

## FINAL REPORT

### PHASE II: DETERMINATION OF HEALTH EFFECTS OF ENVIRONMENTAL POLLUTANTS USING AVIAN MODELS: A HOLISTIC APPROACH



**F.E. Wiley<sup>1</sup>, W.W. Bowerman<sup>1</sup>, E.T. Croisant<sup>2</sup>, P. van den Hurk<sup>3</sup>,  
K.A. Grasman<sup>2</sup>, G. Boehringer<sup>2</sup> and J.G. Sikarskie<sup>4</sup>**

<sup>1</sup>Department of Forestry and Natural Resources, Graduate Program in Environmental Toxicology, Clemson University, Pendleton, South Carolina

<sup>2</sup>Department of Biological Sciences, Wright State University, Dayton, Ohio

<sup>3</sup>Department of Biological Sciences, Graduate Program in Environmental Toxicology, Clemson University, Pendleton, South Carolina

<sup>4</sup>Department of Small Animal Clinical Sciences, Michigan State University, East Lansing, Michigan

**A Report to  
Michigan Great Lakes Protection Fund**

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## SECTION 1.0

### EXECUTIVE SUMMARY

The use of the bald eagle as an ecosystem monitor of Great Lakes Water Quality has been proposed by the International Joint Commission. Recent studies have shown that the eagle is an appropriate model for assessing the spatial and temporal trends of persistent toxic substances and has been selected by the Michigan Department of Environmental Quality (MDEQ) as a biosentinel species. This project furthers the development of health effects biomarkers that could be coupled to the annual survey of persistent toxic compounds in bald eagle blood and feather samples.

Recently, we have found that at least one immune system biomarker, corticosterone levels, is highly correlated with either *p,p'*-DDE (4,4'-DDE) or Total PCB concentrations in blood (Bowerman et al. 2002). Corticosterone functions, in part, in the regulation of fat deposition and storage and in immune system functions. For migratory birds and those that exist in cold climates, alteration of fat deposition could adversely impact survival. Further, a compromised immune system may also decrease survivability. The potential to predict survivability through the use of biomarkers is an encouraging finding. Therefore, we proposed to repeat our field trials and expand the scope of our research by linking the field project to a laboratory study using chickens as a surrogate species for bald eagles.

We report here the results of a two-year grant looking at developing these biomarkers for the bald eagle biosentinel project. This study will attempt to describe a holistic approach to predicting impaired survivability in eagles exposed to persistent, bioaccumulative and toxic contaminants (PBTs). This assessment method will include biomarkers of the endocrine, immune, circulatory, and detoxification systems and general indicators of health including growth rates, body mass, parasitism and birth defects. These indicators will be correlated to exposure to PBTs. Relationships between health indicators and PBT contaminants will be explored using controlled laboratory tests. Those tests that prove to be predictive will be considered for incorporation into the MDEQ Strategic Monitoring Program.

We have analyzed several potential biomarkers in field trials using plasma and fecal swabs from nestling bald eagles. We have also completed two controlled laboratory studies using chickens dosed *in ovo* with PCB 126 and an extract from double-crested cormorant (*Phalacrocorax auritis*) eggs. Results from these studies indicate several physiological analyses that show promise as biomarkers for bald eagles. These include plasma retinyl palmitate (vitamin A) levels, plasma thyroid hormones ratios, and ACTH response. In addition, plasma vitamin E concentrations and the Con-A assay for immune function may prove useful indicators with further study. Indicators that do not seem predictive of contaminant levels in nestling eagles include plasma retinol concentration, total plasma thyroxine (thyroid hormone T4) concentration, and intestinal bacteria.

Several other biomarkers were examined only in the laboratory experiments. Biomarkers that demonstrated good dose-response relationships included vitamin D, cytochrome P450, immune function tests, and growth parameters.

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## SECTION 2.0

### INTRODUCTION

The use of the bald eagle as an ecosystem monitor of Great Lakes Water Quality has been proposed by the International Joint Commission. Recent studies have shown that the eagle is an appropriate model for assessing the spatial and temporal trends of persistent toxic substances and has been selected by the Michigan Department of Environmental Quality (MDEQ) as a biosentinel species. This project furthers the development of health effects biomarkers that could be coupled to the annual survey of persistent toxic compounds in bald eagle blood and feather samples.

