MYCOTOXIN EFFECTS APPARENT WITH CHOLESTERYL TRANSFORMATIONS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

The mycotoxins, termed aflatoxins, are a group of secondary fungal metabolites that were discovered as contaminants of certain lots of animal feeds. These compounds have a high order of acute toxicity to many animal species and have also been shown to possess carcinogenic properties. Their discovery in agricultural commodities and the subsequent demonstration of various biological effects resulting from ingestion of contaminated diets by animals have emphasized the potential hazards which might arise from contamination of the food supply by mycotoxic pollution. These factors have stimulated considerable research activity dealing with many aspects of aflatoxins and other toxic mold metabolites.

Toxicity syndromes of domestic animals ultimately attributed to aflatoxins were first reported in England in 1960 and 1961. The first report of the syndrome in young turkeys was followed within a short time by similar incidents in ducklings and chickens, as well as swine and calves. The common factor in these episodes was a shipment of Brazillian peanut meal used in the animal's rations. It was found that the active ingredient could be extracted from the toxic meal, and this extract reproduced the toxicity in susceptible species. It was later realized that the toxic compound was produced by certain strains of Aspergillus flavus. The name aflatoxin was thus applied to

the group of toxic compounds produced by this fungus. More recently it has been found that aflatoxin may be produced by many fungal species. The toxic properties of the aflatoxins differ, depending upon the test system, dose, and duration of exposure.

Early experimental studies have indicated that the duckling was a species highly susceptible to acute poisoning. When aflatoxin is administered to the duckling at sublethal levels, the result is moderate to severe liver damage. When this is continued over a long period of time, the liver damage may become carcinogenic. Studies have shown this may influence some imbalance of cholesterol and its esters within the lipid fraction of the duckling liver.

The objective of this study was to characterize hepatic cholesteryl liquid crystal transformations as influenced by aflatoxin intake and day-age weight gain of White Pekin ducklings.

CHAPTER II

LITERATURE REVIEW

The aflatoxins represent a group of secondary fungal metabolites that have shown a high order of acute toxicity to many animal species, and have been known to possess potent carcinogenic properties to several animal species (23,38). These metabolites are produced primarily by Aspergillus species common to soil microflora, and to a lesser extent, other species of microorganisms (22,23). Toxicity syndromes of domestic animals caused by aflatoxin were first reported in England in 1961 (7). More than 100,000 turkey poults as well as other species of domestic animals died within a few months from an unknown cause that was then termed "turkey X disease". The factor common to this disease was a shipment of Brazillian peanut meal used in the animal rations. It was found that a substance could be extracted from this peanut meal and this substance reproduced the toxic symptoms in domestic animal species (33). The high toxicity was associated with heavy mold infestion of the peanut meal and Sargeant (33) demonstrated that the toxic compounds were produced by certain strains of Aspergillus flavus isolated from such meals. The generic name "aflatoxin" was subsequently applied to the group of toxic compounds produced by this fungus (26,39).

Isolation of the aflatoxins was greatly facilitated by the discovery that they were strongly fluorescent in long wave ultraviolet light. Due to this property the aflatoxin can be monitored in isolation and puri-

fication procedures, as well as quantitated (39). When the extract is spotted on silica gel thin layer plates and developed in chloroform: methanol (97:3), a series of components are separated. The major common aflatoxins comprise four of these components. Two emit blue visible light and were therefore named aflatoxins B_1 and B_2. Two fluorescent yellow-green light and were named aflatoxins G_1 and G_2. On silica gel plates developed in chloroform:methanol, (97:3), aflatoxin B_1 migrates with an R_f in the order of 0.56; B_2, 0.53; G_1, 0.48; and G_2, 0.46 (39). The amounts and relative proportions of these four components present in culture extracts are variable, depending upon factors such as mold strain, medium composition, and culture conditions. Aflatoxins B_2 and G_2 are usually present in small relative amounts, whereas B_1 usually presents the largest yield (39).

Structures based largely on the interpretation of spectral data were proposed for aflatoxins B_1 and G_1 in 1963 (4,5) and for aflatoxins B_2 and G_2 shortly thereafter (14). These structures are shown in Figure 1. The proposed structure of G_1 has been supported by x-ray crystallography (15).

A rapid, simple, and sensitive method for extraction of aflatoxins from cottonseed products was proposed by Pons and Goldblatt (32). An acetone:water solvent (70:30) was used to yield extracts essentially free of lipids, followed by treatment of the extract with lead acetate to remove gossypol pigments as insoluble lead derivatives. The aflatoxins were then transferred into chloroform and were determined by thin layer chromatography (TLC) on silica gel. Estimation of the aflatoxins at levels as low as 1 ppb (ug/kg) in cottonseed meats, and 4 ppb in meals was possible with this method. Later Pons (31) added a

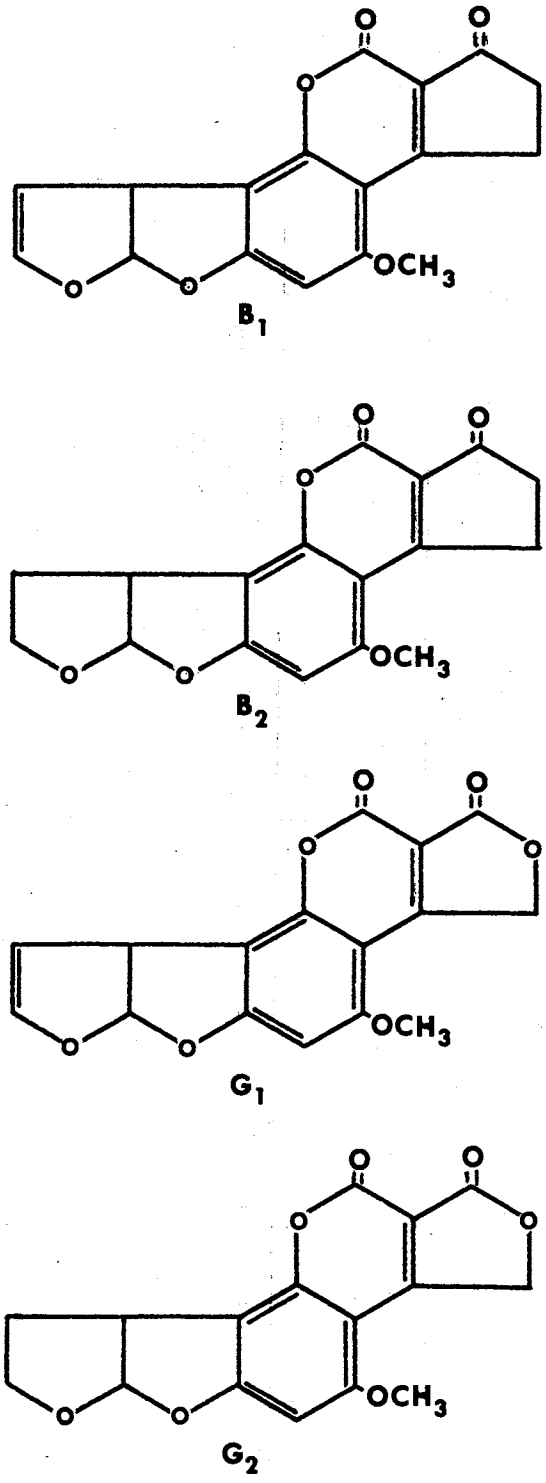


Figure 1. The Structures of Aflatoxins B_1, B_2, G_1, and G_2.

further cleanup step to the procedure to remove most of the interferences. When the final chloroform extract from the original procedure was absorbed on a small silica gel column and washed with diethyl ether, extraneous fluorescent materials and streaking components were removed while the aflatoxins remained on the silica gel column. Subsequent elution with methanol:chloroform (3:97) quantitatively removed aflatoxins and produced extracts for thin layer chromatography which were substantially lower in total solids and residual pigmentation (31). Polaroid photos in ultraviolet light can then be used to permanently record the TLC quantitation (13).

The discovery of the aflatoxins as possible contaminants of animal feeds, and the potential health hazards involved, have stimulated much research concerned with the effect of aflatoxin in various biological test systems. The toxic properties of the aflatoxins depend on the test system, dose, and duration of exposure (39), thus they have been shown to be lethal to animals and animal cells in culture when administered acutely in sufficiently large doses, and to cause histological changes in animals when subacute doses were administered. Chronic exposure for extended periods has resulted in induction of tumors in several animal species (11,21,29,39).

The known pathology and toxicology of aflatoxin poisoning in animals has been extensively reviewed (1,2,6,21,39). Hepatomas have been induced with low aflatoxin dose levels in poultry, cattle, swine, trout, and rodents. Although there is some species variation in susceptibility, the LD_{50} for a single dose of aflatoxin B_1 is in the range of 0.5-1.0 mg/kg body weight for most experimental animals.

Domestic animal species, such as cattle, swine, turkeys, chickens,

and ducks, consuming sublethal amounts of aflatoxin for several days develop a toxic syndrome in which liver damage is the most significant change. Cumulative toxic amounts vary from 0.3 to 15 ppm.

Toxic amounts of aflatoxin B_1 (0.1 mg/kg body weight) incubated daily for five consecutive days induced a bright yellow color in the liver tissue in two to four days in day old ducks (3). A concentration of 1.0 mg/kg of body weight in the ration induced a marked increase in yellowness in this two to four day period. On the other hand, livers of control ducks, tan in color at hatching, became a normal red color in seven to ten days (3).

Armbrecht (3) suggested use of the duckling as a biological assay for aflatoxin. The amount of liver damage was used to quantitate aflatoxin damage. The liver damage, which is a degenerative and regenerative process, fans out from portal areas and is termed "bile duct proliferation" and was graded subjectively as observed on stained sections.

Aflatoxicoses results in swelling, increased firmness in texture, petechial hemorrages, and fatty infiltration of the liver. The livers of ducks that survive two weeks have a yellow ochre color, but livers of ducks previously exposed quickly return to normal color and texture upon feeding a toxin free diet (35). In studies of acute aflatoxicoses in day-ol ducks, the liver becomes pale and autrophic; microscopically there is widespread parenchymal cell necrosis and marked bile duct hyperplasia. Ducks given aflatoxin B_1 for seven to thirty days had nodular hyperplasia of parenchyma cells and massive bile duct hyperplasia (35).

The lesion induced by a single dose of aflatoxin was described by Butler (11). A biliary proliferation rapidly developed reading at

a maximum at three days, which then slowly regressed so that by fourteen days only a slight residual proliferation was present. Some periportal zone necrosis was present by 24-48 hours with the formation of lakes of fat. At fourteen days many mitoses were present in the parenchymal cells, but the birds at this time were growing rapidly (11).

At lower dosage, although the lesion was less severe, there being a poor correlation between the dose and degree of biliary proliferation, and there was great variation in the responses of the animals within groups. At higher dose levels, often a hemorrhagic periportal necrosis was induced. This was studied at ultrastructure level by Theron (38) who suggested that the initial lesion involved the endothelium, however, the dose used was considerably greater than the LD_{50} and resulted in a hemorrhagic necrosis. At LD_{50} doses, no such hemorrhage is seen.

The acute LD_{50} has been estimated by various workers with considerable agreement. Nesbitt (29) estimated the LD_{50} for aflatoxin B_1 to be about 20 ug. per 50 gram duckling. Asaeo (5) estimated it to be 28.2 ug. B_1 per duckling (50 g.) using propylene glycol as solvent in a two day trial; Carnaghan (12), B_1 18.2 ug., G_1 39.2 ug., B_2 84.8 ug., and G_2 172.5 ug., using dimethylformamide as a solvent in a six day trial; Lijensky and Butler (25), B_1 17.5 ug. (0.335 mg/kg) and G_1,45.7 ug. (0.785 mg/kg, with dimethylformamide as solvent in a six day trial).

In a time versus liver damage study (3), observations were made at 1, 2, 4, 8, 16, and 32 days on pairs of survivors receiving 1 mg. of aflatoxin per kilogram. At the end of each period, the respective

groups of birds were sacrificed, and organs and tissues were examined. In the study of minimum liver bile duct proliferation following a single dose of aflatoxin, ten birds were used at each of the two levels, 0.1 and 0.01 mg/kg. Half the birds were killed and autopsied two days after treatment, and the remaining birds were killed and autopsied on the fourth day. These days were selected because previous studies indicated the liver bile duct proliferation was maximal on these days.

The most pronounced macroscopic change observed in the ducklings is the yellow color of the liver developed within one to two days after dosing. Inasmuch as the duckling liver is yellow and fatty at hatching, a comparison of a test bird liver with a control bird of the same age was used to interpret the difference in surface color and hue. The color of the liver of control birds passes through a series of intermediate hues during a seven to ten day period to the usual red liver color. If microscopic observations are made on hematoxylin and eosin stained sections, the color changes will not interfere with the interpretation of aflatoxin poisoning.

When a duckling liver thin section is placed in cross polarized light and viewed with a microscope, a series of crystals can be seen around the area of liver damage. These crystals have the characteristics of liquid crystals, a relatively new designation to describe a particular state of matter (27).

