days, by 24 and 41% at 28 days, and by 32 and 49% at 42 days of age, respectively. The significant reduction in body weight at the 1830 but not the 966 ppb level of aflatoxin at 28 and 42 days of age, infers that the threshold for growth retardation for the chicks used in this

experiment was between these levels.

That aflatoxins depressed body weight was not unexpected (e.g., Briggs *et al.*, 1974 Sharlin *et al.*, 1980). Mechanisms proposed to explain how aflatoxins depress body weight include inhibition of RNA (Clifford and Rees, 1966; Friedman and Wogan, 1966) and DNA synthesis (Rogers and Newberne, 1967), as well as decreased RNA polymerase activity (Gelboin *et al.*, 1966). Direct consequences of partial inhibition of RNA or DNA synthesis is limited protein synthesis which would depress body weight. Also, Sharlin *et al*. (1980) postulated that depressed appetite and/or unpalatability reduced the consumption of feed containing aflatoxin. In this experiment, the decreased consumption of diets containing aflatoxin followed the same pattern as did body weight (Table 1) with the percentage reduction at 42 days of age being 7, 13, 32 and 43 for the 966, 1830, 2890 and 5697 ppb levels, respectively. Dalvi and McGowan (1984) reported that 2500, 5000 and 10,000 ppb of dietary aflatoxin $B_1$ reduced feed consumption in broilers by 39, 38 and 57%, respectively. Poorer feed conversion due to dietary aflatoxin (Table 1) was not observed in this study, however, until the 5697 dosage which is understandable because the heavier control and lower aflatoxin level birds would require more feed for maintenance. At the lower levels of aflatoxin there may

have been buffering since animals which have had their energy intake restricted make more efficient use of the energy ingested and that the improvements in overall energy utilization arise from both greater metabolic efficiency and lower deposition of unwanted tissue (Pearson and Shannon, 1979).

Temperatures

Although there was no influence of body weight on core temperature there was a highly significant positive correlation of .75 between body weight and surface temperature. Overall results were the same whether or not temperatures were corrected for body weight, and only unadjusted data will be presented. There were no differences between populations, sexes or aflatoxin levels for core temperatures (Table 2). HH birds had significantly lower surface temperatures than LH birds while neither was significantly different from the HL and LL populations. Males had significantly higher surface temperatures than females.

Aflatoxin consistently lowered surface temperatures at levels in excess of 966 ppb with the reduction being greater as dosage increased (Table 2). The consistently lower absolute and metabolic body weights associated with higher aflatoxin levels and the subsequent reduction in surface temperature may be attributed to lower feed intake. Even

though core temperatures were maintained with the reduced feed intake, it appears that chickens with aflatoxicosis reduced heat loss by lowering their surface temperatures. Consequently, those fed diets containing 1830 ppb of aflatoxin and greater had a lower heat dissipation. In addition, heat production may have been reduced since aflatoxicosis induces hypothermia through decreased serum glucose and percentage body fat on a dry weight basis (Wyatt et al., 1975). All of these factors may contribute to explaining the observation by Hamilton and Harris (1971) that aflatoxin makes chickens more susceptible to cold and less susceptible to heat.

## Heterophils and Lymphocytes

Neither population nor the interaction of population by aflatoxin dosage was significant for number of heterophils and lymphocytes, or the heterophil/lymphocyte ratio. There was, however, a significant aflatoxin effect in that birds fed 5697 ppb had the highest frequency of heterophils concomitant with fewer lymphocytes than those on the other dietary treatments (Table 3). This result was consistent with that of Tung et al. (1975) who noted heterophils increased and lymphocytes decreased in chickens fed 5000 and higher ppb of aflatoxin. They attributed the modification to aflatoxin-caused hemolytic anemia.

Selye (1953) reported that the characteristic

leucocytic response in birds experiencing stress is lymphopenia with heterophilia. Newcomer (1957) observed that heterophilia is a part of chicken's circulating defense mechanism against stress and Siegel (1972) associated changes in white blood counts in chickens with stressors. The increased circulating heterophils, at the 5697 ppb level is consistent with observations of Hamilton and Harris (1971) and Thaxton et al. (1972) in that aflatoxin causes a physiological stress in chickens.

The phagocytic activity of the large numbers of heterophils observed during aflatoxicosis has been investigated. Chang and Hamilton (1976) reported that the percentage of heterophils phagocytizing during aflatoxicosis is greatly reduced by 625 ppb or greater of aflatoxin. Phagocytosis by macrophages and heterophils is, however, dependent on complement whose concentration is reduced during aflatoxicosis (Richard and Thurston, 1975; Chang and Hamilton, 1976). Complement is a complex series of enzymatic proteins occurring in normal serum that interact to combine with antigen-antibody complex, producing lysis when the antigen is an intact cell. Complement comprises 11 discrete proteins or nine functioning components symbolized as C1 through C9 with C1 being divided into subcomponents, C1q, C1r and C1s. Components C3 and C5 are involved in the promotion of leucocytic chemotaxis. Chang and Hamilton

(1976) reported that chemotaxis by heterophils towards complement (C5a) was significantly inhibited by growth inhibitory levels of 2500 ppb or greater of aflatoxin. Since the components of the complements combine in various sequences to participate in biological activities like antibody-mediated immune lysis, phagocytosis and opsonization, a decrease in complement activity during aflatoxicosis could explain the reduced phagocytic activity observed in this study.

Organs

Liver. Males had significantly larger livers than females with this sexual dimorphism reversed when liver weight was expressed on a body weight basis (Table 4). HH birds had significantly smaller livers than those from the other populations which did not differ (Table 5). When liver weight was adjusted for body weight no consistent pattern was evident among populations.

Aflatoxin had no effect on absolute liver weight until 2890 ppb was fed. At this level, liver size was significantly reduced (Table 6), with a further reduction noted at 5697 ppb. When expressed relative to body weight, the reverse was noted in that the zero and 966 ppb groups had the smallest livers, with weight increasing with each dosage. These results are consistent with previous studies with chicks (Smith and Hamilton, 1970) and roosters (Wyatt

et al., 1973).

Bursa. Male chicks had significantly larger bursae than females (Table 4); however, this sexual dimorphism disappeared when adjustment was made for body weight. The significant population by aflatoxin level interactions (Figure 3) may be attributed to the differential responses of the populations at the lowest and highest levels of aflatoxin. On both an absolute and relative weight basis, bursae of birds from the low antibody line were consistently smaller than those from the other populations (Table 5). Aflatoxin levels greater than 966 ppb resulted in reductions in bursa weight (Table 6; Figure 3), with the changes more dramatic on an absolute than relative weight basis. These results are consistent with previous reports that aflatoxins reduce bursa size (e.g., Thaxton et al., 1974).

Thymus. Males had significantly larger thymi than females (Table 4); however, the difference disappeared when expressed on a relative body weight basis. Thymi of HH birds were significantly smaller than those from the other populations (Table 5). Thymus weights of LH and LL birds did not differ significantly from each other, but they were significantly smaller than that of HL birds. Relative relationships among populations did not change when thymus weights were adjusted for body weights.

Aflatoxin did not reduce thymus weight significantly

until the dietary level fed reached 1830 ppb, with higher levels (2890 and 5697 ppb) causing further reductions in a dose related manner (Table 6). This negative effect of aflatoxin on thymus size was consistent with previous reports (e.g., Thaxton et al., 1974). This pattern existed both on an absolute and relative weight basis and was consistent across sexes and populations as evidenced by a lack of significant interactions among main variables.

Spleen. No significant sexual dimorphism was found for spleen weight (Table 4). Although the population by aflatoxin level interaction for relative spleen weight was significant, there was no consistent pattern among populations for relative weight of the spleen (Table 5), nor did there appear to be an aflatoxin effect (Table 6). On an absolute weight basis, again there was no population effect, however, spleen weight was lower at the two highest aflatoxin levels with the effect greater in the LL than in the other populations (Figure 3).

## Aflatoxin-metabolizing Enzymes

Microsomal enzyme involvement in aflatoxin biotransformation reactions and in glucuronic acid conjugation reactions which render the toxin readily excretable. Consistent with this assumption was the observation that aflatoxin $B_1$ significantly reduced all liver microsomal and soluble enzymes assayed (Table 7). The

quantity of cytochrome P-450/448 was 21% of the control, a value consistent with the 28% reduction noted at 5000 ppb by Dalvi and McGowan (1984). Capability of o-demethylase to demethylate p-nitroanisole was reduced to 30% of control values. Glucuronidation activity of the liver samples showed that detoxification of aflatoxin $P_1$ (a demethylated metabolite of aflatoxin $B_1$) by the glucuronic acid pathway in birds fed 5697 ppb of aflatoxin $B_1$ was 50% of that of livers from chicks on control diets. Furthermore, the activity of a liver cytosolular enzyme, glutathione S-epoxytransferase (GSH-S-transferase), which is involved in epoxide metabolism was reduced by the same dose of aflatoxin to 75% of the controls.

Although the mechanism of aflatoxin oxidation by liver microsomes is not clear, it is believed that the electron transport scheme involving cytochrome P-450/448 functions as an oxygen-activating enzyme for drug (Cooper _et_ _al_., 1965; Omura _et_ _al_., 1965). Sladek and Mannering (1966) provided evidence for two forms of cytochrome P-450 that bind to carbon monoxide, one with a maximal absorbance at 450 nm and the other with a maximal absorbance at 448 nm. Different drugs and toxins bind to different P-450's. Also, it appears that activation of aflatoxin $B_1$ is accomplished through formation of $AFB_1$ 2, 3-epoxide (AFB-epoxide); that is, epoxide is the reactive intermediate of aflatoxin $B_1$

metabolism.

Feeding of 5697 ppb of aflatoxin $B_1$ to chicks had a deleterious effect on their capability to metabolize the toxin. Depressed microsomal enzyme activity by the dietary aflatoxin would increase susceptibility to toxicants detoxified by these enzymes. Inhibition of the action of microsomal enzymes occurs when protein synthesis is inhibited, especially when a bird suffers from starvation, disease or stress. The birds that consumed 5697 ppb of aflatoxin $B_1$ exhibited these symptoms.

Antibody Titers

Significant differences were found among populations for production of antibody to SRBC 5 and 13 days post-immunization. Titers for the HH line were significantly greater than those for the LL line at both ages with means for the crosses being intermediate to those of the parental lines (Table 8), observations consistent for these populations at this antigen dosage (Siegel and Gross, 1980). The phenotypic correlations between antibody titers at days 5 and 13 were 0.38, 0.42, 0.40, and 0.49 from HH, HL, LH, and LL lines, respectively. Each was highly significant. The significant sex by population interaction (Figure 4) was because the male crosses had higher titers than females at both ages while in the parental lines the sexual dimorphism was reversed.

Direct evidence of aflatoxin-induced immunosuppression to SRBC antigen was not obtained in this experiment because there were no differences for this trait among dietary levels of aflatoxin for SRBC antibody (Table 8). This result is consistent with observations in broilers that aflatoxin impaired some component of the immune system other than that forming antibodies (Boonchuvit and Hamilton, 1975). The literature on effect of aflatoxin on antibody production is conflicting. For example, Campbell et al. (1983) did not observe alterations of antibody production to SRBC and Brucella abortus antigen in broiler chicks fed diets containing an aflatoxin-ochratoxin combination. Similarly, Giambrone et al. (1978) did not observe effects of aflatoxin on immunization to rabbit red blood cells in White Leghorns and Adinarayanaiah et al. (1973), and Pier and Heddleston (1970) found no effect of aflatoxin on antibody production to Salmonella pullorum and Pasteurella multocida, in White Leghorns and poults, respectively. Earlier, Pier et al. (1971) reported that aflatoxin did not impair acquired resistance to Newcastle disease. Edds and Bortell (1983) postulated that an aflatoxin-induced impairment of some components of the immune system other than humoral antibodies would explain vaccine failure against Pasteurella multocida during aflatoxicosis despite a lack of alteration of agglutinin levels. For example, a

marked depression in antibody formation to typhoid vaccine in mice was observed when aflatoxin was administered after the beginning of antibody synthesis, while no depression was observed when vaccination was followed by subcutaneous injection of aflatoxin (Galikeev et al., 1968). Immunodepression in chickens fed aflatoxin could be influenced by the age of the SRBC antigen (Thaxton, unpublished) in that old SRBC antigens enhanced immunodepression in chickens fed aflatoxin. Also, the dosage of the antigen influences antibody titer (Gross, 1979; Experiment II of this dissertation). Impairment of the reticuloendothelial system (RES) occurs in a dose related manner during aflatoxicosis (Michael et al., 1973). An impaired RES could reduce further an already decreased ability to remove foreign matter from the circulation and the processing of antigenic components. This reasoning is consistent with the observation (Thaxton et al., 1974) of a dose related decrease in the ability of chicks to form hemagglutinins during aflatoxicosis. The dosages used by Thaxton et al. (1974) bracketed those used in the experiment reported here.