Recently, we have found that at least one immune system biomarker, corticosterone levels, is highly correlated with either *p,p'*-DDE (4,4'-DDE) or Total PCB concentrations in blood (Bowerman et al. 2002). Corticosterone functions, in part, in the regulation of fat deposition and storage and in immune system functions. For migratory birds and those that exist in cold climates, alteration of fat deposition could adversely impact survival. Further, a compromised immune system may also decrease survivability. The potential to predict survivability through the use of biomarkers is an encouraging finding. Therefore, we proposed to repeat our field trials and expand the scope of our research by linking the field project to a laboratory study using chickens as a surrogate species for bald eagles.

We report here the results of a two-year grant looking at developing these biomarkers for the bald eagle biosentinel project. This study will attempt to describe a holistic approach to predicting impaired survivability in eagles exposed to persistent, bioaccumulative and toxic contaminants (PBTs). This assessment method will include biomarkers of the endocrine, immune, circulatory, and detoxification systems and general indicators of health including growth rates, body mass, parasitism and birth defects. These indicators will be correlated to exposure to PBTs. Relationships between health indicators and PBT contaminants will be explored using controlled laboratory tests. Those tests that prove to be predictive will be considered for incorporation into the MDEQ Strategic Monitoring Program.

The Phase I Report that was submitted previously provided results from the first year of this grant. Preliminary field trial results were included in that report and compared among regions (e.g. Great Lakes versus Inland), as the plasma contaminant analysis was not yet complete. Also, results from our first laboratory trial using PCB 126 were provided. In this current report, we are providing results from our second laboratory trial, in which chicken eggs were dosed with an extract taken from double-crested cormorant (*Phalacrocorax auritis*) eggs. We are also reporting final results from the field trial, with the biomarker results compared to plasma contaminant concentrations. Final recommendations will be made on the feasibility of the tested biomarkers, taking all field and laboratory results into consideration.

## SECTION 3.0

### STUDY DESIGN AND METHODS

#### 3.1 BALD EAGLES

Nestling bald eagles (between five and nine weeks of age) were sampled during annual banding activities. The sampling region consisted of the upper and lower peninsulas of Michigan as well as Voyageurs National Park, Minnesota. Nest trees were ascended by a climber who would then lower one nestling to the ground for processing. Blood was collected via the brachial vein using sterile, heparinized syringes fitted with 22 or 25 gauge needles. Samples of whole blood were transferred to heparinized vacuum tubes, kept on ice in coolers, and centrifuged within 48 hours of collection. Blood plasma was decanted and transferred to vacuum tubes and frozen (Bowerman et al. 2003). Age and sex of nestlings was determined by measuring the eighth primary feather and foot pad, respectively (Bortolotti 1984).

Plasma samples were analyzed for organochlorine pesticides and PCBs at Clemson University. One ml of plasma was denatured with methanol, extracted with methylene chloride, and purified with alumina and silica solid phase extraction. Quality control samples were analyzed concurrently and internal gas chromatography standards were added to each sample. Samples were analyzed by gas chromatography with electron-capture detection (GC-ECD). The quantification level was 2 ng/g.

Plasma concentrations of total PCBs and *p,p'*-DDE were compared to vitamin levels, thyroid hormone levels, and ACTH response (corticosterone levels) using three correlation procedures. Correlations were performed with non transformed data, log transformed data, and ranked data. Procedures for identifying the vitamin, thyroid hormone, and corticosterone levels are outlined briefly in the following sections.

##### 3.1.1 *Vitamin Levels*

The fat-soluble vitamins A and E were analyzed from plasma samples. Plasma samples were sent to Michigan State University's Animal Health Diagnostic Laboratory for vitamin analysis. Vitamins were extracted from plasma and analyzed using a high performance liquid chromatography (HPLC) system. For the vitamin A analysis, retinol, retinyl palmitate, and beta-carotene levels were measured.

##### 3.1.2 *Thyroid Hormones*

Thyroid hormones were measured in plasma samples. The thyroid hormones measured were total T3 (triiodothyronine), total T4 (thyroxine), and free T4. All analyses were done at Michigan State University using radioimmunoassays.