The liquid crystalline phase is distinguished from both the solid and liquid phases of matter by first-order phase transitions. It mixes the properties of both the liquid and solid forms and is intermediate between the two (10). This combination of properties yields new characteristics which are found in neither liquids nor solids.

Other unusual behaviors include: (1) formation of monocrystals with application of normal magnetic and electric fields; (2) optical activity in twisted nemantic (or cholesteric) liquid crystals of a magnitude without parallel in either the solid or liquid regime; and (3) sensitivity to temperature which results in color changes.

The first description of what was obviously a mesophase came from a biologist, an Austrian botanist, Reinitzer (8,17) who, in 1888, observed a transition from a solid to a turbid and then a clear fluid in cholesterol benzoate heated from 145 C. to 179 C. It was at least a year later that Lehman (24) described the appearance of the turbid phase and invented the term liquid crystal.

The presence of liquid crystals in biological materials had been suggested as a result of incidental findings in studies of extracted materials (18,28,30,16). A definite claim that liquid crystals entered into the structure of living cells and tissues was made in 1959 (36), when it was shown that complex lipids present in the adrenal cortex, ovaries, myelin, and also in atheromatous arteries existed at body temperature in a characteristic mesophase. This observation arose mainly out of the examination of fresh tissue, for conventional methods of processing tissue for examination, depending as they do upon coagulative fixation, dehydration, organic solvents and other manipulations, usually destroy all the features of a mesophase and probably many other important features as well. This may be why the mesophase had never been identified in classical histology (36).

The renaissance in liquid crystal research in recent years had its beginning with the publication of a review of the field by Brown and Shaw (10) in 1957. International conferences in 1965, 1968, and 1970

have accelerated research. The use of cholesteric liquid crystals as temperature indicators and the discovery of room temperature nematic liquid crystals have catalyzed research. The number of researchers in the field has expanded rapidly in the last ten years, making liquid crystals one of much interest among interdisciplinary research fields (9).

There are two categories of liquid crystals: thermotropic and lyotropic. Thermotropic liquid crystals are prepared by heating certain organic or organometallic compounds. Lyotropic liquid crystals are prepared by mixing two or more components, one of which generally has rather polar molecules. The other component may be an organic or an inorganic compound. The possibilities of preparing a large number of lyotropic liquid crystalline systems are very great; they are found in both animate and inanimate matter. Thermotropic and lyotropic liquid crystals exhibit polymorphism, that is, more than one kind of liquid crystalline phase exists (9,17,19).

The two classes of liquid crystals are smectic and nematic. The term nematic, coined by Friedel from the Greek word meaning thread, describes the threadlike lines that are seen under the microscope. In nematic liquids the only structural restriction is that molecules maintain a parallel or nearly parallel arrangement to each other. The molecules are mobile in three directions and can rotate about one axis. The word smectic, also proposed by Freidel, comes from the Greek word meaning grease or slime. The smectic structure is stratified, with the molecules arranged in layers, their long axis parallel to each other in layers and approximately normal to the plane of layers. The molecules can move in two directions in the plane, and they can rotate about one

axis. Within the layers they can be arranged either in neat rows or randomly distributed. The layers can slide, without much hinderance, over neighboring layers.

Liquid crystals are birefringent, a unique property for one component liquids (8,17,20). Microscopic observations using crossed polarizers show that smectic liquids behave like unaxial crystals, such as calcite. As a consequence of the molecular array, smectic liquids exhibit positive birefringence, ie. the ordinary ray has the lower refractive index. The nematic liquid crystal is also optically positive. The optical axis of the nematic liquid crystal coincides with the preferred direction of the long axis of the molecule. The bulk sample of the nematic liquid crystal is not optically active, but if it is placed between glass surfaces and one surface is rotated slightly, the deformation of the structure by adhesion to the glass surface may result in an optically active system.

In the cholesteric phase, linearly polarized light transmitted perpendicular to the molecular layers will have the direction of its electrical vector rotated progressively along the helical path. The plane of polarization of the light will be rotated through an angle proportional to the thickness of the transmitting material. Such materials are optically active. Cholesteric liquids rotate polarized light to a very large degree (8,17).

The cholesteric phase also exhibits dichroism; ie. one of the polarized components of ordinary light is selectively reflected in the medium much more strongly than the others (37). Depending upon the material, one of the components is transmitted and the other is reflected. It is this property that gives the cholesteric phase its

characteristic iridescent color when it is illuminated by white light (17). The colors exhibited are dependent upon the temperature, the material, the angle of incidence of the light rays, and the angle of observation of the reflected light.

The molecular structure of a cholesteric liquid crystal is very delicately balanced (17) and can be easily upset. Thus, any small disturbance that interferes with the weak forces between the molecules can produce marked changes in such optical properties as reflection, transmission, birefringence, circular dichroism, optical activity, and color.

The presence of lipid, protein, and other substances in various forms of lyotropic mesophases explains many of the properties which distinguish protoplasm from inanimate colloids, or even in more general terms, some of the essential differences between the physical structure of living and non-living matter. In the living cell or tissue, the essential components invariably have a functional as well as structural role, even if they are relatively rigid. Most biochemical reactions proceed normally in liquid phases and, intravitally, are mediated by cell membranes, tissue interfaces, and subcellular particles or organelles such as lyosomes and mitochondria. The existance of the mesophase in these structures offers an explanation of how structure and position can be maintained in the liquid state and, more important, how a preferred orientation of reactive or structurally vital molecules can be maintained in the liquid state (36). Experimental models of biologic mesoforms are optically stable within a limited thermal range of 4 - 42 C., and it seems no accident that this range represents the usual limits of metabolism and growth in all forms of life (9).

As recent as 1964, Fergason (17) said that the function of liquid crystals in living systems is largely unexplored territory. He further stated that so far liquid crystal substances containing cholesterol have not been found in a living animal, but the evidence strongly indicates that such a discovery is in the offing. Brown in 1972 (9) stated that current publications on liquid crystals tend to discuss the biological aspect as chaotic (23), or altogether missing (17). This is surely untrue in the light of evidence obtained. Liquid crystals are not only present in tissue; they would appear to play a singularly important role in that their structure is part of biochemical functions and reactivity, and in at least one major degenerative disease. Slight changes in liquid cyrstal composition and in physical and chemical properties can materially affect the formation, continuation, or cessation of the liquid crystalline state, a delicate balance which is also characteristic of many living processes. A model cell using nematic and smectic liquid crystals has been proposed by Brown and Fergason (8), and its known characteristics have been correlated with the structure and properties of a living cell. The model can explain many functions of cells, including transfer of molecules and ions through their membranes, and the division of cells. Liquid crystals can operate at energy levels limited by thermal fluctuations in the system. Thus, they are good temperature sensors, and one might expect to find them in sensory systems involving sight, smell and taste (36).

Scientists now recognize liquid crystals as real and consider them a legitimate research field. We can expect many worthwhile, maybe startling, developments from studies of this state of matter.

It is not beyond reason to expect that developments in liquid crystals may be as dramatic as those in solid-state research in the last twenty years (9). Their role in living systems will be better understood and, once this is accomplished, more will be known about their function and influence in a living system.

CHAPTER III

MATERIALS AND METHODS

Hile Mammoth White Pekin ducklings used for these experiments were obtained from the Hile Duck Hatchery in Carey, Ohio by one-day air parcel post. These ducklings are well suited to bioassay studies due to their very rapid development and consistent uniform response during the first ten days of age. The newly hatched ducklings were weighed, marked for identification, and randomly assigned to battery pens using five birds per pen, measuring 24" by 29" (61 cm. by 74 cm.). Duckling bioassay reactions were basically the standard formulation for aflatoxin evaluations set by the World Health Organization, with 60% peanut meal which included the aflatoxin addition, 10% casein, 20% sucrose, 5% corn oil, 2% Phillips-Hart mineral salt mixture, and 3% vitamin mixture. High aflatoxin peanut meal was prepared by inoculating ground peanut meal with a spore suspension of Aspergillus parasiticus, Speare (orginally Austwicks Uganda strain 3734/10). The inoculated peanut meal was then incubated at saturated humidity and 30° C. for 96 hours in a Stults model S-30 germinator. The high toxin meal was added to toxin free meal to obtain the appropriate aflatoxin levels. Total aflatoxin levels were 0.988 ug/gram ration and 3.017 ug/gram ration for the low level and high level experiments, respectively. In the low level series, the 0.988 ug. total aflatoxin consisted of 0.593 ug. B_1 and 0.395 ug. G_1 per gram of

ration. For the high level series, the 3.017 ug. contained 1.192 ug. B_1 and 1.825 ug. G_1 per gram of ration. The aflatoxin content was determined using a modification of the extraction method of Pons and Goldblatt (32), and ultraviolet polaroid recordings were used for reference and quantitation (13).

A control group of 25 ducklings were fed a toxin-free diet and 25 ducklings were fed the aflatoxin peanut meal. Also ducklings received in shipment but not used in the experiment (approximately 6-10 ducklings per series) were killed and examined for hepatic cholesterol ester composition at "day zero".

Within the low level experiment, five ducklings were removed and posted at days 2, 4, 6, 8, and 10. For the high level series, three ducklings were removed at each of these days, and the remaining ducklings were moved to an outdoor pen for a long-term study of carcinogenic aflatoxin effects.

A result of aflatoxin ingestion by the duckling is an induced liver damage in various stages. The relative extent of hepatic degeneration injury is utilized as a measure of aflatoxin effects to the duckling. The condition of the liver greatly influences the metabolic transformations of cholesterol esters normally present in hepatic tissue.

At posting of the duckling, the liver was removed intact and weighed. Munsell color ratings were assigned each liver and samples were removed for histological sections. The liver was processed through paraffin, sectioned, and stained with hematoxylin-eosin. A slide was also prepared unstained in paraffin.

Approximately one gram of lyophilized duckling liver tissue was

extracted with petroleum ether three times, then brought to a standard concentration of 10 mg. of extract per ml. of petroleum ether.

Preliminary separation and identification of the petroleum ether extract of lyophilized duckling livers was by thin layer chromatography (TLC), using non-fluorescent silica gel plates. These separations were to distinguish between cholesterol esters, free cholesterol, and fatty acids. The TLC plates were spotted with the liver extract and developed in a solution of isooctane:benzene:acetic acid (65:35:1). The lipid components were then visualized by spraying with a 1% solution of anisaldehyde followed by a 50% sulfuric acid spray. Charring was completed by placing the TLC in an oven at 85^{o} C. for 15 minutes. The components were then visible under long wave ultraviolet light of 640-660 mu. Cholesterol esters are shown as the upper spots with free cholesterol and fatty acids in the lower segment of the TLC. The saturated cholesterol esters (palmitate and stearate) were difficult to separate by thin layer chromatography, so 40 mg. of the extract was methylated and the cholesteryl esters were determined by gas chromatography using a Pekin-Elmer 801 gas chromatograph with a hydrogen flame detector. The gas chromatography also verified other cholesteryl esters previously identified by thin layer chromatography. The individual cholesteryl esters were expressed as percent of petroleum ether extract.

Cholesterol and total cholesteryl esters in the petroleum ether extract of duckling liver were determined by direct screening ferric chloride procedure. In this procedure, the cholesterol and cholesteryl esters are extracted, and proteins are precipitated with acetic acid

containing ferric chloride. The extract when treated with sulfuric acid develops a purple color, and its optical density is read on a colorimeter at 625 nm. Percent cholesteryl esters were determined by approximately the same method, except that free cholesterol was precipitated with digitonin and the cholesteryl esters were extracted with petroleum ether.

The remaining petroleum ether extract was then placed on slides and allowed to crystallize to observe liquid crystal development and to determine visage values for liquid crystal growth. Corresponding photos were obtained to record the structure and extent of liquid crystal development.

Statistical analysis for treatment F values and coefficients of variation were determined using the SAS program (34) for computerized analysis of variance with the IBM 360 Mod 50 computer. Response trends as lines of best fit were determined for each treatment and series for linear, quadratic, cubic, and quartic trends. The predicted values were computed and response of best fit of predicted values were then plotted for highest significant F value trends.

CHAPTER IV

RESULTS AND DISCUSSION

All experiments were conducted in the soil microbiology laboratory at Oklahoma State University. Ducklings were kept in a 10 compartment poultry battery, and water and the experimental rations were made available to the ducklings at all times. The parameters measured in this study are summarized in Tables I and II. These two studies, expressed as high and low toxin series, were used to evaluate the effects of high toxin (3.017 ug/gm feed), low toxin (0.988 ug/gm feed), and zero toxin levels on these birds. The low toxin series included 50 birds, while the high toxin series utilized 30 birds as principals in these studies. Percent coefficients of variation for the high and low toxin series are shown in Tables III and IV. The individual coefficients of variation are for the ducklings within single pen treatments. The correlation from cross products analysis is illustrated in Table V. This relates the correlation between measured parameters and liquid crystalline visage of the petroleum ether extract from duckling livers.