Many of the classical symptoms of aflatoxicosis (e.g., depressed body weight gain, increase in relative weights of liver and spleen, regression in relative weights of bursa of Fabricius and thymus, heterophilia, and lymphopenia) were

observed in this experiment. The processing of antigen and formation of antibodies to SRBC were not, however, impaired in parental lines selected for high and low response to SRBC or reciprocal crosses between them.

# Summary

Four hundred chicks from lines selectively bred for either high (HH) or low (LL) antibody response and 400 crosses (HL and LH) from these parental lines were fed graded levels (0, 966, 1830, 2890 and 5697 ppb) of aflatoxin from 2 to 42 days of age.

Aflatoxin depressed body weights, feed consumption and feed conversion with the effect of the former two occurring at lower dietary levels than that noted for feed conversion. Although there were no differences among aflatoxin levels for body core temperatures, levels of 1830 ppb and higher caused progressive decreases in surface temperatures. Heterophil/lymphocyte ratios increased dramatically at the 5697 ppb dosage as a result of heterophilia and lymphopenia. The absolute and relative weights of bursae and thymi, the absolute weights of the livers and spleens were depressed while the relative weights of livers and spleens were increased by dietary aflatoxin. There was a large decrease in liver metabolism when 5697 ppb of aflatoxin $B_1$ was fed. No effect of aflatoxin was observed in SRBC antibody production from lines genetically selected to differ for this trait. This observation is consistent with that of Boonchuvit and Hamilton (1975) in broilers that aflatoxin impaired some components of the immune system other than that forming antibodies.

Table 1. Means ± standard errors of average feed consumption (g), and feed conversion by population, aflatoxin level, and sex at 42 days of age

| | Feed consumption | Feed conversion |
|---|---|---|
| Population | | |
| HH | 707 ± 44[a] | 2.37 ± .05[a] |
| HL | 763 ± 51[a] | 2.30 ± .06[a] |
| LH | 716 ± 62[a] | 2.32 ± .03[a] |
| LL | 750 ± 62[a] | 2.29 ± .04[a] |
| Aflatoxin $B_1$ (ppb) | | |
| 0 | 908 ± 25[a] | 2.32 ± .03[b] |
| 966 | 845 ± 25[a] | 2.25 ± .03[b] |
| 1830 | 787 ± 20[b] | 2.24 ± .04[b] |
| 2890 | 621 ± 27[c] | 2.34 ± .05[ab] |
| 5697 | 509 ± 32[d] | 2.45 ± .08[a] |
| Sex | | |
| Male | 783 ± 35[a] | 2.30 ± .04[a] |
| Female | 684 ± 37[b] | 2.34 ± .03[a] |

Within main effects, means within a column having the same superscript are not significantly different ($P \leq 0.05$).

Table 2. Means ± standard errors of surface and core temperatures by population, aflatoxin level, and sex at 41 to 42 days of age

| | Temperature | |
|---|---|---|
| | Surface | Core |
| Population | | |
| HH | $29.4 \pm 0.3^{b}$ | $40.3 \pm 0.1^{a}$ |
| HL | $29.9 \pm 0.4^{ab}$ | $40.2 \pm 0.1^{a}$ |
| LH | $30.3 \pm 0.3^{a}$ | $40.4 \pm 0.1^{a}$ |
| LL | $29.8 \pm 0.3^{ab}$ | $40.4 \pm 0.1^{a}$ |
| Aflatoxin $B_1$ (ppb) | | |
| 0 | $32.3 \pm 0.2^{a}$ | $40.4 \pm 0.1^{a}$ |
| 966 | $32.1 \pm 0.2^{a}$ | $40.3 \pm 0.1^{a}$ |
| 1830 | $30.8 \pm 0.3^{b}$ | $40.4 \pm 0.1^{a}$ |
| 2890 | $27.9 \pm 0.3^{c}$ | $40.2 \pm 0.1^{a}$ |
| 5697 | $26.2 \pm 0.2^{d}$ | $40.3 \pm 0.1^{a}$ |
| Sex | | |
| Male | $30.4 \pm 0.2^{a}$ | $40.4 \pm 0.1^{a}$ |
| Female | $29.3 \pm 0.2^{b}$ | $40.3 \pm 0.1^{a}$ |

Within main effects, means within a column having the same superscript are not significantly different ($P \leq 0.05$).

Table 3. Means ± standard errors of number of heterophils, lymphocytes, and heterophil/lymphocyte ratio by aflatoxin level at 18 days of age

| Aflatoxin $B_1$ ppb | Heterophil | Lymphocyte | Heterophil/ lymphocyte |
|---|---|---|---|
| 0 | $13.9 \pm 1.2^{b}$ | $60.5 \pm 2.2^{a}$ | $0.23 \pm 0.03^{b}$ |
| 966 | $14.6 \pm 1.3^{b}$ | $58.1 \pm 2.0^{a}$ | $0.25 \pm 0.03^{b}$ |
| 1830 | $13.9 \pm 1.3^{b}$ | $57.8 \pm 1.9^{a}$ | $0.24 \pm 0.02^{b}$ |
| 2890 | $15.9 \pm 1.7^{b}$ | $55.5 \pm 2.1^{ab}$ | $0.29 \pm 0.04^{b}$ |
| 5697 | $21.1 \pm 1.9^{a}$ | $51.5 \pm 2.3^{b}$ | $0.41 \pm 0.05^{a}$ |

Within main effects, means within a column having the same superscript are not significantly different ($P \leq 0.05$).

Table 4. Means ± standard errors of liver, bursa, thymus and spleen weights by sex at 42 days of age

| Organ[1] | Sex: Male | Sex: Female |
|---|---|---|
| Liver | | |
| Absolute | 11.0 ± 0.2[a] | 9.9 ± 0.1[b] |
| Adjusted | 3.35± 0.1[b] | 3.54± 0.1[a] |
| Bursa | | |
| Absolute | 1.6 ± 0.1[a] | 1.3 ± 0.1[b] |
| Adjusted | 0.43±0.01[a] | 0.41±0.01[a] |
| Thymus | | |
| Absolute | 1.1 ± 0.1[a] | 0.9 ± 0.1[b] |
| Adjusted | 0.29±0.01[a] | 0.29±0.01[a] |
| Spleen | | |
| Absolute | 1.1 ± 0.1[a] | 1.0 ± 0.1[a] |
| Adjusted | 0.33±0.01[a] | 0.32±0.01[a] |

[1]Absolute weights are in g and adjusted weights are per 100 g body weight.

Means within a row having the same superscript are not significantly different ($P \leq 0.05$).

Table 5. Means ± standard errors of liver, bursa, thymus, and spleen weights by population at 42 days of age

| Organ[1] | Population HH | HL | LH | LL |
|---|---|---|---|---|
| Liver | | | | |
| Absolute | 9.9 ± 0.2[b] | 10.8 ± 0.2[a] | 10.7 ± 0.2[a] | 10.6 ± 0.2[a] |
| Adjusted | 3.5 ± 0.1[ab] | 3.3 ± 0.1[c] | 3.6 ± 0.1[a] | 3.4 ± 0.1[bc] |
| Bursa[2] | | | | |
| Absolute | 1.4 ± 0.1 | 1.6 ± 0.1 | 1.5 ± 0.1 | 1.2 ± 0.1 |
| Adjusted | 0.46±0.01 | 0.45±0.01 | 0.4 ±0.02 | 0.34±0.01 |
| Thymus | | | | |
| Absolute | 0.8 ± 0.1[c] | 1.2 ± 0.1[a] | 1.0 ± 0.1[b] | 1.0 ± 0.1[b] |
| Adjusted | 0.24±0.01[c] | 0.32±0.01[a] | 0.29±0.01[b] | 0.30±0.01[ab] |
| Spleen[2] | | | | |
| Absolute | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 |
| Adjusted | 0.33±0.01 | 0.30±0.01 | 0.35±0.01 | 0.32±0.01 |

[1]Absolute weights are in g and adjusted weights are per 100 g body weight.

[2]Comparisons among means not shown because of population by aflatoxin level interaction; see Figure 3.

Means within a row having the same superscript are not significantly different ($P \leq 0.05$).

Table 6. Means ± standard errors of liver, bursa, thymus and spleen weights by aflatoxin level at 42 days of age

| Organ | Aflatoxin $B_1$ (ppb) | | | | |
|---|---|---|---|---|---|
| | 0 | 966 | 1830 | 2890 | 5697 |
| **Liver** | | | | | |
| Absolute | 11.0 ± 0.2$^{a}$ | 11.2 ± 0.2$^{a}$ | 11.3 ± 0.2$^{a}$ | 10.3 ± 0.2$^{b}$ | 8.6 ± 0.2$^{c}$ |
| Adjusted | 2.8 ± 0.1$^{d}$ | 2.9 ± 0.1$^{d}$ | 3.3 ± 0.1$^{c}$ | 3.9 ± 0.1$^{b}$ | 4.3 ± 0.1$^{a}$ |
| **Bursa**[2] | | | | | |
| Absolute | 1.8 ± 0.1 | 1.9 ± 0.1 | 1.6 ± 0.1 | 1.1 ± 0.1 | 0.7 ± 0.1 |
| Adjusted | 0.46±0.02 | 0.49±0.01 | 0.44±0.02 | 0.38±0.01 | 0.34±0.01 |
| **Thymus** | | | | | |
| Absolute | 1.4 ± 0.1$^{a}$ | 1.4 ± 0.1$^{a}$ | 1.0 ± 0.1$^{b}$ | 0.7 ± 0.1$^{c}$ | 0.4 ± 0.1$^{d}$ |
| Adjusted | 0.35±0.01$^{a}$ | 0.37±0.01$^{a}$ | 0.28±0.01$^{b}$ | 0.24±0.01$^{c}$ | 0.20±0.01$^{d}$ |
| **Spleen**[2] | | | | | |
| Absolute | 1.2 ± 0.1 | 1.4 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.1 | 0.6 ± 0.1 |
| Adjusted | 0.32±0.01 | 0.35±0.01 | 0.32±0.01 | 0.33±0.01 | 0.31±0.01 |

[1]Absolute weights are in g and adjusted weights are per 100 g body weight.

[2]Comparisons among means not shown because of population by aflatoxin level interaction; see Figure 3.

Means within a row having the same superscript are not significantly different (P ≤ 0.05).

Table 7. Metabolism in livers of chickens fed aflatoxin $B_1$; means ± standard errors of various measurements

| Trait | Aflatoxin $B_1$ (ppb) | |
|---|---|---|
| | 0 | 5697 |
| Cytochrome P-450/448 (nmol/mg microsomal protein) | 0.33 ± 0.08[a] | 0.07 ± 0.02[b] |
| O-demethylation of p-nitroanisole (nmol p-nitrophenol formed/60 min/100 mg liver) | 215 ± 35[a] | 65 ± 19[b] |
| Glucuronidation of aflatoxin $P_1$ (nmol $P_1$ converted in the liver/30 min) | 12 ± 4[a] | 6 ± 1[b] |
| Glutathione-S-epoxytransferase (change in absorbance units/10 min/ml supernatant) | 0.20 ± .01[a] | 0.15 ± .01[b] |

Means within a row having the same superscript are not significantly different ($P \leq 0.05$).