### 3.1.3 *Corticosterone*

The ACTH stimulation test was used as a measure of the stress response. During normal stress, animals secrete ACTH (adrenal corticotropin hormone) from the pituitary gland, which in turn stimulates secretion of corticosterone. Exposure to toxicants can reduce the normal stress response. The purpose of the ACTH test is to determine if the contaminant load causes a reduction in plasma corticosterone levels.

Blood was collected from 33 nestling bald eagles in Michigan and Minnesota in 2000 and 2001. Sterile techniques were used to collect up to 12 ml of blood from the brachial vein with heparinized syringes fitted with 22 or 24 gauge needles prior to administration of ACTH ( $T_0$ ). Samples of whole blood were transferred to EDTA vacuum tubes (1.5 ml) and kept on ice in coolers. After initial venipuncture, 0.125 mg ACTH was administered intramuscularly into the pectoral muscle. After 30 minutes, a second venipuncture occurred and 1.5 ml of blood was collected and transferred to a second EDTA vacuum tube ( $T_{30}$ ). Blood was centrifuged within 45 minutes of collection. Blood plasma was decanted, placed in cryovials and stored in a  $-80^{\circ}\text{C}$  freezer until transport to Michigan State University for analysis. Plasma corticosterone levels were analyzed by a solid-phase radioimmunoassay. ACTH response was calculated as the difference in corticosterone levels between  $T_0$  and  $T_{30}$ .

### 3.1.4 *Intestinal Bacteria*

This endpoint was examined as a master's research project by Faith Wiley. Preliminary results from the PCB 126 study were reported in the Phase I Report. Final results of the project, including all field and laboratory data, are reported in Ms. Wiley's thesis, which has been included here as Appendix I. All materials and methods, as well as an introduction to the project, are included in the thesis.

## 3.2 CHICKENS – DOUBLE-CRESTED CORMORANT EGG EXTRACT

### 3.2.1 *Dosing, Hatch, and Colony Care*

White leghorn chickens eggs were dosed with four concentrations of the double-crested cormorant egg extract delivered in a sunflower oil carrier. The double-crested cormorant (DCC) egg extract was provided by Dr. Donald Tillitt of the USGS. This extract was taken from double-crested cormorant eggs collected from Green Bay, Wisconsin in 1988, and has been analyzed previously (Powell et al. 1997). The extract contains a mixture of contaminants including PCBs, dioxins, and furans, and the principle component is PCB 126. Dose groups included control (non-inject), vehicle control (pure sunflower oil), and the following extract concentrations: 0.0625 cormorant egg-equivalents (egg-EQ), 0.125 egg-EQ, 0.1875 egg-EQ, and 0.250 egg-EQ. Egg-equivalent concentrations and corresponding TEQs and PCB 126 concentrations are shown in Table 3.2.1.1. The 0.0625 and 0.125 egg-EQ solutions, as well as the pure sunflower oil solution for vehicle control dosing, were prepared by Dr. Tillitt at USGS. The other two doses were prepared at Clemson by dilution of a 1.00 egg-EQ solution provided by Dr. Tillitt.

Table 3.2.1.1. Doses of double-crested cormorant egg extract used in the study, expressed as cormorant egg-equivalents, Toxic Equivalents, and ng/g of PCB 126.

Dose	Egg-EQ <sup>1</sup>	TEQs (pg/g) <sup>2</sup>	ng/g PCB 126 <sup>3</sup>
Control	0	0	0
Vehicle Control	0	0	0
Dose 1	0.0625	20.13	0.210
Dose 2	0.125	40.25	0.421
Dose 3	0.1875	60.38	0.631
Dose 4	0.250	80.50	0.842

<sup>1</sup>Doses expressed in units of cormorant egg-equivalent/egg. (The concentration contained in one cormorant egg would be 1 egg-EQ).

<sup>2</sup>Dose expressed in Toxic Equivalents, relative to 2,3,7,8-TCDD (in pg/g of cormorant egg)

<sup>3</sup>Doses expressed in ng/g PCB 126.