The most obvious effect of aflatoxin injection by ducklings was the difference in weight gain of toxin free and aflatoxin fed birds (Figure 2). The weight increase of toxin free birds was near exponential, with weight increasing from near 50 grams at day zero, to near 350 grams at ten days of age. Weight of toxin fed birds with the low

TABLE I

EFFECT OF DAY-AGE AND TOXIN INTAKE ON GROWTH AND CHARACTERISTICS OF HEPATIC LIQUID CRYSTALLINE COMPONENTS*

LOW TOXIN SERIES											
Day Age	Gain	Liver	Pet Eth Extract	Chole-sterol	Chol ester	Cholesteryl Ester Composition					Visage
Treatment	Grams	%	%	%	%	Palm %	Stearic %	Oleic %	Linol %	Arach %	Rating
2 N	49.52	5.43	41.22	1.75	13.01	24.30	11.38	45.66	14.82	3.18	0.6
2 T	31.14	3.87	34.96	0.80	27.38	12.56	12.54	44.38	21.76	7.82	8.4
4 N	79.00	4.32	25.96	1.65	8.43	17.60	16.24	39.58	13.90	10.80	2.6
4 T	55.20	3.51	28.19	0.50	11.94	13.92	17.82	35.80	21.48	10.62	2.6
6 N	138.98	4.56	22.12	1.35	2.36	19.62	15.28	31.18	16.68	15.02	4.2
6 T	82.22	4.16	33.65	1.05	5.43	16.06	15.26	36.78	26.44	7.48	1.6
8 N	236.70	3.86	11.84	1.10	11.18	18.20	17.66	29.34	17.10	17.74	3.8
8 T	125.14	4.92	33.00	0.65	7.75	16.14	9.16	37.54	29.52	7.68	2.2
10 N	273.38	4.01	11.72	0.92	12.32	24.32	18.42	25.94	18.24	13.04	4.4
10 T	153.94	4.24	26.18	0.76	9.80	16.42	15.10	33.52	25.00	9.94	2.4
Age-Trt F value	5.38	5.22	4.08	0.61	8.78	4.06	4.88	2.68	1.61	5.77	29.32
P>F	.0018	.0021	.0074	.6622	.0001	.0076	.0030	.0445	.1899	.0012	.0001

*Each value is the mean from 5 ducklings within each day-age-toxin treatment. Low aflatoxin diet level was 0.988 ug/g feed (0.593 ug B_1 and 0.395 ug G_1). Liquid crystalline cholesteryl visage rated as: 10, most iridescent brilliance with cross-polarized microscopy, to 1, least apparent anisotrophism.

TABLE II

EFFECT OF DAY-AGE AND TOXIN INTAKE ON GROWTH AND CHARACTERISTICS OF HEPATIC LIQUID CRYSTALLINE COMPONENTS*

	HIGH TOXIN SERIES										
Day Age	Gain	Liver	Pet Eth Extract	Chole-sterol	Chol ester	Cholesteryl Ester Composition					Visage
Treatment	grams	%	%	%	%	Palm %	Stearic %	Oleic %	Linol %	Arach %	Rating
2 N	30.27	5.28	47.09	1.35	17.41	25.13	10.10	45.63	16.13	3.20	6.3
2 T	11.33	3.70	56.97	1.87	22.33	8.37	8.50	60.10	15.47	7.53	8.7
4 N	68.47	6.67	45.29	0.89	7.48	24.37	9.83	45.97	16.40	3.47	2.7
4 T	18.60	4.25	36.92	2.12	29.15	12.47	10.23	49.47	21.83	6.03	7.0
6 N	118.20	4.80	30.99	1.36	6.94	22.40	13.07	43.07	16.07	5.40	1.3
6 T	25.47	3.32	53.78	1.82	17.34	14.00	13.70	47.07	21.97	3.27	3.3
8 N	212.90	4.47	17.89	1.28	8.75	21.33	15.17	38.30	16.47	8.77	3.3
8 T	40.17	3.70	40.33	1.64	19.62	14.80	12.53	41.33	25.87	5.43	2.7
10 N	303.97	3.94	10.43	2.14	9.62	21.87	16.70	38.57	16.83	6.03	5.7
10 T	31.03	3.23	48.28	1.36	11.67	12.56	11.87	45.50	27.70	2.40	2.7
Age-Trt F value	27.96	1.68	4.36	2.19	3.06	2.69	5.13	3.48	9.11	2.50	3.41
P>F	.0001	.1932	.0107	.1061	.0401	.0602	.0054	.0256	.0004	.0749	.0278

*Each value is the mean from 3 ducklings within each day-age-treatment. High aflatoxin diet level was 3.017 ug/g feed(1.192 ug B_1 and 1.825 ug G_1). Liquid crystalline cholesteryl visage rated as: 10, most iridescent brilliance with cross polarized microscopy, to 1, least apparent anisotropism.

TABLE III

PERCENT COEFFICIENTS OF VARIATION OF MEASURED PARAMETERS BY DAY-AGE TREATMENT*

LOW TOXIN SERIES

Day Age Treatment	Gain grams	Liver %	Pet Eth Extract %	Chole-sterol %	Chol ester %	Cholesteryl Ester Composition Palm %	Stearic %	Oleic %	Linol %	Arach %	Visage Rating
2 N	10.51	10.04	15.21	73.54	40.18	12.44	16.18	7.16	15.09	37.82	91.29
2 T	9.03	8.99	3.10	46.35	9.51	25.62	21.22	10.50	15.45	25.51	18.05
4 N	10.02	7.81	32.56	99.70	50.69	31.66	9.21	23.70	20.17	39.26	58.33
4 T	28.12	5.56	25.58	35.36	44.42	30.64	24.60	14.70	23.74	34.44	64.36
6 N	16.04	10.02	29.64	51.74	36.88	8.28	20.42	21.05	17.35	32.98	26.08
6 T	27.35	42.66	18.90	45.80	63.48	6.00	19.79	10.42	11.30	44.00	83.85
8 N	10.21	5.42	41.04	101.19	13.25	3.71	13.06	18.74	11.18	25.79	11.79
8 T	14.44	16.49	52.87	43.52	21.38	19.96	55.38	9.30	4.11	69.33	74.69
10 N	29.19	6.61	28.78	98.67	52.36	15.07	11.30	12.08	14.16	21.76	12.45
10 T	28.38	6.19	42.01	48.67	20.02	5.37	13.53	12.83	12.60	43.07	55.90

*Percent coefficient of variation values were derived from measured parameters and day age of 50 ducklings within the low toxin series.

TABLE IV

PERCENT COEFFICIENTS OF VARIATION OF MEASURED PARAMETERS BY DAY-AGE TREATMENT*

HIGH TOXIN SERIES

Day Age Treatment	Gain grams	Liver %	Pet Eth Extract %	Chole-sterol %	Chol ester %	Cholesteryl Ester Composition Palm %	Stearic %	Oleic %	Linol %	Arach %	Visage Rating
2 N	8.04	13.50	15.53	38.62	25.89	22.72	11.16	9.53	10.78	54.49	9.12
2 T	11.75	11.95	2.56	15.16	19.09	39.65	5.88	5.21	5.19	5.36	6.66
4 N	9.48	16.44	16.41	35.90	9.67	8.15	10.97	4.69	9.81	40.01	21.65
4 T	18.66	16.24	31.12	58.12	37.06	34.37	14.70	7.13	14.49	25.91	14.29
6 N	35.25	14.56	46.80	28.26	18.22	7.31	5.80	0.48	8.27	42.07	43.30
6 T	9.07	4.87	4.86	11.63	8.24	5.00	3.34	0.68	2.96	12.37	69.28
8 N	19.86	4.47	55.32	29.00	12.36	10.57	8.71	14.10	13.38	51.44	96.44
8 T	45.86	12.68	25.92	6.73	38.13	8.86	16.40	8.50	5.90	25.79	114.56
10 N	12.18	10.11	44.17	48.24	19.32	14.92	6.25	8.30	11.65	110.60	36.74
10 T	54.59	31.65	37.99	31.92	64.95	7.97	12.51	0.79	6.63	23.20	78.06

*Percent coefficient of variation values were derived from measured parameters and day age of 30 ducklings within the high toxin series.

TABLE V

CORRELATION FROM CROSS PRODUCTS ANALYSIS OF DUCK AGE-TREATMENT WITH LIQUID CRYSTALLINE VISAGE AND MEASURED PARAMETERS*

	Gain	Liver %	Pet Eth Extract %	Chole-sterol %	Chol ester %	Cholesteryl Ester Composition Palm %	Stearic %	Oleic %	Linol %	Arach %
LOW TOXIN SERIES										
Day Age	-0.29	0.75	0.63	0.39	0.86	0.52	0.70	0.62	-0.26	-0.84
Treatment	-1.00	-1.00	1.00	-1.00	1.00	-1.00	-1.00	1.00	1.00	-1.00
Duck (Age Trt)	-0.21	-0.32	-0.53	0.01	0.37	0.05	0.02	0.10	-0.05	0.02
HIGH TOXIN SERIES										
Day Age	-0.55	0.36	0.64	0.18	0.77	-0.71	-0.81	0.82	-0.69	-0.03
Treatment	-1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	1.00	1.00	-1.00
Duck (Age Trt)	0.08	0.11	-0.02	-0.06	-0.31	-0.06	0.01	0.25	-0.26	-0.05

*Cross products analysis correlation values were derived from measured parameters and liquid crystalline visage ratings of 50 ducklings within the low toxin series and 30 ducklings within the high toxin series.

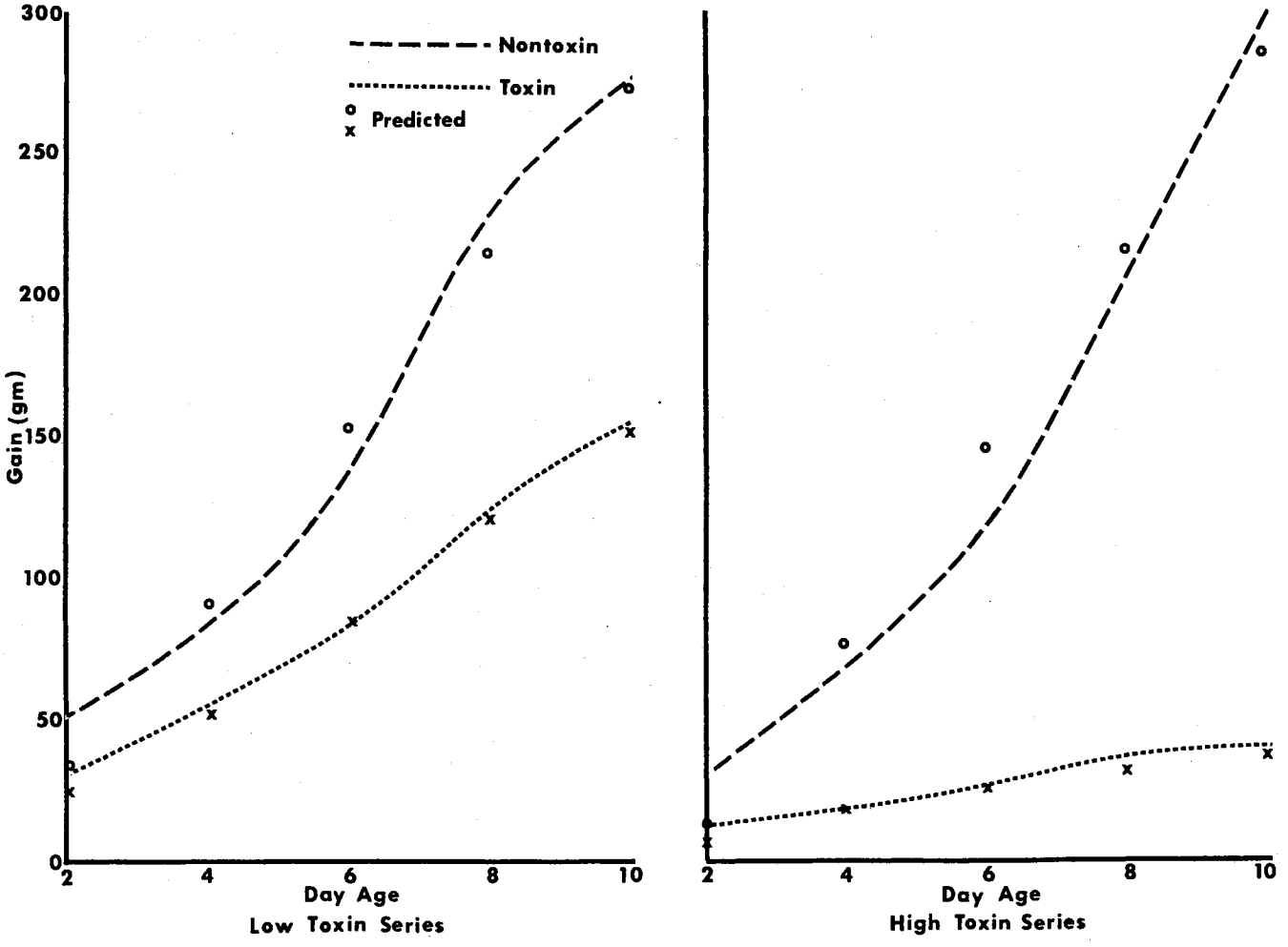


Figure 2. Trend Estimates and Predicted Means Relating Weight Gain with Duckling Day Age and Toxin Level.

toxin ration increased near linear from 50 grams to almost 200 grams. Within the high toxin series the weight was approximately the same until eight days of age, then weight decreased at 10 days of age. This was probably due to the toxin dose level approximating the LD_{50} dose level previously reported for this species.