Table 8. Means ± standard errors of antibody titers ($\log_2$) at 5 and 13 days after intravenous inoculation of sheep erythrocyte antigen by population and aflatoxin level

| | Days post-inoculation | |
|---|---|---|
| | 5 | 13 |
| Population | | |
| HH | $6.93 \pm 0.29^{a}$ | $2.90 \pm 0.19^{a}$ |
| HL | $4.52 \pm 0.23^{bc}$ | $1.94 \pm 0.18^{b}$ |
| LH | $5.07 \pm 0.27^{b}$ | $2.34 \pm 0.15^{b}$ |
| LL | $3.82 \pm 0.21^{c}$ | $1.44 \pm 0.15^{c}$ |
| Aflatoxin $B_1$ (ppb) | | |
| 0 | $5.04 \pm 0.33^{a}$ | $2.07 \pm 0.16^{a}$ |
| 966 | $4.87 \pm 0.25^{a}$ | $1.95 \pm 0.17^{a}$ |
| 1830 | $4.96 \pm 0.32^{a}$ | $1.89 \pm 0.18^{a}$ |
| 2890 | $5.16 \pm 0.29^{a}$ | $2.40 \pm 0.22^{a}$ |
| 5697 | $5.33 \pm 0.35^{a}$ | $2.43 \pm 0.23^{a}$ |
| Sex | | |
| Male | $5.01 \pm 0.18^{a}$ | $2.22 \pm 0.12^{a}$ |
| Female | $5.13 \pm 0.21^{a}$ | $2.07 \pm 0.13^{a}$ |

Means within a row having the same superscript are not significantly different ($P \leq 0.05$).

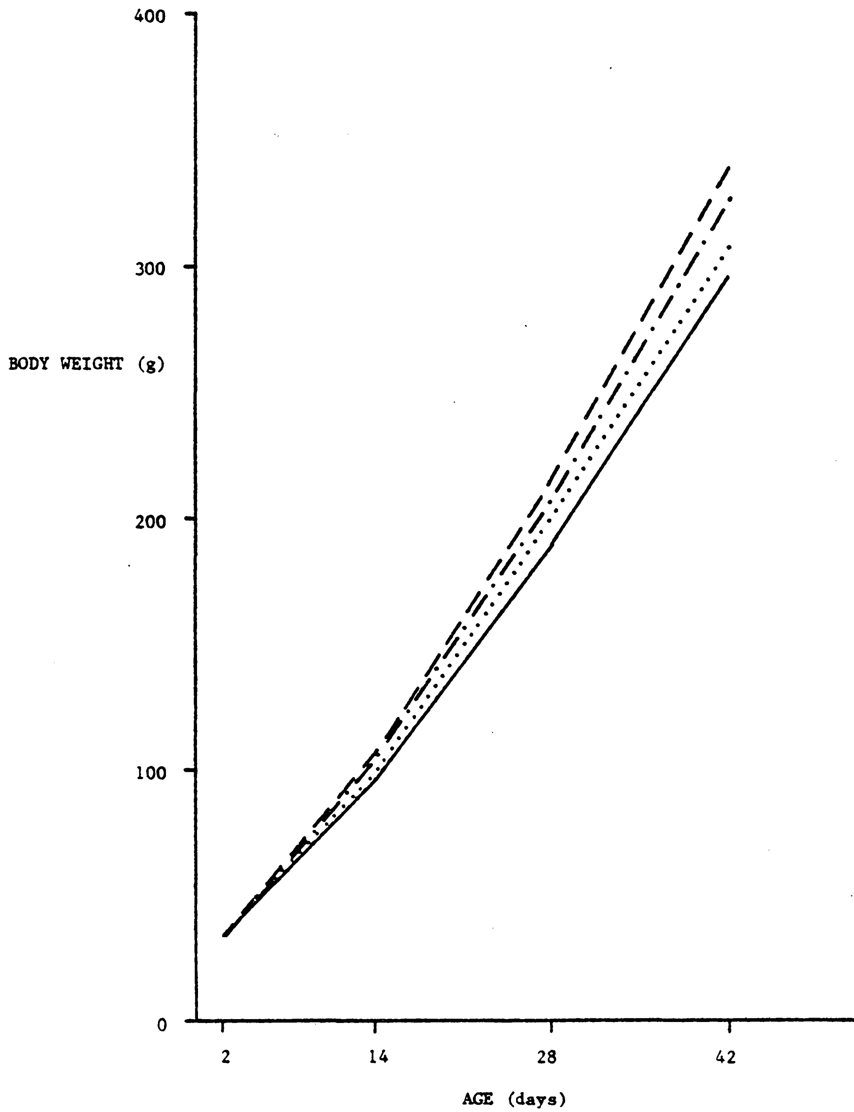


Figure 1. Body weight at 2, 14, 28, and 42 days of age by population (HH, HL, LH, and LL)

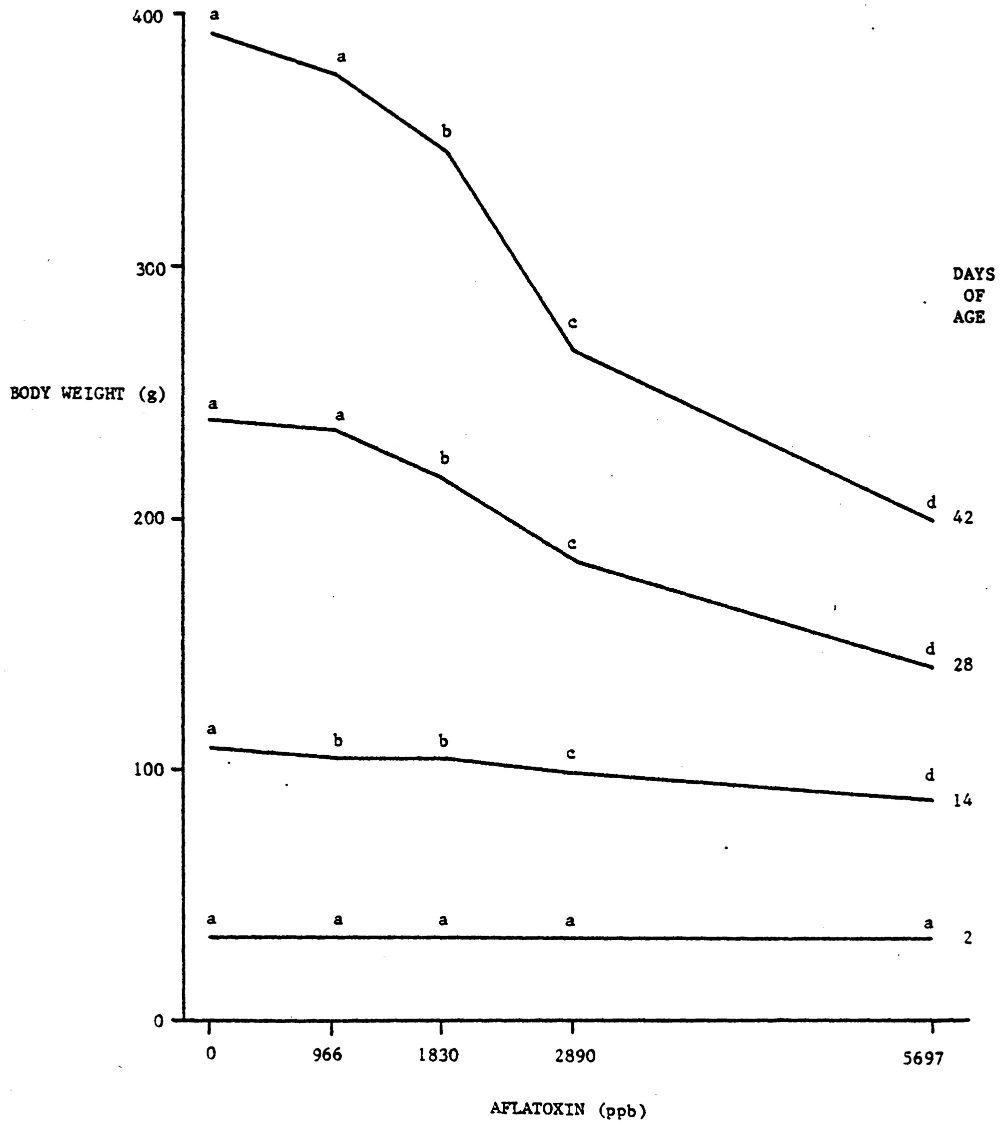


Figure 2. Body weight at 2, 14, 28, and 42 days of age at various aflatoxin levels

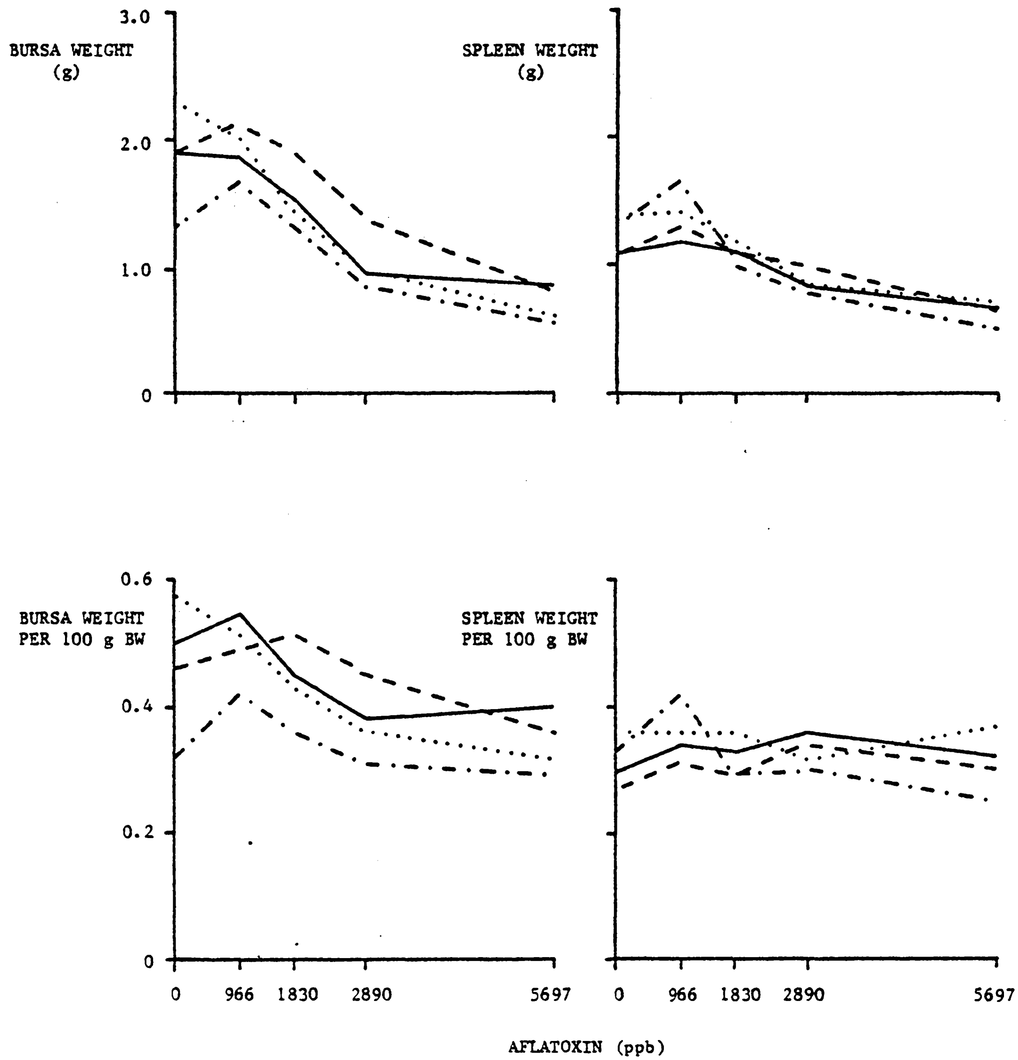


Figure 3. Bursa and spleen weights at 42 days of age where population by aflatoxin level interactions were significant