Dosing took place over two days, 10-11 September 2002. Fertilized chicken eggs were obtained from the Clemson University Morgan Poultry Center on the first day of dosing. Eggs were transported from the Poultry Center to the Clemson Institute of Environmental Toxicology (CIET), where they were randomized and assigned to dose groups. Eggs were candled to determine placement of the air cell, which was marked with a pencil, and all eggs that had cracks or displaced air cells were discarded. Eggs were weighed to one tenth of a gram. Prior to injection, the egg shell surface above the air cell was wiped with 70% ethanol and a small hole was made above the air cell using a dissection probe (sterilized by dipping in alcohol and running through a flame). Eight  $\mu$ L of the appropriate dosing solution was then injected into the air cell using a 10  $\mu$ L Hamilton® syringe. The hole was covered with a thin layer of paraffin wax. Eggs were kept at room temperature until all dosing was complete. Eggs were transported back to the Clemson University Morgan Poultry Center on the morning of 12 September where

they were placed in a commercial incubator set at 37.6°C and 58% humidity. Table 3.2.1.2 shows the number of eggs dosed per group. The number of eggs dosed per group was based on previous egg dosing experiments with PCB 126 and an initial experiment with the DCC extract.

Table 3.2.1.2. Total numbers of eggs dosed per group.

Dose Groups	# Eggs Dosed
Control	54
Vehicle Control	42
0.0625 egg-EQ	47
0.125 egg-EQ	84
0.1875 egg-EQ	102
0.250 egg-EQ	116
Total	445

Eggs were transported to a hatcher set at 37.2°C and 50% humidity on Day 18 of incubation (30 September). Eggs were candled on 19 September and 30 September to identify any non-viable eggs, which were removed at that time and examined for stage of death and any malformations. Eggs began to hatch on 3 October but were slow in their progress. Chicks were not removed from the hatcher until 4 October. Hatched birds were weighed and wing tagged for identification. They were placed randomly in a brooder and immediately given food and water. Chicks remained at the Poultry Center for the duration of the experiment. All eggs that did not hatch were examined for stage of death on 4 October and any malformations were noted. Ten control birds were sacrificed on 4 October for cloacal swab analysis. Euthanasia was accomplished by placement in a CO<sub>2</sub> chamber.

Food and water were given *ad libitum* and checked daily. The food was a non-medicated, standard chicken feed prepared at the Clemson University Morgan Poultry Center. Cage liners were changed daily and birds were observed for any signs of distress. Weights were taken weekly on each chick.

Chicks were euthanized for necropsy in two groups, at either two weeks or five weeks of age. Chicks were randomly assigned to one of these age groups. Two-week necropsies took place 16-18 October and five-week necropsies were done 6-8 November. Necropsies took place over three days because of the large number of birds that had to be processed. Ten birds in each dose group were sacrificed at both necropsies, with the exception of 12 control birds being sacrificed at two weeks.

Birds were euthanized by CO<sub>2</sub> chamber. Immediately following euthanasia, a blood sample was taken by heart stick and placed in an EDTA tube. These samples were centrifuged to separate the plasma from blood cells. One ml of plasma was put into each of two cryovials when total volume permitted. If volume was inadequate, one cryovial was filled to one ml of plasma and the remainder placed in the second vial. These samples were then placed in a -80°C freezer. Following blood collection, cloacal swabs were taken from the birds for intestinal flora analysis,

as described in Appendix I. The organs were then harvested from the birds and weighed. The brain, liver, heart, right and left thymi, and bursa were harvested from all birds and gall bladders were taken from the five-week birds. Half of the brain and the entire heart were placed in a single container of 10% buffered formalin solution. The other half of the brain and the entire liver were wrapped in foil and placed in a -80°C freezer. Gall bladders were placed in cryovials and stored in the -80°C freezer. The bursa and thymi were disposed of after weights were recorded. Sex of the birds was recorded at five-weeks of age and was determined by examining gonads.

In addition to these procedures, thymocyte and bursal cell density was calculated at the two-week necropsy, as described in Section 3.6. Analysis of all immune function tests was done at Wright State University. One cryovial of plasma was sent to Michigan State University for thyroid hormone and vitamin analysis. The remaining cryovials will be sent to the University of Florida for estrogen, testosterone, and vitellogenin analysis. Intestinal bacteria and liver enzyme levels were analyzed at Clemson University.