Palatability of the ration is apparently not a factor, as previous studies have shown that ducklings transferred from the control diet to the aflatoxin diet ingested normal amounts of feed for one to two days. Intubation of 6 mg. of aflatoxin B_1 per 100 grams body weight daily for five days also causes the characteristic lethargy, loss of appetite, and reduction in weight gain.

The percentage liver (Figure 3) was calculated as the fresh weight of the duckling liver divided by the final body weight before posting. The nontoxin birds in both series showed a decline in percent liver as age increased. This decrease was approximately 1.5 to 2.0 percent. The low level of toxin caused birds to develop a larger liver in relationship to duckling body weight. The liver has the capacity to regenerate normal tissue when hepatic tissue is damaged. The aflatoxin damaged duckling liver tissues to an extent that resulted in liver size increases of regenerated hepatic tissues. With the high toxin level, the percent liver remained approximately the same with a decrease of approximately 0.5 percent. This was probably due to the high dose approaching the LD_{50} for the ducklings, and, although the liver was severely damaged, the ducklings were not capable of hepatic tissue regeneration sufficient to compensate for the high toxin intake and resulting increased rate of liver damage.

The percent petroleum ether extract of duckling liver was

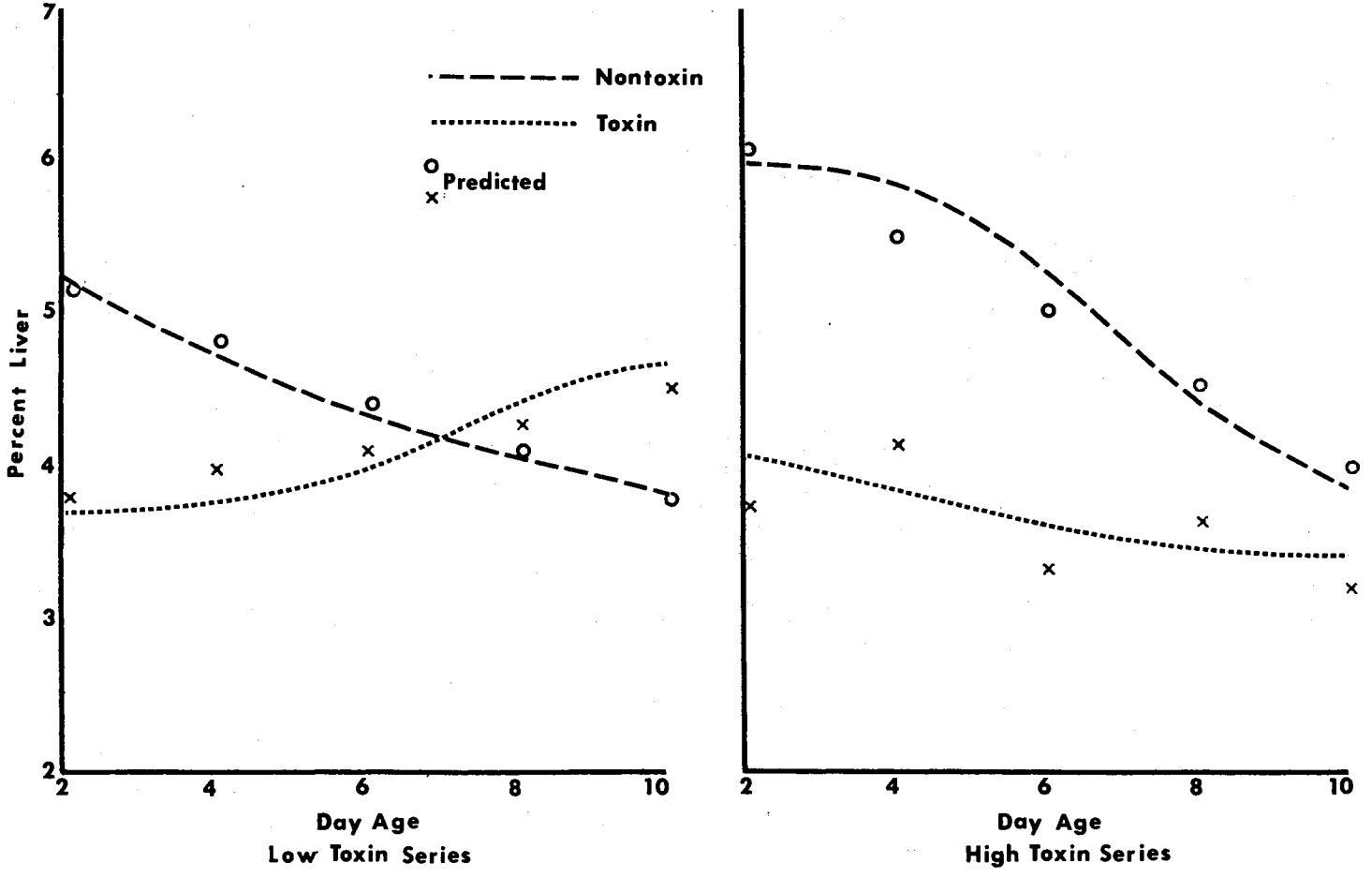


Figure 3. Trend Estimates and Predicted Means Relating Percent Liver with Duckling Day Age and Toxin Level.

markedly influenced by aflatoxin fed to the ducklings (Figure 4). The normal toxin free livers contained 41-48 percent extractable lipid at 2 days age. As day age increased, the percent lipid decreased to approximately 10 percent at 10 days of age. With the toxin fed birds the percent pet ether extract was 28-34 percent for the low toxin level and 40-55 percent at the high toxin level. Generally lipid percentage decreased slightly as day age increased for both the high and low toxin levels as contrasted to the normal large decreases in liver lipid content with day age of ducklings with the nontoxin diet.

The percent total cholesterol (Figure 5) with both high and low toxin levels was greatest at day 2, with the low toxin level 22% and the high toxin level 27%. The low toxin series showed a more rapid decrease in percent total cholesterol, with a minimum at day 8, while the trend in the high toxin level was still decreasing at 10 days age. Nontoxin birds had a relatively constant percent total cholesterol, showing only a slight decrease in percent total cholesterol at approximately day age 6.

Free cholesterol was determined by subtracting cholesteryl esters from total cholesterol (Figure 6). With the nontoxin birds, the percent free cholesterol was decreased approximately 1.7% in the low toxin series, and increased 1.0% in the high toxin series. The birds fed the low toxin ration had almost no change in percent free cholesterol during the 10 day period with the level remaining at approximately 0.7%. The level of free cholesterol in the high toxin series was initially 2% and this decreased to 1.4% at 10 days age.

Percent cholesteryl esters followed the same trend as total cholesterol (Figure 7). The relative change in percent free

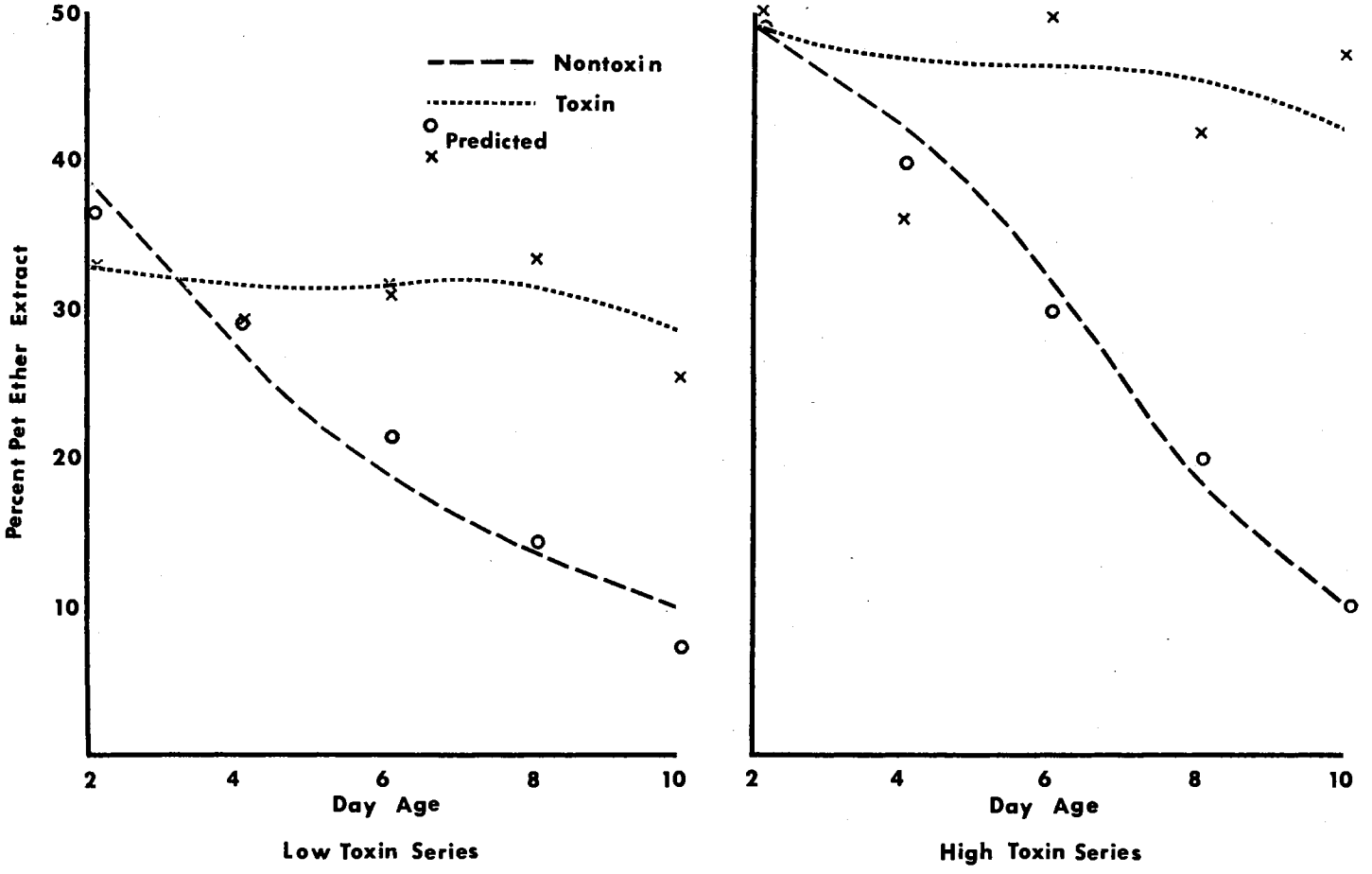


Figure 4. Trend Estimates and Predicted Means Relating Percent Pet Ether Extract with Duckling Day Age and Toxin Level.

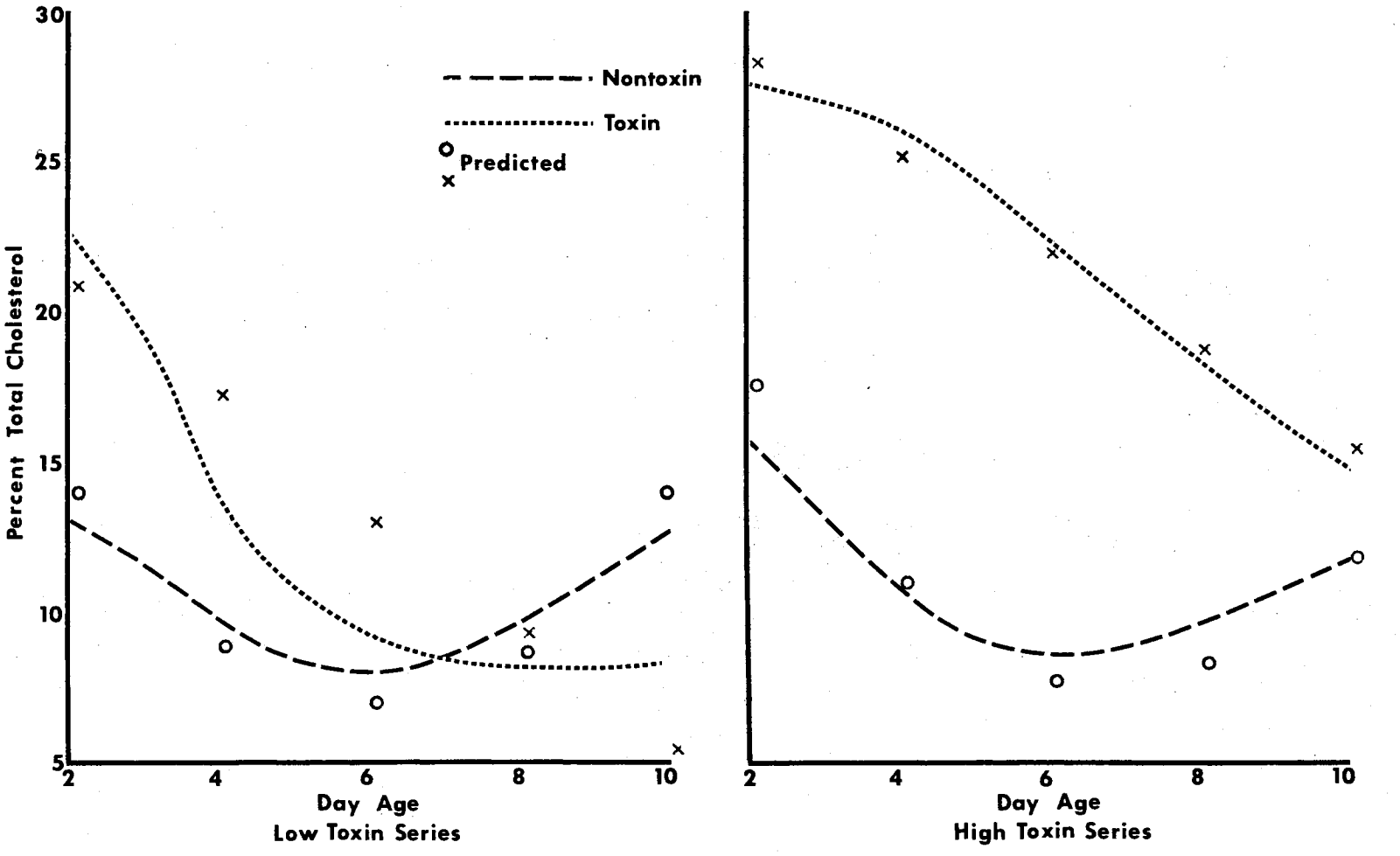


Figure 5. Trend Estimates and Predicted Means Relating Percent Total Cholesterol with Duckling Day Age and Toxin Level.