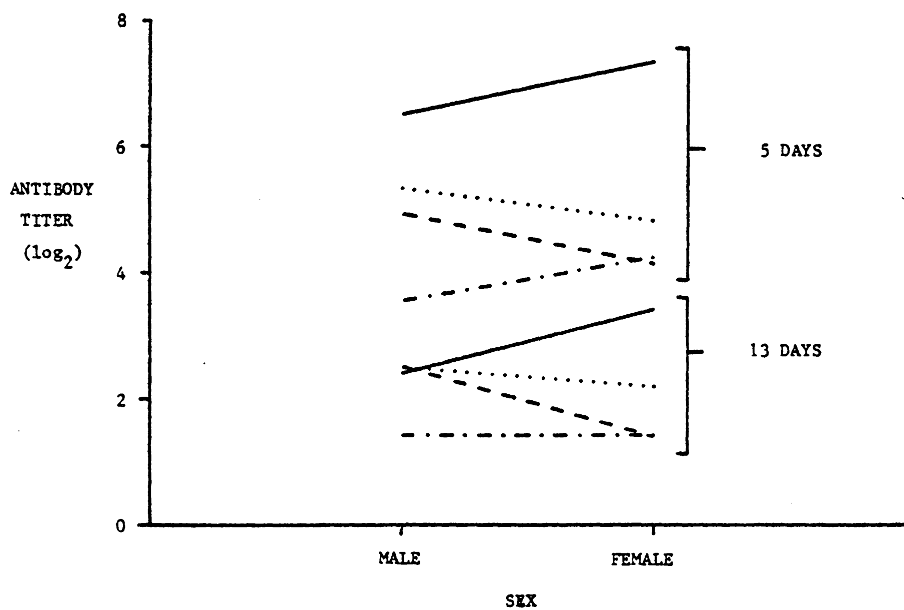


Figure 4. SRBC antibody titers 5 and 13 days after immunization, where population by sex interactions were significant

## EXPERIMENT II

# DIVERGENT SELECTION FOR ANTIBODY PRODUCTION TO SHEEP ERYTHROCYTES: KINETICS OF PRIMARY AND SECONDARY ANTIBODY RESPONSES

## Introduction

Genetic differences in the magnitude and kinetics of the primary response of chickens to sheep erythrocytes (SRBC) were reported by van der Zijpp (1983). The relationship between primary and secondary antibody responses varied in a temporal context (van der Zijpp *et al.*, 1983). The implications of such information may be relevant to vaccination programs. Uhr and Finkelstein (1963) inoculated guinea pigs with an above threshold dose of ϕ174 bacteriophage and noted that the relative rate of 19S formation was maximal and dose-dependent; below this concentration slower relative rates were obtained. The role of concentration of antigen was recently demonstrated in the chicken (Siegel *et al.*, 1983). Using *Salmonella pullorum* antigen, they showed that environmental stressors could affect the expression of maximum primary antibody responses if high antigen doses were not used in immunization. In mice duration and intensity of the immune response were dependent upon the time the antigen persisted in the recipient (Hanna and Peters, 1971). Siegel and Gross (1980) selected lines of White Leghorn chickens that differed in antibody production 5 days post-primary immunization with 0.1 ml of 0.25% SRBC antigen. Although line differences in SRBC antibody titers were also present 21 days post-immunization, detailed temporal agglutinin patterns to an

initial SRBC antigen and booster challenges are lacking. Also, there is a void of information on antibody production by these populations in response to concentrations of SRBC antigen above and below those used as the selection criterion.

The objective of this experiment was to measure the kinetics of primary and secondary antibody responses to varying concentrations of SRBC antigen in parental lines and reciprocal crosses of the parental lines of chickens selected for high and low antibody response to SRBC antigen.

## Materials and Methods

Two trials were conducted using $S_9$ generation parents to produce progeny from the parental lines and reciprocal $F_1$ crosses between them. In all matings, the sire line is denoted first and the dam line second (e.g., HL = high line sires and low line dams). Trial 1 was designed to measure primary and secondary antibody responses over time to SRBC antigen administered via the same protocol under which selection was practiced in the development of the selected lines. Trial 2 was designed to measure primary and secondary responses to concentrations of SRBC antigen that were higher and lower than that under which selection was practiced.

### Trial 1

Forty-five chicks from each parental and reciprocal $F_1$ populations were hatched on the same day, vaccinated for Marek's disease, wingbanded and reared in floor pens. Sexes and lines were intermingled in the same flock and feed and water were provided *ad libitum*.

At 35 days of age, all birds were immunized by injecting 0.1 ml of 0.25% SRBC into the brachial vein. During the first 10 days post-primary immunization (PPI), blood was collected daily from the brachial vein of a random sample of 15 birds from each of the 4 populations for SRBC

antibody determination. Blood was obtained from the same sample of birds every fourth bleeding. From 11 to 34 days PPI, 15 birds per population were bled every other day for antibody determination. On day 24 PPI a random sample of 13 birds from each population was given a booster dose of 0.1 ml of 0.25% SRBC administered intravenously. Remaining birds were maintained as the non-booster control.

SRBC antibody was determined by the microtiter procedure of Wegmann and Smithies (1966). Each bird's serum sample was titrated, and its antibody level expressed as $\log_2$ of the highest dilution giving a visible agglutinin. Analyses of variance were used to test for differences among populations within days post-immunization using the statistical model:

$$Y_{ij} = \mu + L_i + e_{ij}$$

where i = 1, 2 . . . 4 populations, and j = 1, 2 . . . 15 individuals per subclass. When significant differences were found, means were separated by Duncan's Multiple Range Test.

Trial 2

Forty-five chicks from each of the 4 populations were randomized at 35 days of age into 3 antigen dose treatment groups. Each chick within a group received via the brachial vein in 0.1 ml, one of the three antigen doses: 0.025, 0.25 and 25% suspension of SRBC for primary immunization. Twenty-four days later, all birds received a booster dose of

0.1 ml of 0.25% SRBC. Husbandry procedures were the same as in Trial 1. Antibody was measured in blood obtained from the brachial vein of each bird on days 6, 13, and 20 PPI and 3, 7 and 10 days after the booster was administered. Assay procedures were the same as those used in Trial 1 and data were analyzed within days post-immunization using the statistical model:

$$Y_{ijk} = \mu + L_i + T_j + (LT)_{ij} + e_{ijk}$$

where i = 1, 2 . . . 4 populations, j = 1, 2, 3 antigen dose treatments, and k = 1, 2 . . . 15 individuals per subclass. Where significant differences were found among main effects, means were separated by Duncan's Multiple Range Test. Correlations between peak primary and secondary antibody titers were calculated within populations.

## Results and Discussion

### Trial 1

Commencing about 3 days PPI, antibody production increased rapidly in all of the populations, reaching a maximum at about 6 days and maintaining this level to about 12 days after which it declined (Figure 5). Significant differences were noted between parental populations from day 4 through day 24 PPI after which they disappeared. During that period the crosses were intermediate to parental lines and not significantly different from each other. Means and standard errors for agglutinin responses were 3.5 ± 0.5 and 1.1 ± 0.3 for HH and LL, respectively, on day 4 PPI resulting in approximately a 5-fold difference in agglutinin titers between parental lines. By day 6, the HH population mean of 7.5 ± 0.7 was 11 times greater than the LL population mean of 4.0 ± 0.4. The drop in agglutinin response on day 10 in the LL line cannot be readily explained since all populations were intermingled in the same flock and nothing unusual was noticed. Levels for this line returned to the previous reading on the next bleeding.

Although differences were noted among the populations in antibody levels to a primary immunization with SRBC antigen, temporal patterns were similar across populations. A very different situation emerged in the secondary response to SRBC antigen. Although all populations responded

similarly in a temporal sense, there were no differences in antibody titers.

Peak titers for PPI and secondary responses occurred at about the same time (i.e., by 6 days for the former and 3 days for the latter), regardless of population. Yet, there were differences among populations in antibody produced during the PPI response, while no differences were observed in the secondary response inferring that selection did not phenotypically influence the magnitude of the secondary immune response. Little or no evidence of an anamnestic response existed in any population except the LL line.

Trial 2

This trial was conducted to determine if the general lack of anamnestic response observed in Trial 1 was dose-related. Primary and secondary responses to intravenous inoculation of 0.1 ml of 0.25% SRBC antigen followed the same general pattern as that observed in Trial 1 for this dosage (Figure 6b). The PPI response was greater in HH than LL chicks with the reciprocal crosses intermediate and not different from each other. There were no differences between the populations for secondary response; only LL chicks showed an anamnestic pattern. Lack of differences among populations for secondary response may be attributed to the lack of a correlated secondary response to genetic selection for antibody production to SRBC antigen day 5 PPI.

Within population phenotypic correlation coefficients of .10, .39, .00 and .29 between peak primary and secondary titers (day 6 post-primary and day 3 post-secondary immunization) were not different from zero for progeny from HH, HL, LH and LL matings, respectively.

When chicks were given an initial intravenous immunization of 0.1 ml of 25% SRBC antigen, high antibody titers were produced in all the populations (Figure 6c). Although the temporal pattern was the same as that observed at the dosage under which selection was practiced, mean agglutinin responses among populations did not differ significantly from one another. This result supports those of Gross (1979) that differences in antibody production among stocks of chickens are less pronounced at higher doses of SRBC. My results also support those of van der Zijpp (1978, 1983) that a very high SRBC antigen dosage produced the maximal attainable response in chickens.

Agglutinin responses of the populations to post-secondary immunization (PSI) showed that priming with 0.1 ml of 25% SRBC resulted in a very modest secondary response when the booster was 0.1 ml of 0.25% SRBC (Figure 6c). As with the 0.25 primary challenge, nonsignificant correlations were obtained between PPI and PSI peak titers were not different from zero in any population. Masking of a secondary response by the high primary dosage agreed with

the reports of Grantham (1972) and Benner *et al*., (1974) who observed that while primary inoculation of mice with a high SRBC antigen resulted in a clear primary response, secondary response was poor. Moller and Wegzell (1965) and Grantham and Fitch (1975) reported that antibody produced during primary immunization of mice with SRBC acted through a feedback mechanisms that limited the magnitude of the secondary response to subsequent antigenic challenge both *in vivo* and *in vitro*. Hanna and Peters (1971) postulated from data in mice that a large immunizing dose of SRBC antigen converted most of the sensitive cells all the way to antibody-producing cells during the primary response leaving only a small pool of sensitized cells to respond to secondary challenge.

When chicks were given 0.1 ml of 0.025% SRBC antigen intravenously, the general pattern of primary responses was for all populations to exhibit low antibody production. Yet, there was evidence of different mean titers among populations at day 6 PPI (P = .07). As seen in Figure 6a, mean antibody titer for HH progeny was several-fold higher than those for the HL, LH, and LL progeny which were closely grouped. At 13 and 20 days of age, however, means were similar for all populations. Agglutinin responses of the crosses at this lower dosage were similar to line LL, rather than intermediate to the low parental lines.

Agglutinin responses of the populations to PSI showed that priming with 0.1 ml of 0.025% SRBC antigen, followed with an 0.1 ml of 0.25% SRBC antigen resulted in agglutinin titers of HH and HL progeny that were significantly larger than those of LH and LL chickens 3 days after the booster was given (Figure 6a). Agglutinin responses on day 7 PSI showed that the HH population produced significantly larger titers than LH and LL populations, while mean titers of HL, HH and LL did not differ from each other. The HH population produced significantly higher titers on day 10 PSI than the other populations which did not differ significantly. As with other primary dosages, the correlations within populations between day 6 PPI and day 3 PSI titers were not significantly different from zero.

General

Results obtained in this experiment were consistent with previous findings that initial low dosage immunization of antigen resulted in a greater secondary response to subsequent antigenic challenge than when a large priming dose was used. For example, Grantham (1972) showed that initial immunization of mice with a low dose of SRBC antigen resulted in a greater secondary response to subsequent challenge than immunization with a large antigen dose that was optimal for the production of the primary response.