Dose groups were analyzed for significant differences in both mortality and deformities. A chi-square analysis was used to determine significance in mortality data and Fisher's Exact test was used to determine significant differences in incidence of deformities.

### 3.2.2. *Growth and Organ Weights*

Birds were weighed at hatch and each following week until they were euthanized. Therefore, three weights were obtained for all birds and six weights were recorded for the half euthanized at five weeks of age. Due to higher than expected hatchability in several of the dose groups, there was a group of birds that were not included in the necropsies. However, their weights were recorded throughout the experiment and they were included in this analysis. All weights were recorded to one tenth of a gram. Mean growth weights at each week as well as mean weight gain (percent of body weight at hatch) were analyzed among dose groups. Influence of gender on bird weight was also analyzed in the five-week-old birds.

All organs harvested at both necropsies had weights recorded to one thousandth of a gram. These organs included the brain, liver, heart, left and right thymi, and bursa. Gall bladders were not weighed. Mean relative organ weights (organ mass/body mass) were analyzed for significant differences among dose groups.

A Kruskal-Wallis test was used to determine overall significant differences among dose groups. A multiple comparison test was used to determine which dose groups were significantly different from one another.

### 3.2.3 *Vitamin Levels*

Vitamin D levels were analyzed in the plasma samples using the DiaSorin 25-OH-D assay. This procedure measures the amount of 25-hydroxyvitamin D (25-OH-D) and other hydroxylated vitamin D metabolites in plasma to assess vitamin D sufficiency.

Vitamins A and E were not measured in this experiment as they were in the PCB 126 study. No significant differences were found among dose groups for these vitamin levels in the PCB 126 study and because plasma samples would have to be combined to obtain enough material to test, it was determined not to examine these endpoints for this study.

A Kruskal-Wallis test was used to determine overall significant differences among dose groups. A multiple comparison test was used to determine which dose groups were significantly different from one another.

### 3.2.4 *Thyroid Hormones*

Thyroid hormones were measured in plasma taken at the two-week and five-week necropsies as an indicator of thyroid function. The thyroid hormones measured were T<sub>3</sub> (triiodothyronine), T<sub>4</sub> (thyroxine), and free T<sub>4</sub>. All analyses were done at Michigan State University using radioimmunoassays. A Kruskal-Wallis test was used to determine overall significant differences among dose groups and a multiple comparison test was used to determine which dose groups were significantly different from one another.

### 3.2.5 *Corticosterone*

The ACTH stimulation test was used as a measure of the stress response. During normal stress, animals secrete ACTH (adrenal corticotropin hormone) from the pituitary gland, which in turn stimulates secretion of corticosterone. Exposure to toxicants can reduce the normal stress response, so the purpose of this test was to determine if the dose concentrations caused a reduction in plasma corticosterone levels.

The test was performed on five birds in each dose group prior to the five-week necropsies. An initial blood sample (T<sub>0</sub>) was obtained from the jugular vein of each bird at 29 days of age (1 November). Because limited blood volume can be taken from birds of this size at any one time, the ACTH injection was not done until 33 days of age (5 November). At this time, 2.5 IU Cortosyn® was injected into the breast muscle and a second blood sample was obtained 30 minutes post-injection (T<sub>30</sub>). In both cases, blood samples were immediately placed in EDTA tubes and centrifuged for plasma separation. Plasma was placed in cryovials and stored in a -80°C freezer until transport to Michigan State University for analysis. Plasma corticosterone levels were analyzed by a solid-phase radioimmunoassay. ACTH response was calculated as the difference in corticosterone levels between T<sub>0</sub> and T<sub>30</sub>. A Kruskal-Wallis test was used to determine overall significant differences among dose groups and a multiple comparison test was used to determine which dose groups were significantly different from one another.

### 3.2.6 Immunotoxicology

Several immune function tests were performed on the hatched birds, including the phytohemagglutinin (PHA) skin response test, the sheep red blood cell (SRBC) hemagglutination assay, and measures of immune organ mass and cell density.