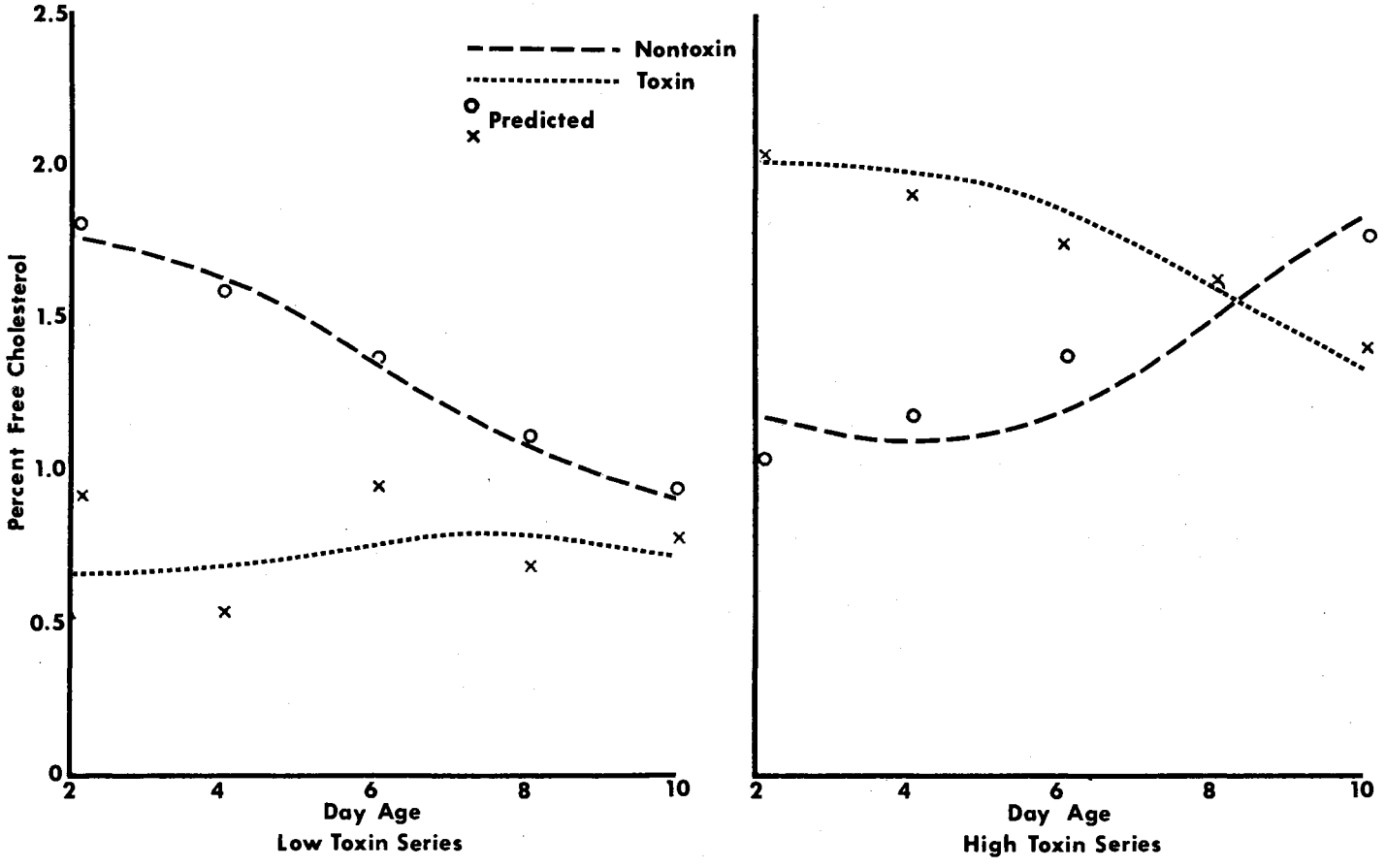


Figure 6. Trend Estimates and Predicted Means Relating Percent Free Cholesterol with Duckling Day Age and Toxin Level.

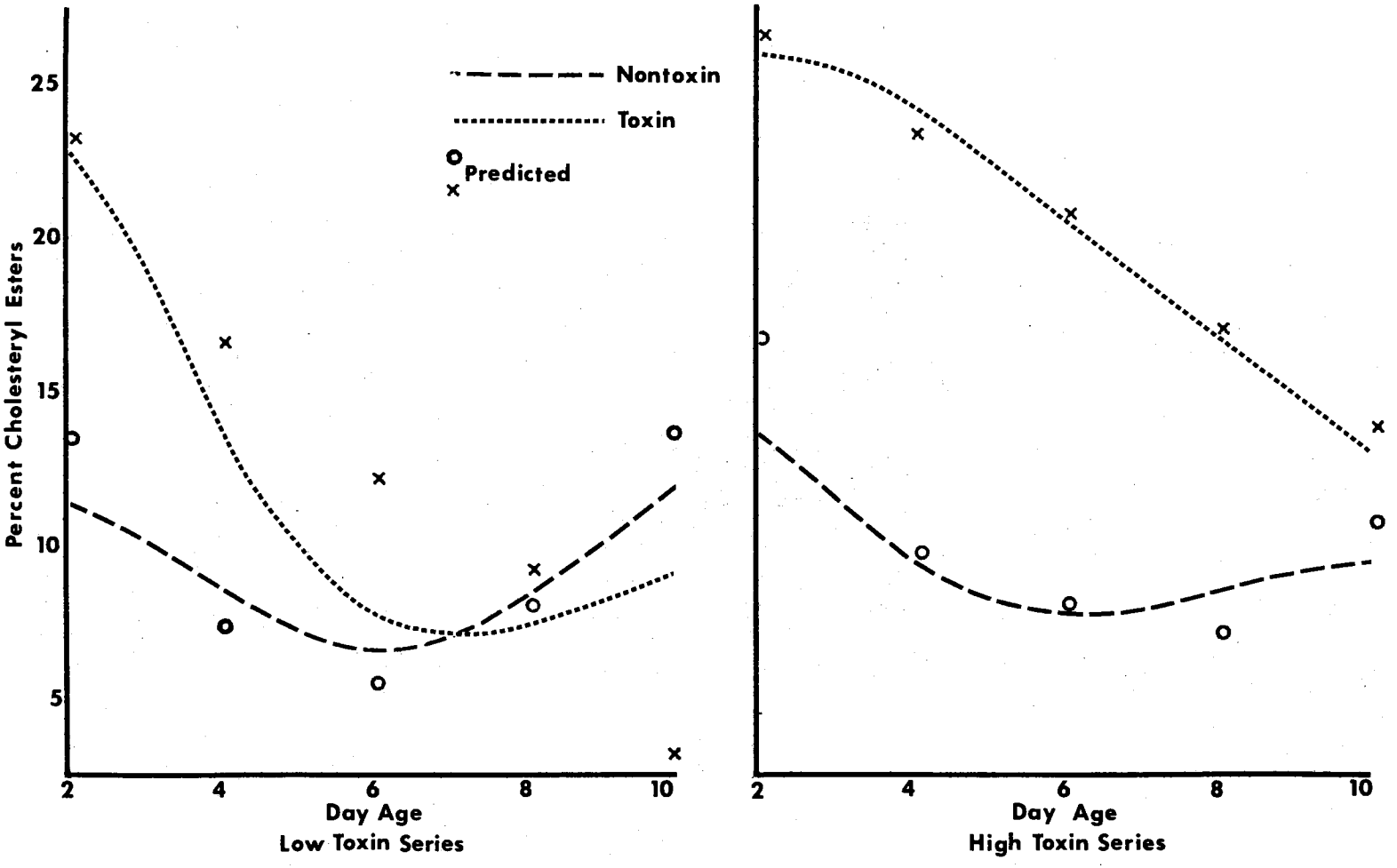


Figure 7. Trend Estimates and Predicted Means Relating Percent Cholesteryl Esters with Duckling Day Age and Toxin Level.

cholesteryl was small as compared to total cholesterol. The highest percent cholesteryl esters was at day age 2, with both nontoxin and toxin birds. The nontoxin check birds had a lower level of cholesteryl esters than toxin birds. Also the percent cholesteryl esters decreased slightly from 12% at 2 days to 7% at 6 days age, then increased at 10 days to approximately the two day values. The low toxin series decreased more rapidly to a minimum of 7% at 8 days age as contrasted to the high toxin series that showed a gradual decrease from 25% to 15% during the 10 day period.

Percent cholesteryl arachidonate as influenced by day age is shown in Figure 8. The greatest change is shown in the low toxin series with an increase from 5% at 2 days age to 16% at 8 days age. Within the higher toxin series, the increase was only from 3% at day age 2, to a maximum of 7% at 10 days age. The nontoxin check birds remained at approximately the same level in both series, with a small decrease in percent cholesteryl arachidonate in the check birds of the high toxin series (Figure 9).

The relationship between percent cholesteryl oleate and day age is shown in Figure 10. In the nontoxin check series, the percent cholesteryl oleate decreased from 45% to 25% and 47% to 38% in the low and high level experiments respectively. Within the toxin fed series at the low level, the decrease was near linear from 42% to 35%. The high toxin series showed a greater difference with a decrease from 57% to 42% cholesteryl oleate during the 10 day bioassay period.

The cholesteryl palmitate content of petroleum ether extracts of duckling liver is shown in Figure 11. Generally the nontoxin check ducklings had a much higher level of cholesteryl palmitate than the

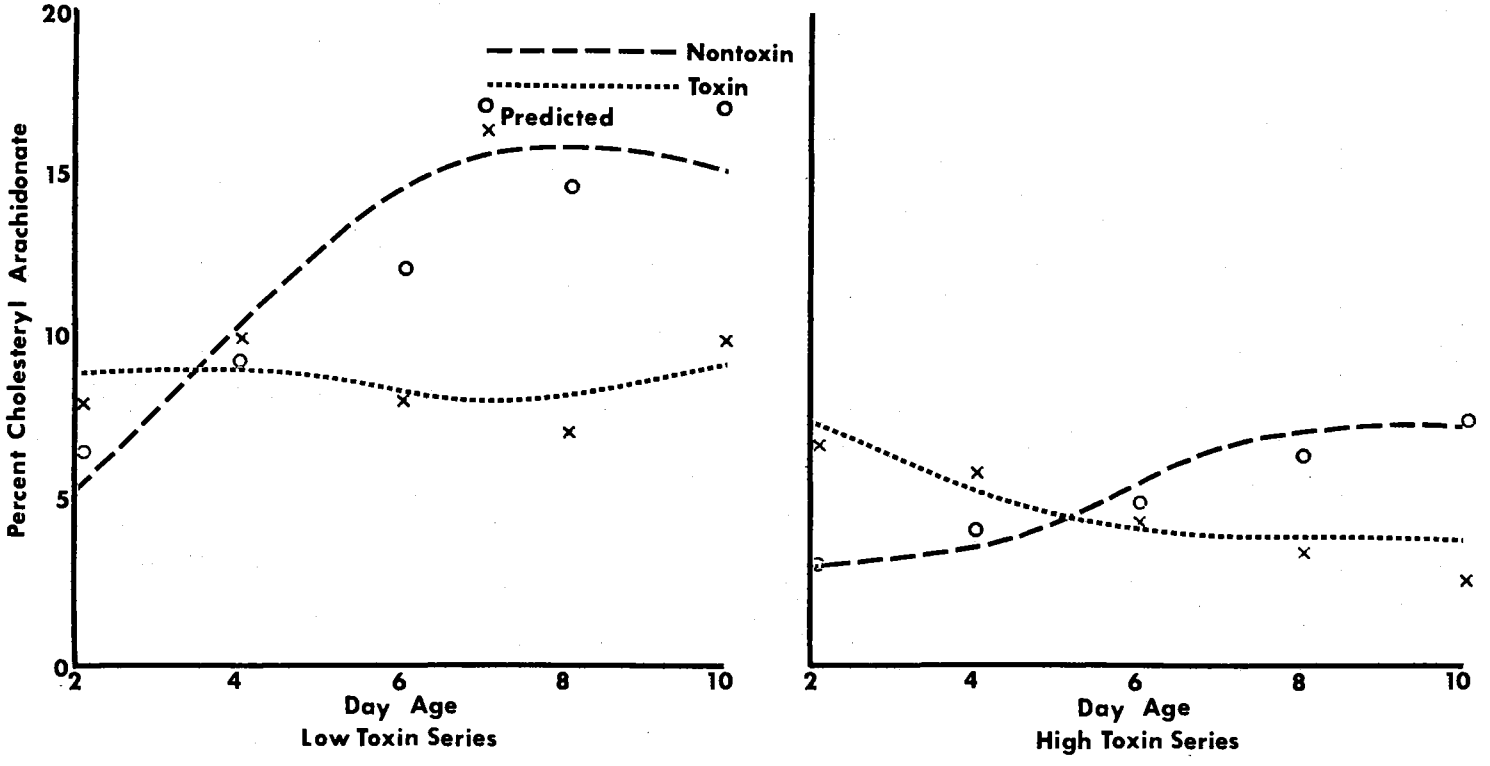


Figure 8. Trend Estimates and Predicted Means Relating Percent Cholesteryl Arachidonate with Duckling Day Age and Toxin Level.