A consistent lack of correlation between the peaks of

primary and secondary responses, regardless of line and primary dose, differed from the observations of van der Zijpp et al. (1983). Although there was inconsistency in correlations among various ages, phenotypic and genetic correlations between day 5 PPI and day 3 PSI were positive and of a modest magnitude in chickens inoculated with SRBC antigen. Whether the differences between her results and ours are due to pleiotrophy, linkage and/or nongenetic factors is unknown.

The experiments reported here showed that selection for high and low antibody response 5 days PPI to 0.1 ml of 0.25% SRBC antigen produced distinctly different lines for this dosage. Although these populations and crosses between them had similar temporal patterns in their primary and secondary antibody responses, genetic differences in the magnitude of response between these populations could be masked by increasing or decreasing the primary dose of SRBC antigen. Selection for a primary response had no influence on the secondary response probably because the antigenic challenge dose used in the selection program was intermediate. High dosages may override the systems, and low dosages may not be sufficient to activate thresholds associated with antibody production.

It is tempting to infer that higher immunization dosages may be needed to provide adequate protection in

disease prevention programs involving meat-type poultry. This is because the magnitude of the response to 0.1 ml of 0.25% SRBC antigen obtained in the LL line is similar to that observed in broiler stocks (Siegel *et al*., 1984), response to SRBC antigen is negatively associated with growth (Siegel *et al*., 1982; van der Zijpp, 1983a), antibody response to *Salmonella* antigen administered at higher dosages is less affected by stressors (Siegel *et al*., 1983), and correlations between primary and secondary antibody responses found in this experiment are low.

Summary

This experiment consisted of two trials involving chicks from lines selectively bred for either high (HH) or low (LL) antibody response to sheep erythrocytes (SRBC) and from reciprocal crosses (HL and LH) of these parental lines. In Trial 1, chicks were immunized intravenously with 0.1 ml of 0.25% SRBC antigen at 35 days of age and half of them received a booster dose of 0.1 ml of 0.25% SRBC on day 24 post-primary immunization. In Trial 2, 35-day old chicks were immunized intravenously with 0.1 ml of either 0.025, 0.25% or 25% SRBC. On day 24 post-primary immunization, all chicks were reimmunized with 0.1 ml of 0.25% SRBC. Results showed that (1) selection for primary antibody response did not influence secondary response, (2) dosage of primary response influenced secondary response, (3) peak titers occurred at about the same time for primary (by 6 days) and booster (by 3 days) immunization regardless of line, and (4) line differences appeared as early as day 4 and persisted until day 24 post-primary immunization.

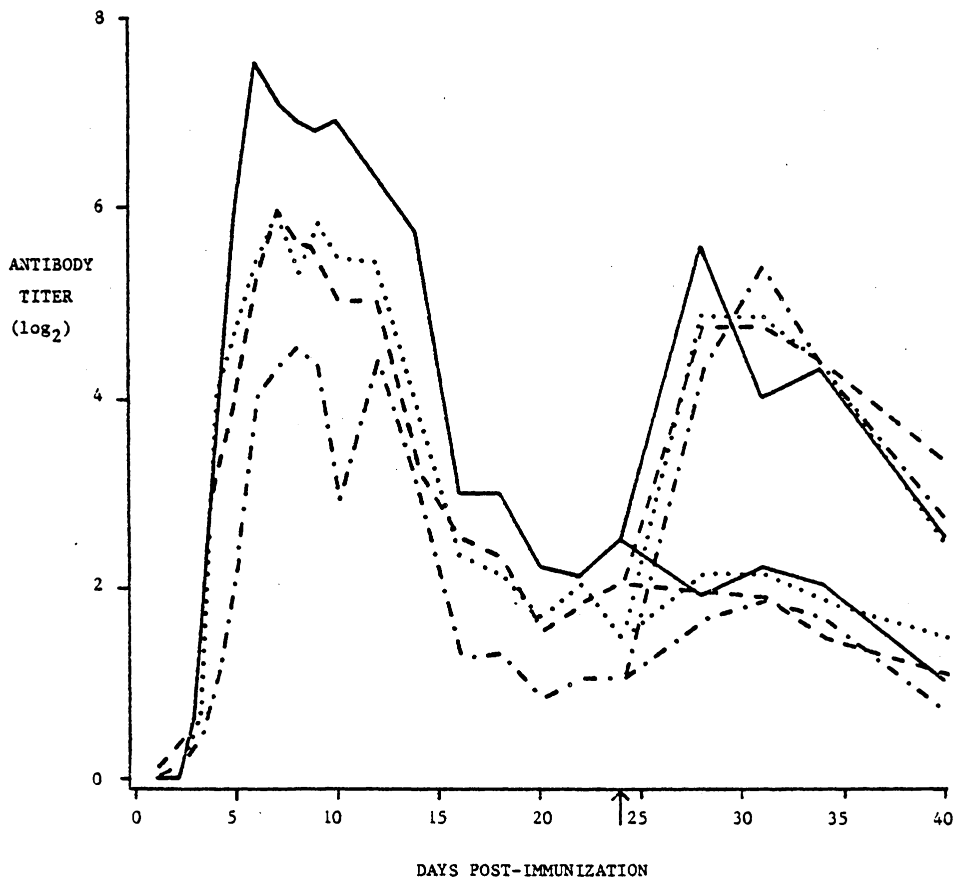


Figure 5. Primary and secondary responses to SRBC antigen in HH, HL, LH, and LL populations.

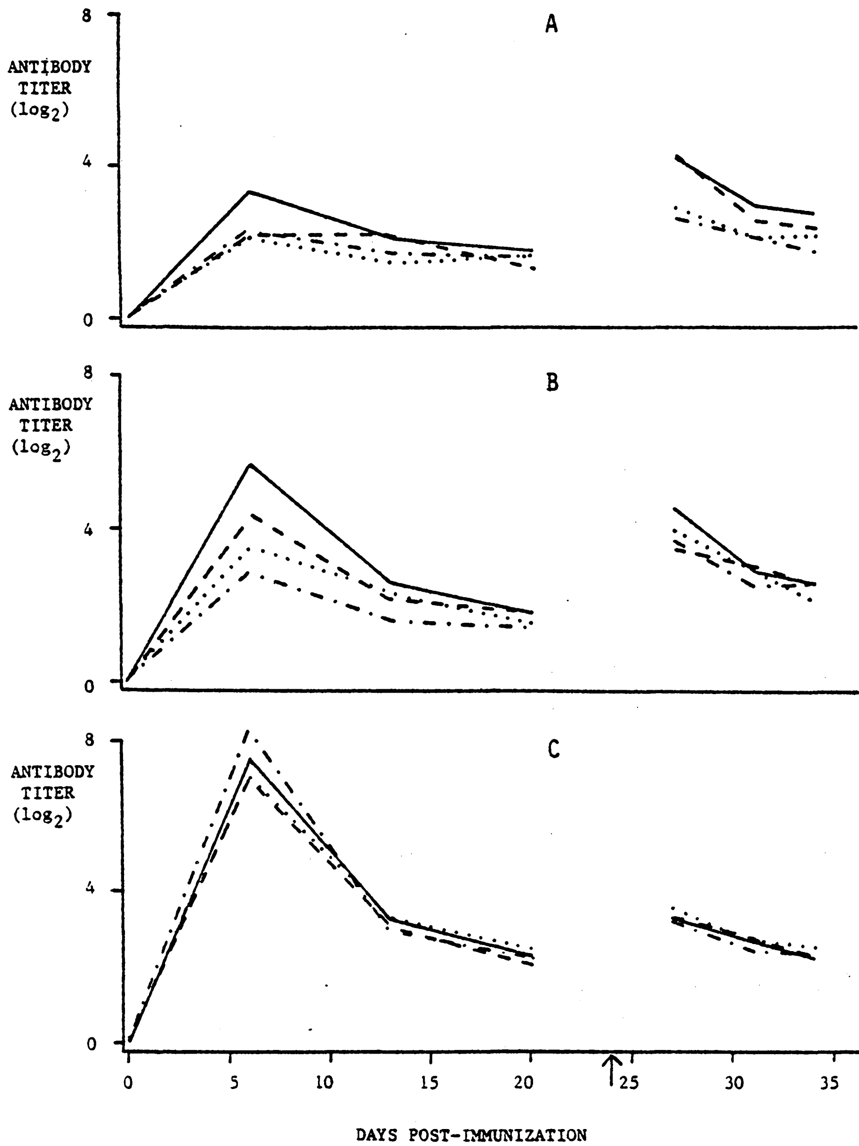


Figure 6. Primary and secondary responses to SRBC antigen in HH, HL, LH, and LL populations. Primary doses were 0.025%, 0.25%, and 25% while the booster dose was 0.25%

## EXPERIMENT III

## DIVERGENT SELECTION FOR ANTIBODY PRODUCTION TO SHEEP ERYTHROCYTES: AGE EFFECT IN PARENTAL LINES AND THEIR HYBRIDS

# Introduction

Siegel and Gross (1980) developed lines of chickens differing in antibody production to sheep erythrocyte antigen (SRBC). High line (HH) chickens had better immunity to Newcastle disease, were more resistant to *Mycoplasma gallisepticum*, *Eimeria necatrix*, splenomeglia virus, and northern feather mite, while less resistant to *Escherichia coli* and *Staphylococcus aureus* than low line (LL) chickens (Gross *et al*., 1980). Other correlated responses showed that LL chickens were heavier, reached sexual maturity at earlier ages, had greater intensity of egg production and higher mortality from nonspecific causes than those from the line HH (Siegel *et al*., 1982). Also, high line birds shake their heads more (Dunnington *et al*., 1983; Cherry *et al*., 1984) and air peck less (Cherry *et al*., 1984) than those from the low line.

In their selection experiment, Siegel and Gross (1980) administered the antigen intravenously between 41 and 51 days of age, and measured antibody production 5 days later. In subsequent experiments, they noted that the relative differences between the lines in production of antibody were also present when erythrocyte antigen was given to adults (Siegel *et al*., 1982). The experiment presented here measured antigen production to SRBC antigen, sizes of various organs associated with immunoresponsiveness in

selected lines and reciprocal crosses between them.

## Materials and Methods

Chickens used in this experiment were obtained from matings among the 9th generation of lines divergently selected for response to 0.1 ml of a 0.25% suspension of SRBC antigen (Siegel and Gross, 1980). Progeny were obtained from matings within the parental lines and reciprocal crosses between them. The sire line is denoted first and the dam line second in identifying a mating (e.g., HL = a high line sire and a low line dam).

### Trial 1

Individual birds were wing-banded at hatching and reared as contemporaries on litter in sex and line-intermingled flocks. A sample of blood was obtained at 14 days of age from the brachial vein of 10 randomly selected chicks from each population and tested for the presence of maternal SRBC antibody. At 7-day intervals from 14 to 35 days of age, 10 randomly selected chicks per population were intravenously immunized with 0.1 ml of 0.25% suspension of SRBC antigen in buffered saline. Five days after an immunization (19, 26, 33 and 40 days of age), blood was obtained via the brachial vein and antibody titers measured by the microtiter procedure (Wegmann and Smithies, 1966). Titers were expressed as the $\log_2$ of the highest dilution giving visible agglutinin. After bleeding, each chick was

weighed, sacrificed by cervical dislocation, and its bursa, spleen and thymus removed and weighed to the nearest 0.1 g.

Data were analyzed by analysis of variance using the statistical model:

$$Y_{ijk} + \mu + L_i + I_j + (LI)_{ij} + e_{ijk}$$

where i = 1, 2 . . . 4 populations, j = 1, 2 . . . 4 age groups, and k = 1, 2 . . . 10 individuals per subclass.

Prior to analysis, organ weights were expressed relative to body weight (i.e., organ weight/100 g of body weight). When significant differences were observed, means were separated by Duncan's multiple range test.

Trial 2

The results of Trial 1 dictated the desirability of comparing the same 4 populations for their ability to produce SRBC antibody at younger ages and at different dosages of SRBC antigen. Husbandry procedures were similar to those used in Trial 1 except that the chicks were battery reared.

At 7 days of age, 10 birds from each population were sampled for presence of maternal SRBC antibody. All were negative. An initial test was then initiated whereby 10 randomly selected chicks per population were intravenously inoculated with 0.1 ml of 0.25% suspension of SRBC. Blood was collected via the brachial vein for SRBC antibody assays on the fifth day post-immunization. A second test involved

the same design as the first except that chicks were immunized with 0.1 ml of 12.5% suspension of SRBC. The same protocol was used in the third test except that there were 12 chicks per population and the dosage was 0.2 ml of 12.5% suspension of SRBC. In a fourth test, 10 chicks randomly selected from each population at 1 and 7 days of age (the 22nd day of incubation was considered as 1 day of age) were individually weighed, sacrificed by cervical dislocation, and their bursa, spleen, and thymus removed and weighed to the nearest 0.1 g.

Within each of the first 3 tests, Chi-square analyses were used to compare populations for frequencies of responders and nonresponders to SRBC immunization. Analysis of variance was used for comparison involving those birds that produced SRBC antibody. When significant differences were found, population means were separated by Duncan's multiple range test. The following model was used to analyze body, bursa, spleen, and thymus weights.

$$Y_{ijk} + \mu + L_i + I_j + (LI)_{ij} + e_{ijk}$$

where i = 1, 2 . . . 4 populations, j = 1, 2 age groups, and k = 1, 2 . . . 10 individuals per subclass. Prior to analysis, organ weights were expressed relative to body weight (i.e., organ weight/100 g of body weight). When significant differences were observed among populations, means were separated by Duncan's multiple range test.

<u>Histology</u>

Bursae were removed from 5 HH and 6 LL chicks at 21 days of age, and fixed in 10% buffered formation. Five μm sections were stained in hematoxylin and eosin and photographs taken at 37x. A grid plate with 4 x 4 cm inner and 6 x 6 cm outer dimensions were placed on 3 locations of each photograph. Number and size of bursa lymphoid follicles were determined using a Zeiss microprocessor with opto-stylus. Only follicles within or touching the inner grid line were counted and measured. Values for the 3 locations of an individual grid were pooled and data analyzed by analysis of variance using a complete randomized design.

## Results and Discussion

### Trial 1

SRBC antibody. No SRBC maternal antibody was detected in any of the chicks tested. When SRBC antigen was given, age effects and age by population interactions were not significant. HH birds had significantly higher antibody titers than LL birds and the reciprocal crosses while always intermediate to their parental lines, did not differ from the LL line (Table 9). Parental line differences were consistent with those obtained in the $S_3$ generation (Siegel and Gross, 1980). However, unlike the earlier generations, the titers of the crosses are now lower, rather than higher, than their mid-parent average. Biozzi *et al*. (1979) postulated that antibody production to SRBC antigen between high and low selected lines of mice were due to a faster rate of multiplication and differentiation of B-lymphocytes in the high line and a stronger catabolic activity of macrophages in the low line.

The lack of significant differences in antibody production among ages indicate that serological maturity for precipitins to 0.1 ml of 0.25% SRBC antigen was attained by 14 days, an age prior to that when selection was made (41 to 51 days of age). Previously, it was shown that differences in antibody response of these lines existed in adults (Siegel *et al*., 1982). Such observations are consistent

with those obtained in New Hampshire chickens by McCorkle and Glick (1980).

Body and organ weight. The age by population interaction was not significant for body and organ weights. As expected, 19, 26, 33 and 40 day-old birds had body weights that differed significantly from one another (Table 9). Comparisons among the populations showed that LL birds and the crosses were significantly heavier than HH birds. The difference in body weight between the parental lines was consistent with that noted in previous generations (Siegel and Gross, 1980; Siegel *et al*., 1982), and with the negative genetic correlation between body weight and hemagglutinin antibody titers to SRBC antigen (van der Zijpp, 1982).

The age by population interaction was not significant for bursa weight. At 26, 33 and 40 days of age, chicks had significantly heavier than bursae at 19 days (Table 10). Bursae of LL birds were significantly smaller than those of other populations with bursae of the crosses not different from each other. When compared to the HH line, the bursae of HL but not the LH crosses were significantly larger than those of that parental line.

The role of the bursa in antibody production has been confirmed numerous times since the initial publications (Glick *et al*., 1956; Chang *et al*., 1957). Although small bursae of LL chicks is consistent with the positive

association between bursa size and antibody production (e.g., Glick, 1956; Sadler and Glick, 1962), the relationship between bursa size and production of SRBC antibody is far from clear. For example, in lines selected for large and small bursae size, the peak antibody titers to SRBC was greater in the small than large bursa lines (Landreth and Glick, 1973; Yamamoto and Glick, 1982). Earlier, Jaffe and Jaap (1966) found no differences in antibody titers to Salmonella typhimurium in 2 breeds of chickens which differed in bursa size. The inconsistency in antibody production among stocks known to differ in bursa size demonstrates that caution should be exercised in making inferences on antibody production from differences in bursae size among lines. This is because it is the presence of lymph follicles, not the size of the bursa per se, that determines antibody production (May and Glick, 1964). Means and standard errors of number of follicles per square cm were 1.54 ± .16 and 1.46 ± .11 for HH and LL lines, respectively. Areas of follicles were 1.28 ± .13 and 1.49 ± .06 square mm for HH and LL lines, respectively. None of the differences between lines were significant. Since, however, HH birds had larger bursa than LL birds they would have more active lymphoid follicles that could affect future antibody potential. This reasoning is consistent with that proposed by May and Glick (1964).

Although neither age nor the age by population interaction was significant, significant differences were found among populations for spleen weights per 100 g body weight. Spleens of the HH and cross populations while not different from one another, were heavier than those from LL chickens. Glick (1978) reported that more than 90% of the lymphocytes in the bursa were bursal lymphocytes and that the number of lymphocytes in the spleen is divided almost equally between T and B-cells. Selection for high and low antibody production has resulted in concomitant changes in both bursa and spleen size.

Significant differences were found among populations and ages for thymus weights per 100 g of body weight, while the age by population interaction was not significant. Relative thymus weight increased with age, being significantly smaller at 26 days and significantly larger at 40 days of age. Thymus weights of the reciprocal crosses were similar and significantly larger than those of line HH and smaller than those of line LL. This pattern of an intermediate spleen size for the crosses suggests additive genetic variation for this trait. A biological explanation for differences between selected lines for thymus weight is not readily apparent and may be due to genetic drift. A basis for this reasoning is that normal antibody production to bovine serum albumin was observed in 6 to 9-week old

neonatally thymectomized chickens (Warner and Szenberg, 1964). Because the thymus is particularly associated with cell mediated immune reactions, it may be that the LL line is more responsive in cell-mediated immunity than the HH and the cross populations. Determining whether or not this is the case requires additional experimentation.

Trial 2

SRBC antibody. When 0.1 ml of 0.25% of SRBC was intravenously administered at 7 days of age, the percentages of birds showing detectable antibody titers were 30, 10, 0, and 0 for the HH, HL, LH and LL populations, respectively. Chi-square analysis comparing frequencies of responders and non-responders showed no significant differences between populations. Increasing the dosage of SRBC to 12.5% produced detectable SRBC antibody in 80, 40, 40, and 30% of the HH, HL, LH and LL chicks, respectively. The HH and LL chicks differed significantly in frequency of responders, while the crosses differed neither from each other nor their parental lines. The means and standard errors of the antibody titers for responders were 2.0 ± 0.4, 1.2 ± 0.6, 1.2 ± 0.5, and 1.0 ± 0.4 for HH, HL, LH, and LL, respectively. None of the differences among populations was significant. Increasing the volume of antigen administered to 0.2 ml of 12.5% SRBC produced detectable titers in essentially all of the HH and cross chicks (HH-12/12,

HL-12/12, and LH-11/12). In contrast, only 4 of 12 LL chicks produced antibody. Chi-square analysis showed that the frequency of responders was significantly lower for the LL than for the other populations. Means and standard errors of antibody titers for responders were 3.3 ± 0.2, 2.8 ± 0.2, 2.6 ± 0.3, and 0.7 ± 0.4 for HH, HL, LH, and LL, respectively, with means for the HH, HL and LH birds being significantly higher than those of LL birds. Thus, not only was there a difference between populations for frequency of responders but the overall pattern among populations persisted in the titers of responsive individuals.

Body and organ weight. Population by age interactions were not significant for body and relative weights of bursa, spleen, and thymus. As expected, birds were significantly heavier at 7 than 1 day of age (Table 11). There were no differences among populations for body weight, inferring that the differences in body weight observed in Trial 1 and by Siegel and Gross (1980) appeared after the decline of the large maternal effect of egg weight.

Differences among populations for relative weights of bursa, spleen and thymus were not present at 1 and 7 days of age. Age comparisons revealed no difference between these ages for bursa weight while significant differences were noted for spleen and thymus. Spleen weight relative to body weight was greater at 7 than at 1 day of age, while the

reverse pattern was noted for thymus weight.

General

Differential growth patterns were observed for organs associated with immunoresponsiveness in chickens. Examination of relative growth across ages (Tables 10, 11) shows a spurt in bursa weight relative to body weight at about 26 days and then a plateau. The rapid increase in relative bursa weight during the period studied was evident in all populations and is consistent with observations in White Leghorns (Glick, 1956) and White Rocks (Wolfe *et al*., 1962). Spleen weight relative to body weight doubled during the first week, doubled again during the second week, and then remained constant in the HH, HL and LH lines. The second doubling was not observed in line LL. These data bracket the three-fold increase in relative spleen weight observed through 42 days of age in White Rocks (Wolfe *et al*., 1962). Calculations made from their data show that relative to body weight, spleen weight plateaus at about 42 days. Relatively, body growth was greater than thymus growth during the first week post hatching. Thereafter, increases in thymus weight were progressively greater than those of body weight. These results are consistent with those calculated from the data of Wolfe *et al*. (1962).

Serological maturity of chicks immunized with 0.1 ml of 0.25% SRBC was attained at 14 days of age and did not

increase further with age. Furthermore, the relationship between the parental lines and crosses between them followed the same pattern for the concentration and dosage of SRBC used. At 7 days of age, a majority of the chicks failed to respond or responded very weakly to 0.1 ml of 0.25% SRBC antigen, suggesting immaturity of certain components of immune systems (e.g., bursa, spleen and thymus) or insufficient numbers of antibody producing cells. Low antibody titers to SRBC antigen were produced in the HH and cross chicks by a 2-fold increase in antigen administered and a 50-fold increase in antigen concentration. These data suggest that while the chicks were capable of producing antibody at 7 days of age, they were serologically immature because maximum titers were not produced after the threshold level is reached. Perhaps more important from an application viewpoint is that the difference in the frequency of responders of the LL from the HH and cross lines indicates genetic differences in the thresholds. The similarity between the high line and the crosses suggest dominant genetic effects on the threshold. This observation is consistent with the observation of dominance by Balcavora et al. (1973) in the capability for formation of antibody in mice.

## Summary

Two trials were conducted to measure age dependency for the onset of immunological maturity in populations known to differ in their capacity to produce SRBC antibody. Populations included lines selected for high and low response to SRBC and reciprocal crosses between them. In Trial 1, chicks from each population were intravenously immunized with a single injection of 0.1 ml of 0.25% suspension of SRBC in buffered saline at 14, 21, 28 and 35 days of age. In Trial 2, chicks from each population were intravenously immunized at 7 days of age with a single injection of either 0.1 ml of 0.25%, 0.1 ml of 12.5% or 0.2 ml of 12.5% SRBC antigen and antibody measured 5 days later. At this time, body, bursa, spleen and thymus weights were obtained. A separate hatch was necessary to obtain weight data at 1 and 7 days of age.

At 14 days of age and older, HH line birds had larger bursal and spleen weights and smaller thymal weights relative to body weight than those from LL line. Relative to body weight, spleen and bursa grew at a faster rate to 19 and 25 days of age, respectively, and then plateaued. In contrast, there was a progressive increase in thymus weight relative to body weight through 40 days of age.