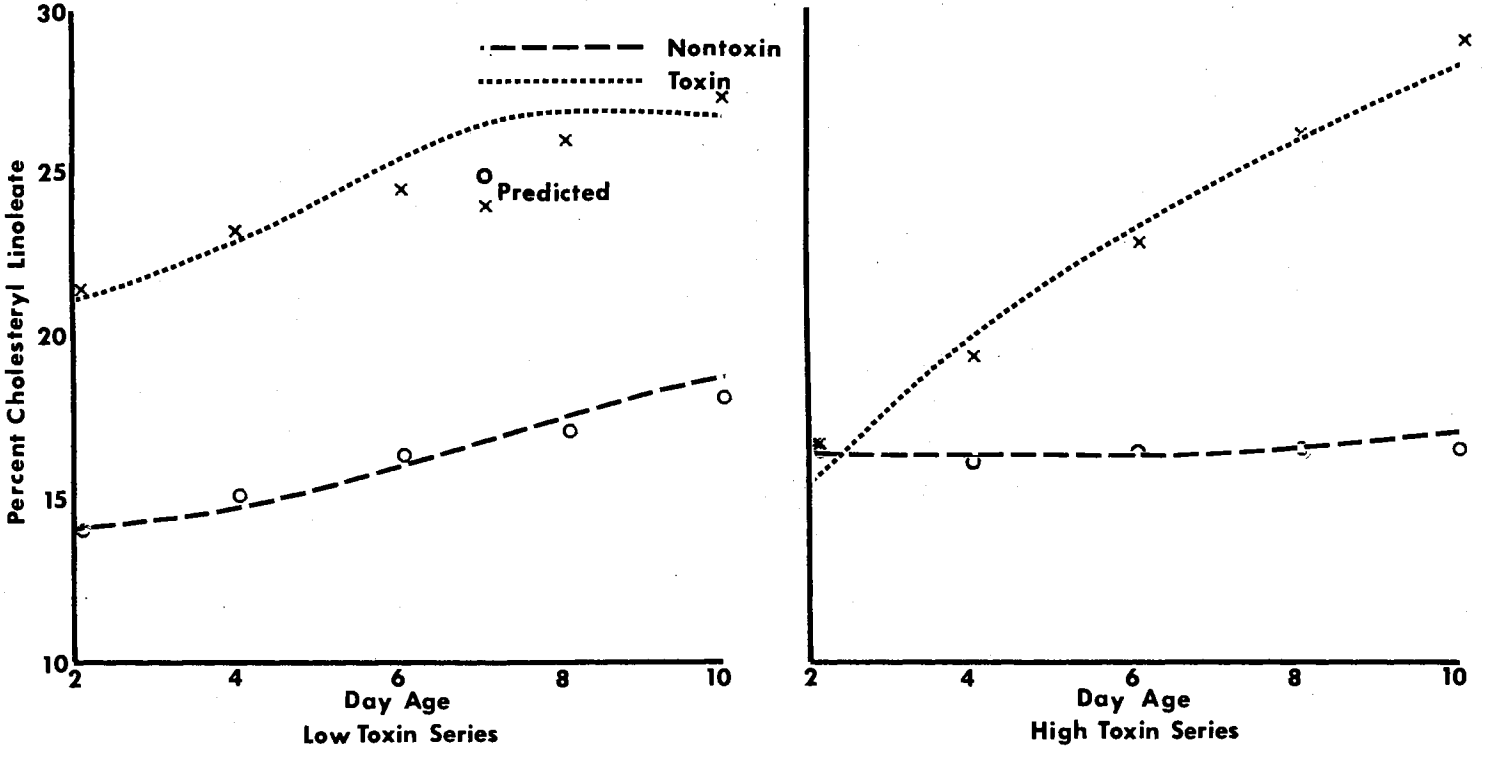


Figure 9. Trend Estimates and Predicted Means Relating Percent Cholesteryl Linoleate with Duckling Day Age and Toxin Level.

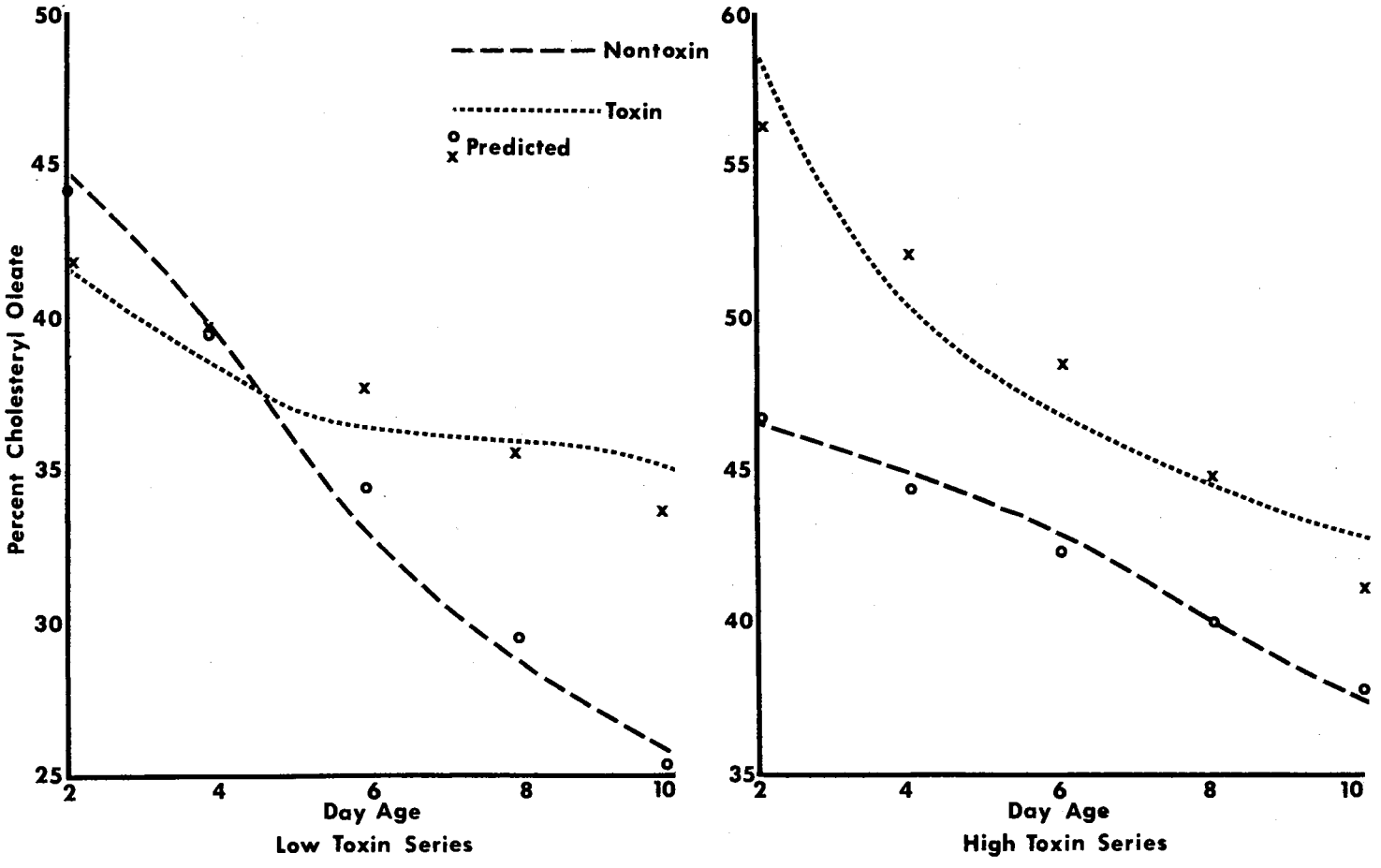


Figure 10. Trend Estimates and Predicted Means Relating Percent Cholesteryl Oleate with Duckling Day Age and Toxin Level.

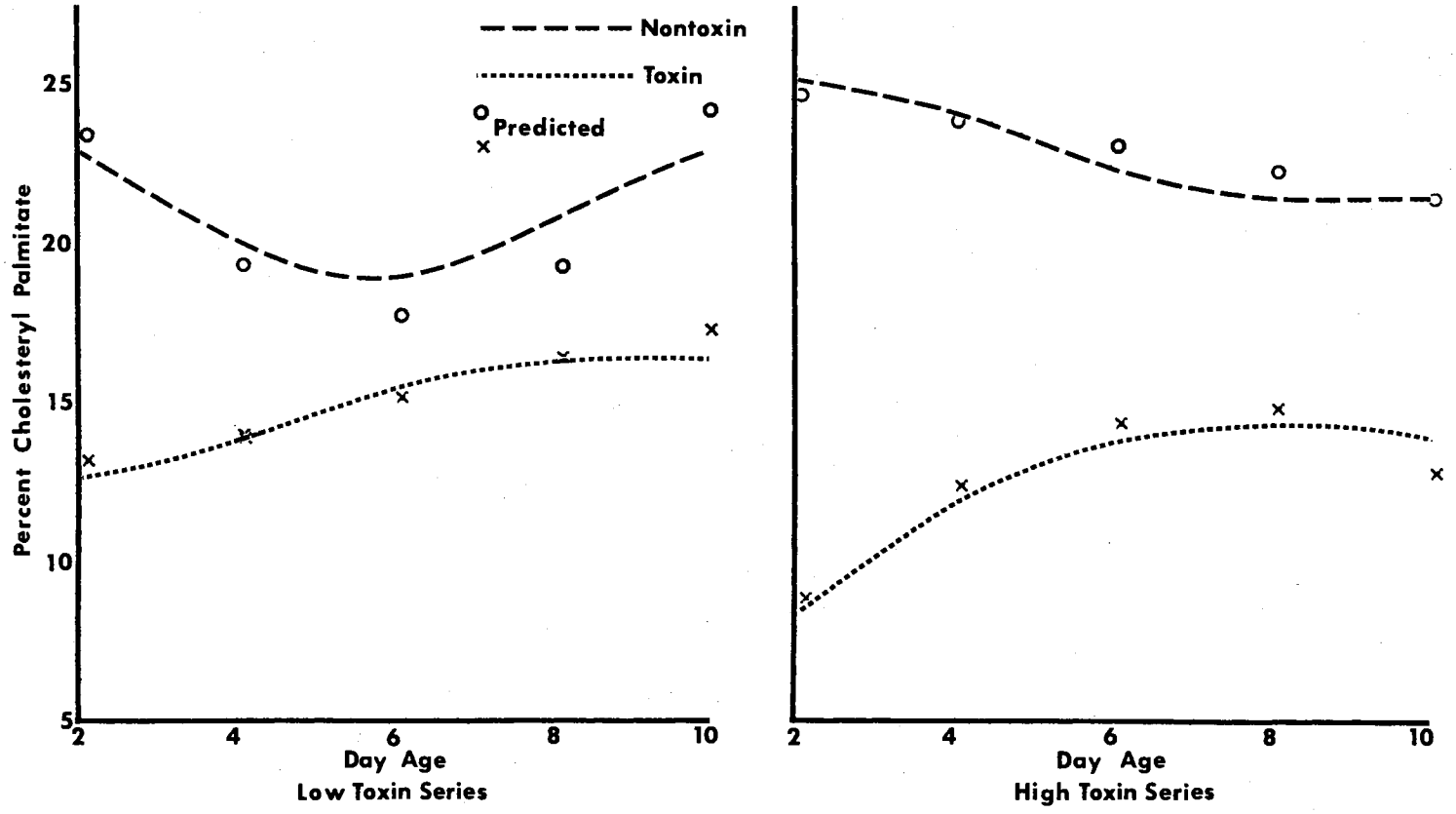


Figure 11. Trend Estimates and Predicted Means Relating Percent Cholesteryl Palmitate with Duckling Day Age and Toxin Level.

toxin treatment, with approximately 22% and 24% cholesteryl palmitate in the nontoxin fed birds of low and high level series respectively. The toxin treated ducklings showed a slight increase of 2% and 7% in the low and high toxin levels respectively, and both toxin treatments were in the 10%-15% range.