Differences among populations for frequency of responders to SRBC antigen were found at 7 days of age.

This qualitative pattern persisted in the quantitative context of the antibody titers of those who responded, demonstrating genetic differences in both the event and subsequent levels of antibody production has been reached. Although birds from the HH line produced significantly higher titers than those from the LL and cross lines, all populations reached their respective serological maturity by 14 days of age.

Table 9. Means and standard errors for antibody production to SRBC and body weight by age and population

| Age (days) | Populations | | | | |
|---|---|---|---|---|---|
| | HH | HL | LH | LL | Pooled |
| Titer ($log_2$) | | | | | |
| 14 | 6.6 | 4.0 | 5.0 | 3.6 | 4.8±0.3[a] |
| 21 | 5.9 | 5.1 | 4.6 | 3.1 | 4.6±0.3[a] |
| 28 | 5.3 | 4.1 | 4.4 | 4.1 | 4.5±0.3[a] |
| 35 | 5.7 | 4.8 | 3.9 | 3.5 | 4.5±0.5[a] |
| Pooled | 5.9±0.4[a] | 4.5±0.4[b] | 4.5±0.3[b] | 3.6±0.3[b] | |
| Body wt. | | | | | |
| 19 | 74 | 80 | 73 | 83 | 78 ± 2[d] |
| 26 | 181 | 210 | 196 | 180 | 192 ± 6[c] |
| 33 | 227 | 285 | 290 | 274 | 269 ± 8[b] |
| 40 | 293 | 390 | 363 | 354 | 350 ± 12[a] |
| Pooled | 194 ± 15[b] | 241 ± 19[a] | 230 ± 19[a] | 223 ± 18[a] | |

Means within a row or column having the same superscript are not significantly different ($P \leq .05$).

Table 10. Means and standard errors for bursa, spleen and thymus (per 100 g body weight) by age and population

| Age (days) | Populations | | | | |
|---|---|---|---|---|---|
| | HH | HL | LH | LL | Pooled |
| **Bursa** | | | | | |
| 19 | 0.25 | 0.29 | 0.24 | 0.24 | 0.26±0.01[b] |
| 26 | 0.46 | 0.48 | 0.50 | 0.37 | 0.45±0.02[a] |
| 33 | 0.46 | 0.50 | 0.58 | 0.41 | 0.48±0.02[a] |
| 40 | 0.45 | 0.56 | 0.45 | 0.39 | 0.46±0.02[a] |
| Pooled | 0.40±0.03[b] | 0.46±0.02[a] | 0.44±0.02[ab] | 0.35±0.02[c] | |
| **Spleen** | | | | | |
| 19 | 0.25 | 0.20 | 0.22 | 0.10 | 0.19±0.01[a] |
| 26 | 0.21 | 0.17 | 0.21 | 0.11 | 0.18±0.01[a] |
| 33 | 0.19 | 0.21 | 0.20 | 0.12 | 0.18±0.01[a] |
| 40 | 0.21 | 0.20 | 0.22 | 0.13 | 0.19±0.01[a] |
| Pooled | 0.22±0.01[a] | 0.20±0.01[a] | 0.21±0.01[a] | 0.12±0.01[b] | |
| **Thymus** | | | | | |
| 19 | 0.25 | 0.31 | 0.23 | 0.41 | 0.30±0.02[c] |
| 26 | 0.36 | 0.43 | 0.43 | 0.50 | 0.43±0.02[b] |
| 33 | 0.32 | 0.47 | 0.43 | 0.49 | 0.43±0.02[b] |
| 40 | 0.38 | 0.55 | 0.48 | 0.56 | 0.49±0.02[a] |
| Pooled | 0.32±0.02[c] | 0.44±0.02[b] | 0.40±0.02[b] | 0.49±0.02[a] | |

Means within a row or column having the same superscript are not significantly different ($P \leq .05$).

Table 11. Means and standard errors for body weight and relative bursa, spleen and thymus (per 100 g body weight) by age and population

| Age (days) | Populations | | | | |
|---|---|---|---|---|---|
| | HH | HL | LH | LL | Pooled |
| Body wt. | | | | | |
| 1 | 33 | 32 | 31 | 32 | 32 ± 1[b] |
| 7 | 44 | 46 | 47 | 44 | 45 ± 1[a] |
| Pooled | 38 ± 1[a] | 39 ± 2[a] | 39 ± 2[a] | 38 ± 2[a] | |
| Bursa | | | | | |
| 1 | 0.17 | 0.20 | 0.20 | 0.17 | 0.18±0.02[a] |
| 7 | 0.24 | 0.22 | 0.24 | 0.16 | 0.22±0.02[a] |
| Pooled | 0.20±0.02[a] | 0.21±0.02[a] | 0.22±0.02[a] | 0.16±0.02[a] | |
| Spleen | | | | | |
| 1 | 0.05 | 0.05 | 0.05 | 0.04 | 0.05±0.01[b] |
| 7 | 0.11 | 0.10 | 0.10 | 0.09 | 0.10±0.01[a] |
| Pooled | 0.08±0.01[a] | 0.08±0.01[a] | 0.08±0.01[a] | 0.06±0.01[a] | |
| Thymus | | | | | |
| 1 | 0.31 | 0.29 | 0.31 | 0.25 | 0.29±0.01[a] |
| 7 | 0.26 | 0.20 | 0.23 | 0.20 | 0.22±0.01[b] |
| Pooled | 0.28±0.02[a] | 0.25±0.02[a] | 0.27±0.02[a] | 0.23±0.02[a] | |

Means within a row or column having the same superscript are not significantly different ($P \leq .05$).

# GENERAL SYNTHESIS

This dissertation consists of a series of experiments conducted with White Leghorn chickens from lines selected for high and low antibody production to sheep erythrocyte antigen (SRBC) and reciprocal $F_1$ crosses between the lines. Experiments were designed to study (1) the effects of feeding varying levels of aflatoxin $B_1$ on growth and immunological traits, (2) the kinetics of primary and secondary antibody response to SRBC antigen, and (3) the ontogeny of production of antibody to SRBC antigen.

Regardless of line, aflatoxin $B_1$ depressed body weight, feed consumption, and feed conversion with the effect on the former occurring at lower dietary levels than that for feed conversion. Bursa, liver, spleen, and thymus weights were reduced when aflatoxin was fed. When adjusted for body weight, however, dietary aflatoxin increased liver weight, decreased bursa, and thymus weight, and had no effect on spleen weight. Dietary aflatoxin reduced surface temperatures, liver enzymes, and increased heterophil to lymphocyte ratios. Although such changes can influence resistance to infectious agents, there was no direct evidence that aflatoxin induced suppression for antibody to SRBC antigen.

Antibody response to 0.1 ml of 0.25% SRBC antigen indicated that selection for response at this dosage 5 days

post immunization had little, if any, association with the secondary response observed at this dosage. Correlations between peak initial and secondary responses were not significant. The dosage of SRBC antigen given in the initial immunization, however, influenced the response relationship among populations, and asymptotic values of primary and secondary responses which are 6 and 3 days post immunization, respectively.

Comparisons involving the ontogeny of antibody response to SRBC antigen showed differences among populations as early as 4 days postimmunization. Although chickens from the HH line produced significantly higher titers than those from LL and $F_1$ crosses, all populations reached their respective serological maturity at 14 days of age. There were differences among populations for frequency of responders by 7 days of age and the qualitative pattern persisted in the quantitative context of the antibody titers of the responders. Chickens from the high antibody line had significantly larger bursal and spleen weights and smaller thymal relative weights than those from the low line. This difference was present at 14 days of age and older. Relative to body weight, the spleen and bursa grew at a faster rate to 19 and 25 days of age, respectively, and then plateaued.

The above results provide a basis for future

experimentation. For example, differences have been observed among the antibody lines for development of immunity or resistance to Newcastle disease, Mycoplasma gallisepticum, Eimeria necatrix, splenomeglia virus, northern feather mite, Escherichia coli, and staphylococcus aureus (Gross et al., 1980). Also, it has been shown that aflatoxins influence susceptibility to various infectious agents (Hamilton and Harris, 1971; Boonchuvit and Hamilton, 1975). Lacking is experimentation with concurrent infection of various avian pathogens and aflatoxicosis in stocks known to differ in their resistance to these pathogens. Investigations designed to simulate field situations should use White Leghorn chickens to complement the extensive aflatoxin studies that have involved meat-type stocks. This point is cogent because our results suggest that White Leghorns may, in general, exhibit greater resistance than meat-type stocks to aflatoxins. Varying doses of dietary aflatoxin should be used to observe acute and subclinical avian aflatoxicosis complicated by common pathogens. The levels of aflatoxin $B_1$ used in Experiment I, as well as higher and lower levels, should be evaluated to determine their effect on reproduction. This reasoning is particularly cogent because of the dramatic effect noted on liver microsomal enzymes and the role of the liver in egg production.

Experiments on the kinetics of antibody production should provide information on both total circulating antibody as well as the types of immunoglobulins present, e.g., IgM and IgG to identify 2-mercaptoethanol sensitive or resistant immunoglobulins. Such data should be obtained from the parental lines and crosses between them using chicks primed with various dosages of SRBC antigens and given various booster doses. Lastly in future experiments with the antibody lines, information should be obtained on both humoral and cell-mediated immunity at different ages and under different environmental conditions. Integration of the results from such investigations could contribute important insights into the development of poultry breeding and production programs.

## SUMMARY AND CONCLUSIONS

This dissertation consisted of a series of experiments conducted with White Leghorn chickens from lines selected for high and low antibody production to sheep erythrocyte antigen (SRBC) and reciprocal $F_1$ crosses between the lines. Graded levels (0, 966, 1830, 2890 and 5697 ppb) of aflatoxin $B_1$ fed from 2 to 42 days of age depressed body weights, feed consumption, and feed conversion. Feed conversion, however, exhibited a less sensitive response to aflatoxin than either body weight or feed consumption. Levels of 1830 ppb and higher caused progressive decreases in surface but not core temperatures. Heterophilia and lymphopenia were not observed until the dietary level reached 5697 ppb. A major effect of dietary aflatoxin involved liver metabolism. Although no effect of aflatoxin for levels fed was observed in SRBC antibody production, the toxin reduced bursa and thymus weights both on an absolute and relative body weight basis.

The kinetics of initial and secondary antibody responses to SRBC antigen were measured in parental line and $F_1$ progeny. Chicks were immunized intravenously with 0.1 ml of 0.25% SRBC antigen at 35 days of age and a half of each group was given 0.1 ml of 0.25% SRBC booster 24 days later. In a second trial, chicks were immunized intravenously with 0.1 ml of either 0.025, 0.25 or 25% SRBC at 35 days of age

and reimmunized with 0.1 ml of 0.25% SRBC 24 days later. The results showed (1) correlations between initial and secondary antibody response peaks within populations and primary dosages were not different from zero, (2) dosage of the initial immunization influenced the secondary response, (3) peak titers occurred at about the same time for primary (by 6 days) and booster (by 3 days) regardless of population, and (4) differences among populations were observed as early as day 4 and persisted until day 24 post-primary immunization.

At 7 days of age, there were differences among populations for frequency of responders to SRBC antigen. Differences were also noted in antibody levels among those who responded. These observations demonstrate genetic variation for both the event and for subsequent levels of antibody production. Although line HH birds produced significantly higher titers than those from the other populations, all reached their respective serological maturity by 14 days of age inferring that selection for high and low antibody response has not modified this trait.

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# APPENDIX A

Appendix Table 1. Analyses of variance for liver, bursa, spleen, and thymus weights at 42 days of age, Experiment I

| Source of Variation | df | Absolute Weight | | | |
|---|---|---|---|---|---|
| | | Liver | Bursa | Spleen | Thymus |
| Line | 3 | 16.55** | 3.89** | 0.36 | 2.48** |
| Sex | 1 | 120.67** | 6.56** | 1.20** | 2.18** |
| Dose | 4 | 96.79** | 21.79** | 7.20** | 15.95** |
| L x S | 3 | 0.75 | 0.47 | 0.05 | 0.10 |
| L x D | 12 | 2.31 | 0.68** | 0.34* | 0.17 |
| S x D | 4 | 1.46 | 0.15 | 0.08 | 0.14 |
| L x S x D | 12 | 2.62 | 0.42 | 0.16 | 0.08 |
| Error | 356 | 2.94 | 0.30 | 0.16 | 0.13 |

*$P \leq .05$, **$P \leq .01$.

Appendix Table 2. Analyses of variance for liver, bursa, spleen, and thymus weights at 42 days of age, Experiment I

| Source of Variation | df | Organ wt./100 g body wt. Liver | Bursa | Spleen | Thymus |
|---|---|---|---|---|---|
| Line | 3 | 1.77** | 0.29** | 0.05** | 0.11** |
| Sex | 1 | 3.46** | 0.02 | 0.03 | 0.002 |
| Dose | 4 | 34.54** | 0.31** | 0.03 | 0.39** |
| L x S | 3 | 0.24 | 0.03 | 0.01 | 0.01 |
| L x D | 12 | 0.60* | 0.04** | 0.03** | 0.01 |
| S x D | 4 | 0.28 | 0.01 | 0.01 | 0.01 |
| L x S x D | 12 | 0.44 | 0.03 | 0.01 | 0.01 |
| Error | 356 | 0.28 | 0.02 | 0.01 | 0.01 |

*P ≤ .05, **P ≤ .01.

Appendix Table 3. Analyses of variance for heterophil (H), lymphocyte (L), heterophil/lymphocyte (H/L) ratio, and SRBC antibody titers, Experiment I

| Source of Variation | df | Heterophil | Lymphocyte | H/L[2] | SRBC antibody titers Day 5 | Day 13 |
|---|---|---|---|---|---|---|
| Line | 3 | 75.1 | 122.6 | 0.06 | 170.2 | 37.4 |
| Dose | 4 | 297.9** | 424.5* | 0.38** | 2.9 | 5.5 |
| L x D | 12 | 135.4 | 208.9 | 0.07 | 6.4 | 1.8 |
| Error[1] | 168 | 79.0 | 160.3 | 0.07 | 6.0 | 2.7 |

*$P \leq .05$, **$P \leq .01$.
[1]361 and 360 for days 5 and 13 SRBC antigen, respectively, for df.
[2]Analyzed using arc sin transformation.

Appendix Table 4. Analyses of variance for feed consumption and conversion at 42 days of age, Experiment I

| Source of Variation | df | Feed Consumption | Feed Conversion |
|---|---|---|---|
| Line | 3 | 2,913,436* | 0.01 |
| Sex | 1 | 16,708,148** | 0.02 |
| Dose | 4 | 82,695,085** | 0.06* |
| L x S | 3 | 831,900 | 0.04 |
| L x D | 12 | 1,121,029 | 0.02 |
| S x D | 4 | 654,207 | 0.03 |
| L x S x D | 12 | 769,587 | 0.01 |

*$P \leq .05$, **$P \leq .01$.

Appendix Table 5. Analyses of variance for surface and core temperatures at 42 days of age, Experiment I

| Source of Variation | df | Temperature Surface | Temperature Core |
|---|---|---|---|
| Day | 1 | 198** | 84.42** |
| Met. body wt. | 1 | 102** | 0.01 |
| Line | 3 | 12** | 0.78* |
| Sex | 1 | 31** | 0.34 |
| L x S | 3 | 10** | 0.80** |
| Dose | 4 | 80** | 0.43 |
| L x D | 12 | 5* | 0.54** |
| S x D | 4 | 18** | 0.38 |
| L x S x D | 12 | 9** | 0.74** |
| Error | 323 | 2.5 | 0.20 |

*P ≤ .05, **P ≤ .01.

Appendix Table 6. Analyses of variance for body weight (log transformations) at 14, 28, and 42 days of age, Experiment I

| Source of Variation | df | Days of age[1] 14 | 28 | 42 |
|---|---|---|---|---|
| Line | 3 | 9.56** | 11.11** | 13.29** |
| Sex | 1 | 28.04** | 57.88** | 87.23** |
| Dose | 4 | 19.37** | 136.37** | 229.73** |
| L x S | 3 | 0.43 | 1.65* | 0.51 |
| L x D | 12 | 0.39 | 1.08** | 0.39 |
| S x D | 4 | 1.14** | 0.86 | 1.08 |
| L x S x D | 12 | 0.64* | 2.00** | 1.18* |
| Error | 708 | 0.30 | 0.48 | 0.62 |

*$P \leq .05$, **$P \leq .01$.
[1]Values are $10^{-2}$.

Appendix Table 7. Analyses of variance for organ[1] and body weights for two age comparisons, Experiment II

| Source of Variation | df | Body wt. | Spleen[2] | Spleen[3] | Thymus[2] | Thymus[3] | Bursa[2] | Bursa[3] |
|---|---|---|---|---|---|---|---|---|
| | | 0-7 day organ and body weights | | | | | | |
| Line | 3 | 10 | .019 | 0.11 | 0.20 | 1.36 | 0.28* | 1.39 |
| Age | 1 | 3432** | 1.68** | 4.90** | 0.09 | 9.99** | 3.12** | 1.76 |
| L x A | 3 | 26 | 0.004 | 0.01 | 0.08 | 0.13 | 0.15 | 0.53 |
| Error | 72 | 19 | 0.011 | 0.06 | 0.09 | 0.60 | 0.08 | 0.54 |
| | | 14-40 day organ and body weights | | | | | | |
| Line | 3 | 16773** | 35.51** | 0.41** | 1.90** | 0.18** | 1.38** | 0.08** |
| Age | 3 | 530413** | 1.06 | 1.88** | 16.35** | 0.26** | 15.47** | 0.44** |
| L x A | 9 | 3530 | 2.78 | 0.04** | 1.03** | 0.01 | 0.40** | 0.02 |
| Error | 140 | 2048 | 4.47 | 0.02 | 0.14 | 0.01 | 0.15 | 0.01 |

*P ≤ .05, **P ≤ .01.
[1]Values are x $10^{-2}$.
[2]Analysis based on absolute weight.
[3]Analysis based on organ weight per 100 g body weight.

# APPENDIX B

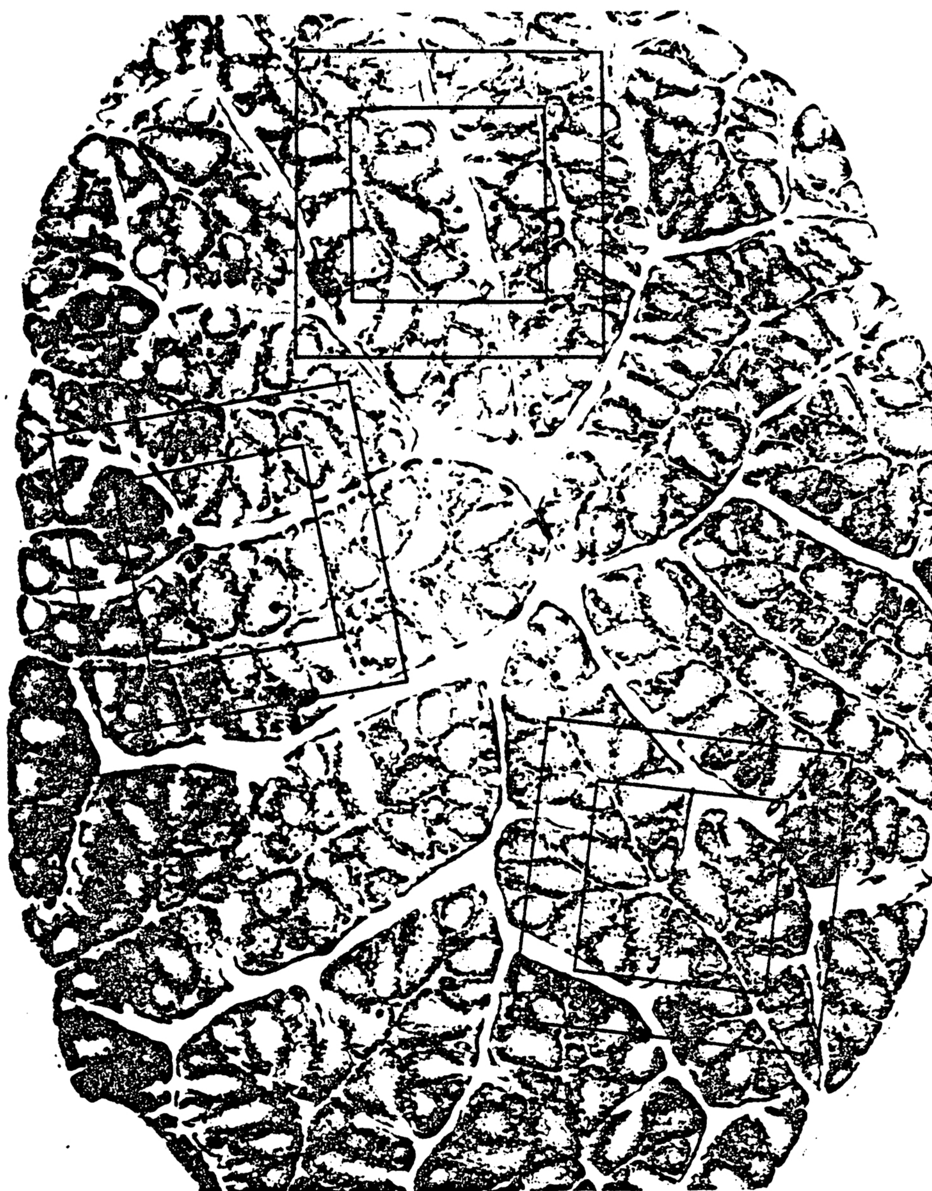

Plate 1. Photomicrograph (35% reduction) of bursa of Fabricius from HH line birds taken at 21 days of age. The numbers and areas of the bursal lymphoid follicles were determined in 3 grid positions 37 x.

Plate 2. Photomicrograph (35% reduction) of bursa of Fabricius from LL line birds taken at 21 days of age. The numbers and areas of the bursal lymphoid follicles were determined in 3 grid positions 37 x.

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# THE ONTOGENY OF THE IMMUNE RESPONSE TO SHEEP ERYTHROCYTES AND RESISTANCE TO AFLATOXINS IN CHICKENS

by

Charles Obidigbo Ubosi

(ABSTRACT)

Experiments were conducted to study the ontogeny, kinetics, and the influence of aflatoxin $B_1$ on antibody response to sheep erythrocyte (SRBC) antigen in White Leghorn chickens. In the first experiment, chickens from the parental lines and reciprocal crosses between them were fed diets containing graded levels, from 0 to 5697 ppb of aflatoxin $B_1$. Aflatoxin depressed body weights, feed consumption and feed conversion, with feed conversion being depressed less than either body weight or feed consumption. Although there were no differences among aflatoxin levels for body core temperatures, levels of 1830 ppb and higher caused progressive decreases in surface temperatures. Heterophilia, lymphopenia and reduced liver metabolism were observed at the 5697 ppb level. Although bursa and thymus weights were smaller in the aflatoxin-fed birds, there was no reduction in their SRBC antibody levels.

The second experiment was designed to measure primary and secondary antibody response to intravenous immunization of SRBC antigen. Treatments included immunization at the

dosage of SRBC antigen under which selection was practiced, and higher and lower concentrations. Although the dosage of primary immunization influenced the magnitude of the secondary response within population-primary dosage correlations between peak primary and secondary antibody response were not different from zero. Differences among populations in antibody levels appeared as early as day 4 and persisted until day 24 post-primary immunization. Yet, the general response patterns were the same for all populations with respective peaks occurring at the same time.

The ontogeny of post-hatching production of antibody SRBC antigen and growth of bursa, thymus and spleen were measured in the third experiment. Both parental lines and reciprocal crosses between them reached serological maturity by 14 days of age. By 7 days, there were differences among populations for frequency of responders to SRBC antigen and magnitude of titers, inferring genetic variation for both the event and subsequent levels of antibody production.