The influence of aflatoxin on percent cholesteryl stearate of duckling liver extracts is shown in Figure 12. The percent cholesteryl stearate increased from 12%-17% and 10%-17% in the nontoxin check birds. In both low and high toxin series, there was first an increase in cholesteryl stearate percentage, then a decrease. The low toxin level series had a maximum of 16% cholesteryl stearate at 4 days age and a minimum of 12% at 10 days age. The high toxin level series had a minimum of 8% cholesteryl stearate at 2 days age and a maximum of 13% at 7 days age.

The composition ratio of cholesteryl oleate to the total saturated cholesteryls (palmitate plus stearate) is shown in Figure 13. In general, the percent saturated cholesteryl esters increased with day age of normal nontoxin birds, and the percent unsaturated cholesteryl oleate decreased. An opposite trend was apparent with day age as toxin levels increased. This interrelationship indicated that the ratio of cholesteryl oleate to saturated cholesteryl esters was significant and reflected some influence of toxin intake on normal metabolism of these lipids. The magnitude and comparative brilliance of the liquid crystalline fraction was apparently related to this ratio and was found to be positively related to the resultant liquid crystalline visage with cross polarized microscopy. Characteristics and optical activity of liquid crystalline components within the

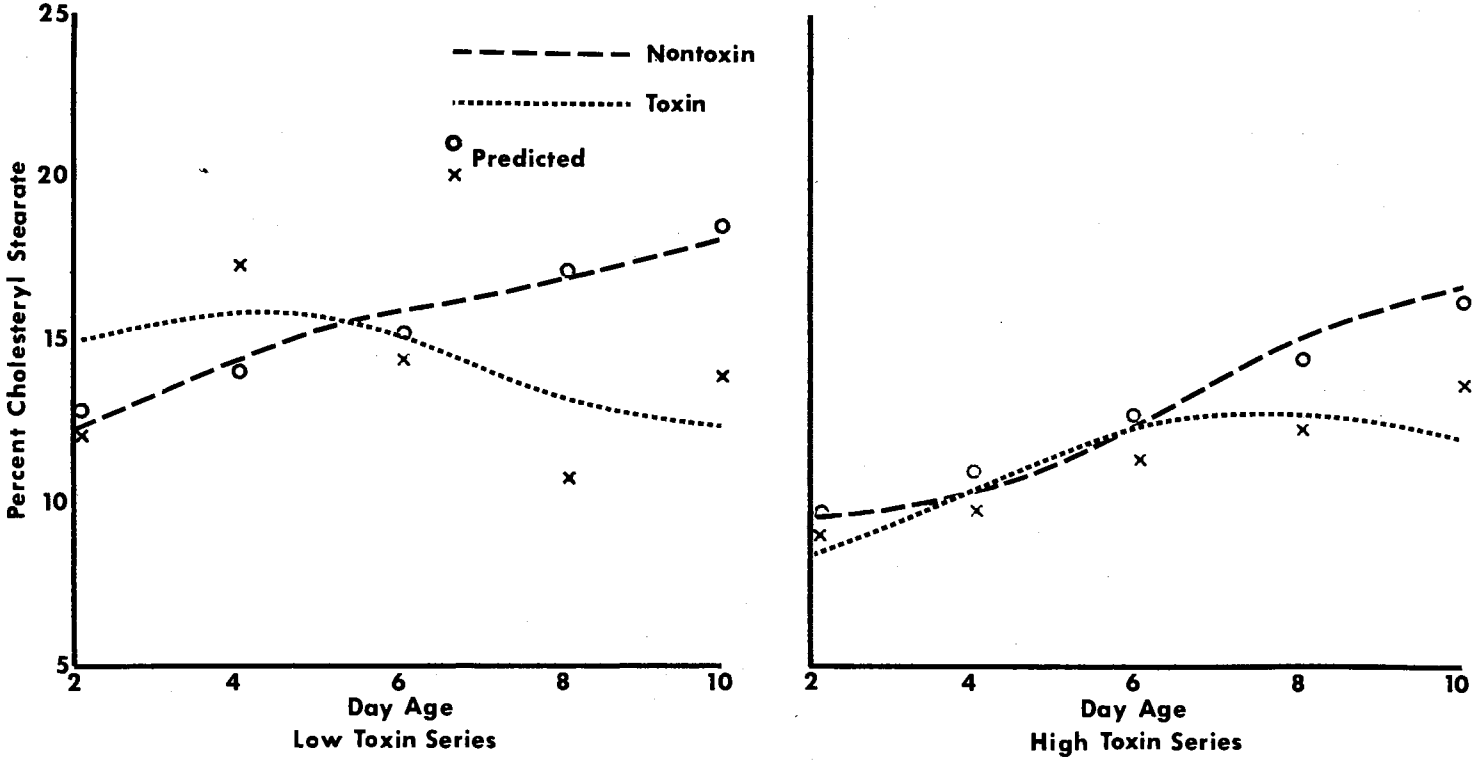


Figure 12. Trend Estimates and Predicted Means Relating Percent Cholesteryl Stearate with Duckling Day Age and Toxin Level.

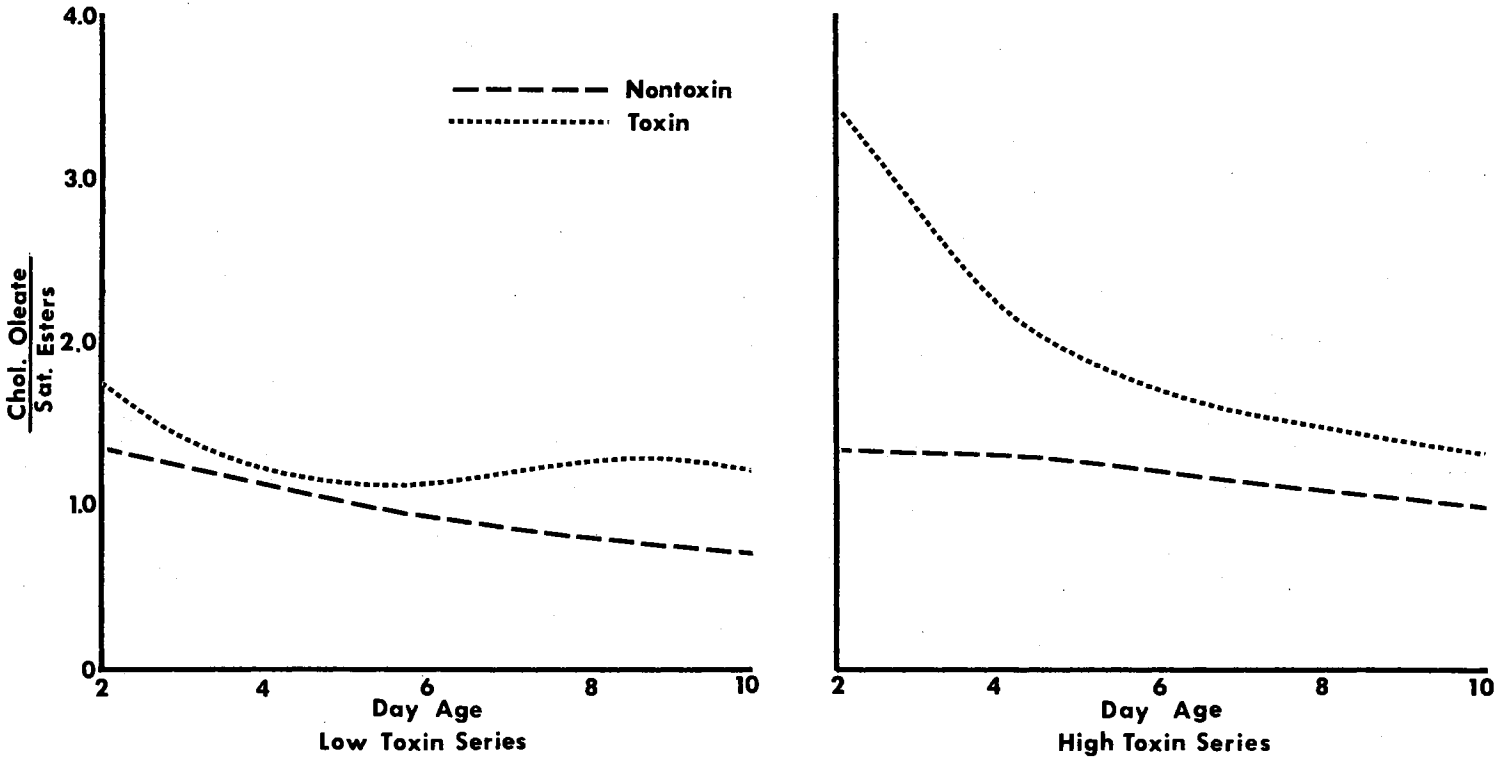


Figure 13. Trend Estimates Relating the Ratio of Cholesteryl Oleate to Saturated Cholesteryl Esters with Duckling Day Age and Toxin Level.

petroleum ether extract of duckling livers were determined by allowing the extract to evaporate on a glass slide and observing the structure and brilliance with polarized light microscopy. The relative visage was assigned a rating of 1 to 10, with 1 as very little crystal development, and 10 as maximum optical acitvity. An example of the general trend of visage values as influenced by toxin intake and day age is shown in Figure 14. Generally, as day age increased, visage rating values increased in toxin free birds and decreased with toxin fed birds. Examples of representative liquid crystalline visage ratings with day age and toxin level intake are shown in Figures 15 to 24. At the two day age (Figures 15, 16) the nontoxin treatment had a visage rating of 8, with the toxin treatment having a visage rating of 10. The principle differences apparent in composition indicated cholesteryl palmitate lower and cholesteryl oleate higher within the toxin series.

At four day age (Figures 17, 18) the liquid crystal visage value was much lower in the nontoxin series and the visage value for the toxin series remained relatively unchanged. At the four day age, the greatest change in composition was an increase of cholesteryl palmitate within the nontoxin series.

At 6 days age (Figures 19, 20) the composition and visage values of liquid crystals remained about the same as the 4 day levels for both the toxin and nontoxin series although some differences were apparent in the general crystal configurations. This was also noted for visage values at 8 days age (Figures 21, 22). Even though both the toxin and nontoxin visage values were similar at day age 8, there was an apparent change in the liquid crystal configurations. The principal

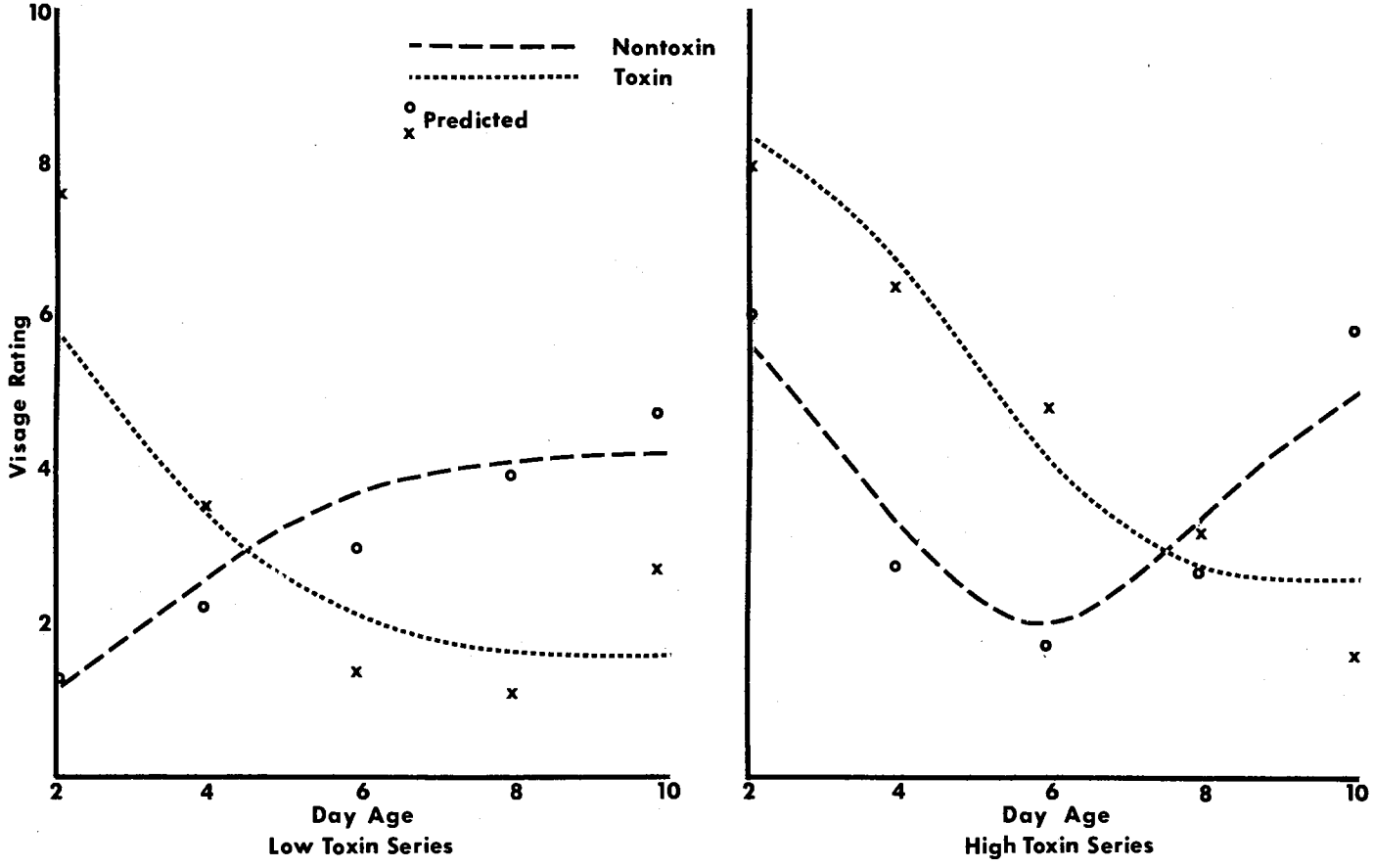


Figure 14. Trend Estimates and Predicted Means Relating Visage Rating with Duckling Day Age and Toxin Level. Low Level: Age, Quadratic P>0.0050; Age x Treatment, Linear P>0.0001. High Level: Age, Quadratic P>0.0013; Age x Treatment, Linear P>0.0045.

Figure 15. Liquid Crystal Visage Rating 8, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2545, Nontoxin 2 Day Age, % Cholesteryl Esters: Palmitic 18.7, Stearic 11.4, Oleic 50.0, Linoleic 14.8, and Arachidonic 5.2.

Figure 16. Liquid Crystal Visage Rating 10, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2552, Toxin 2 Day Age, % Cholesteryl Esters: Palmitic 5.5, Stearic 9.0, Oleic 62.2, Linoleic 15.4, and Arachidonic 7.9.

Figure 17. Liquid Crystal Visage Rating 2, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2548, Nontoxin 4 Day Age, % Cholesteryl Esters: Palmitic 26.1, Stearic 8.6, Oleic 44.3, Linoleic 17.9, and Arachidonic 3.1.

Figure 18. Liquid Crystal Visage Rating 7, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2553, Toxin 4 Day Age, % Cholesteryl Esters: Palmitic 7.7, Stearic 11.8, Oleic 51.7, Linoleic 21.1, and Arachidonic 7.7.

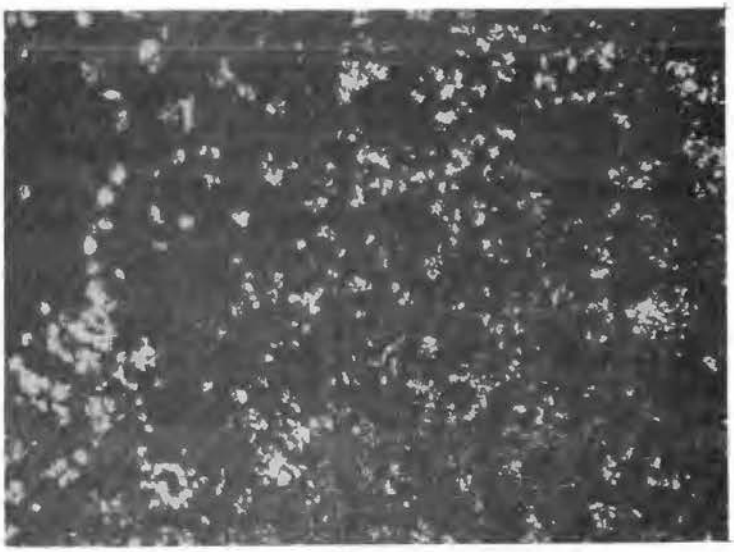
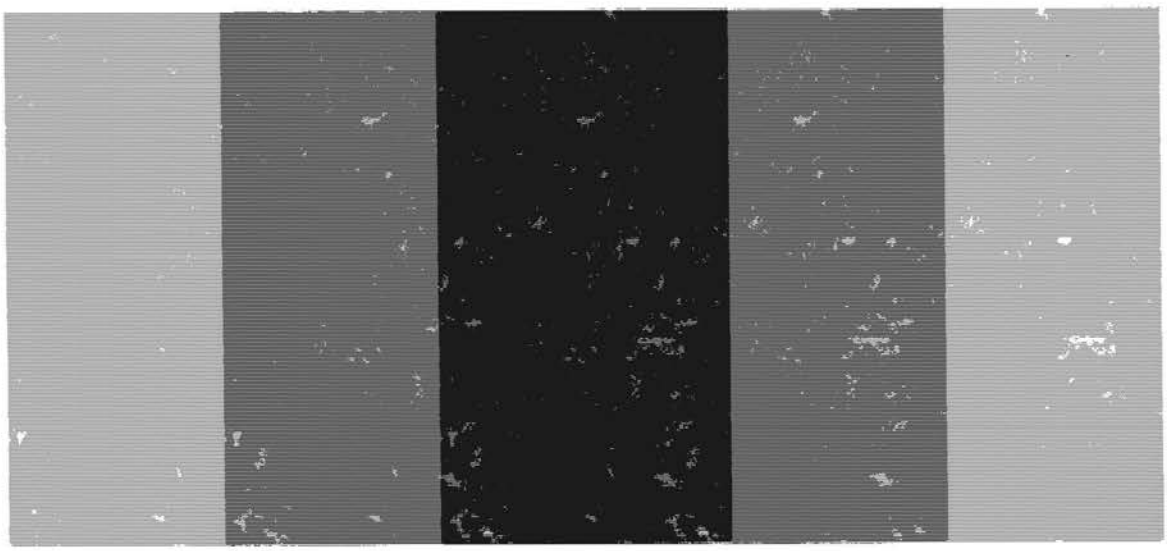
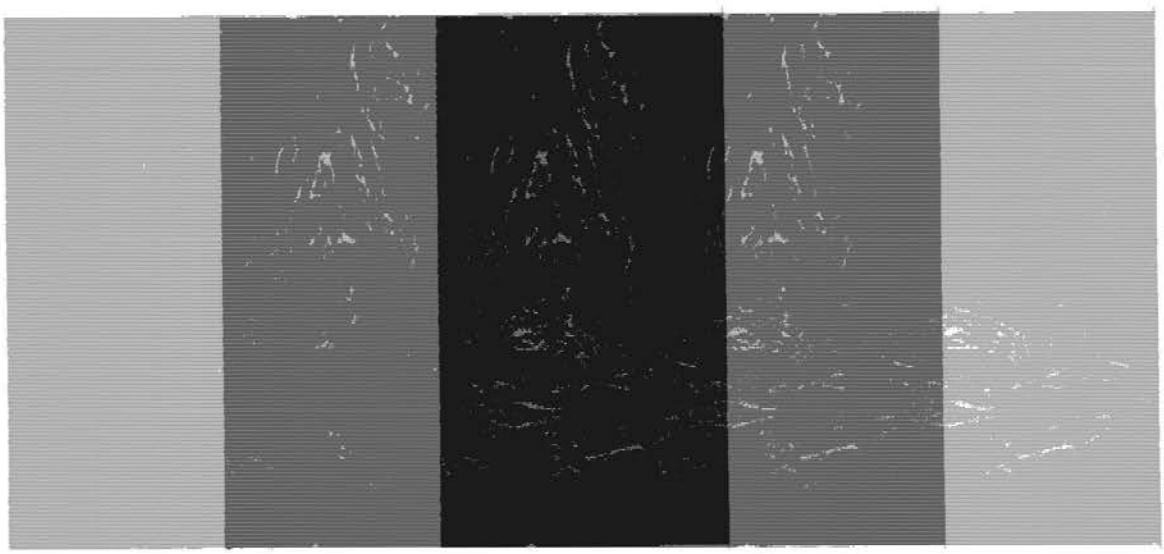


Figure 19. Liquid Crystal Visage Rating 3, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2561, Nontoxin 6 Day Age, % Cholesteryl Esters: Palmitic 22.0, Stearic 13.4, Oleic 42.9, Linoleic 15.3, and Arachidonic 6.4.

Figure 20. Liquid Crystal Visage Rating 5, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2567, Toxin 6 Day Age, % Cholesteryl Esters: Palmitic 14.7, Stearic 13.6, Oleic 46.7, Linoleic 21.3, and Arachidonic 3.7.



e 21. Liquid Crystalline Characteristics of Partially Purified Hepatic Li
Crystalline Characteristics of Partially Purified Hepatic Li
Day Age, % Cholesterol, % Phospholipids, Palmitic 23.5, Stearic
14.0, Oleic 41.0, Linoleic 4.0, Arachidonic 4.1.



e 22. Liquid Crystalline Characteristics of Partially Purified Hepatic Li
Crystalline Characteristics of Partially Purified Hepatic Li
Age, % Cholesterol, % Phospholipids, Palmitic 32.7, Stearic
Oleic 41.0, Linoleic 4.0, Arachidonic 5.5.

differences in composition of these crystals was in cholesteryl palmitate levels. The percentage cholesteryl palmitate in the toxin series was approximately one half that of the nontoxin series. At day age 10 (Figures 23, 24) the liquid crystals in the nontoxin series began to assume a "featherlike" form, and the relative brightness increased slightly from day age 8. At the 10 day age in the toxin series, the liquid crystals began to lose form and intensity, and their structure was not as well defined as observed with the previous day age levels.

It should be noted that the configurations of liquid crystals are sensitive to changes in temperature, humidity, extraction, and evaporation procedures. In this study these factors were kept as near constant as possible and the photographs shown are representative of the liquid crystalline visage for a specific day age and toxin level.

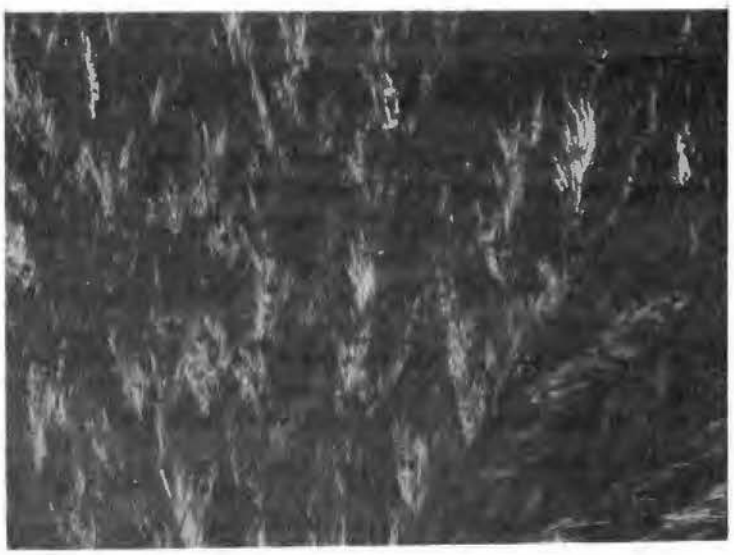


Figure 23. Liquid Crystal Visage Rating 7, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2572, Nontoxin 10 Day Age, % Cholesteryl Esters: Palmitic 18.1, Stearic 17.2, Oleic 34.9, Linoleic 16.6, and Arachidonic 13.2.

Figure 24. Liquid Crystal Visage Rating 4, for Nonpolar Hepatic Liquid Crystalline Characteristics. Duckling 2577, Toxin 10 Day Age, % Cholesteryl Esters: Palmitic 13.6, Stearic 13.5, Oleic 45.6, Linoleic 25.6, and Arachidonic 1.8.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objective of this study was to identify cholesteryl ester composition of duckling liver and to characterize hepatic cholesteryl liquid crystal transformations as influenced by aflatoxin intake and day-age weight gain of White Pekin Ducklings.

The most obvious effect of aflatoxin injection by ducklings was a decrease in weight gain as the aflatoxin dose level increased. Ducklings fed aflatoxin had a larger percent liver than toxin free ducklings due to the regeneration of the damaged liver after injury by aflatoxin.

The percent petroleum ether extract of duckling liver was also affected by aflatoxin fed to the ducklings. The percent lipid remained approximately the same in the toxin birds as day age increased, but in the nontoxin series, the percent lipid decreased as day age increased.

Initially, approximately half of the petroleum ether extract consisted of cholesteryl oleate. This percentage decreased during the study but the decrease in percent cholesteryl oleate was greater in nontoxin birds as day age increased.

The relative liquid crystal visage depended primarily upon the percent cholesteryl oleate in the hepatic tissue extract of duckling liver, This was further related to the total saturated cholesteryls,

(stearic and palmitic). As the ratio of saturated cholesteryl esters to cholesteryl oleate increased, the relative visage also increased. The general trend of liquid crystalline visage rating values increased with toxin free birds and decreased in toxin fed birds.

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