The PHA skin test measures a T-cell dependent inflammatory response (Stadecker et al. 1977, Corrier and DeLoach 1990). This response was measured in birds at the age of 11 days in the interdigital skin web between the 3<sup>rd</sup> and 4<sup>th</sup> toes (Corrier and DeLoach 1990). 15 birds in each dose group were sampled. The thicknesses of the skin webs to be injected were measured using calipers with precision to 0.01mm (Dyer Company). PHA-P (Sigma, St. Louis, MO) was prepared in sterile phosphate buffered saline. Thirty microliters of 3333 µg/mL PHA (100µg) was injected sub-cutaneously using 30 gauge needles. A saline control was administered into the opposing skin web. Twenty four hours later (+/- 2 hours) the thickness of each injected skin web was again measured. The stimulation index was calculated as the change in thickness of the PHA injected skin web minus the change in thickness of the saline injected skin web.

A subset of chicks received an immunization of 0.1 ml of a 1% sheep red blood cell (SRBC) suspension in sterile normal saline at 22 days of age. Blood samples (1% or less of body weight) were collected at peak antibody titer (about six days post-immunization) and total and 2-mercaptoethanol (2-ME) resistant (IgG) antibody titers were measured using a hemagglutination test adapted from that of Grasman et al. (1996). To measure total anti-SRBC titers, 50 µL of plasma from each bird was added to 50 µL of normal saline and serially diluted across one row of a 96 well, V-bottom microtiter plate. Fifty microliters of a 1% suspension of SRBC was added to each well and plates incubated at 37°C for 3 hours. To measure 2-ME resistant titers (IgG), 50 µL of plasma from each bird was added to 50 µL of 0.2M 2-ME in the first column of a 96 well, V-bottom microtiter plate and incubated at 37°C for one hour. Samples were then serially diluted across the plate in normal saline, 50 µL of a 1% suspension of SRBC was added to each well and plates incubated at 37°C for 3 hours. Antibody titers were noted as the highest dilution showing agglutination (spreading of SRBC around the well as opposed to a round button of SRBC in the bottom of the well). SRBC from the same sheep were used for both immunization and hemagglutination. All samples were assayed in duplicate. IgM (2-ME sensitive) titers were calculated by subtracting IgG titers from total titers.

At time of necropsy of 12-14 day old chicks, counts of thymocytes and bursal cells were made. Chicks were euthanized over days 12-14 of the experiment, therefore chicks varied in age at time of cell counts. Individual left thymi or bursae were homogenized in 1.5ml of phosphate buffered saline in a 2ml Kontes tissue grinder. Lymphoid cells were counted using trypan blue exclusion on a hemacytometer at 400X magnification.

Using Statistica version 5 (Statsoft, Tulsa, OK), ANOVA with Tukey's test and regression were used to analyze the endpoints. The non-parametric Jonckheere test was used to detect significant dose-response trends.

### 3.2.7 *Cytochrome P450*

Cytochrome P450-1A activity was measured in liver samples using the EROD assay. All analyses were done at Clemson University. Liver samples were kept at -80° C until use. After thawing, tissues were homogenized in sucrose/Tris-HCl buffer (pH 7.8) with a mechanical tissue homogenizer. Microsomes and cytosol were separated by differential centrifugation (100,000 g) and were stored at -80° C in buffered media with 20 % glycerine. Protein content of the samples was measured with the bicinchoninic colorimetric assay, using bovine serum albumine as a standard (Pierce).

Cytochrome P450-1A activity was measured with ethoxyresorufin as a substrate (Pohl & Fouts, 1980). Microsomes (100 µg/ml) were incubated with substrate at room temperature and fluorescence of the increasing product resorufin was measured over 30 min in a 96-well plate reader, using 530 and 585 nm as excitation and emission wavelengths.

Significant differences ( $p < 0.05$ ) between treatment groups were detected using one-way ANOVA on log transformed data. Significant linear increases were measured as a post-hoc test, using Prism software.

### 3.2.8 *Intestinal Bacteria*

This endpoint was examined as a master's research project by Faith Wiley. Preliminary results from the PCB 126 study were reported in the Phase I Report. Final results of the project, including all field and laboratory data, are reported in Ms. Wiley's thesis, which has been included here as Appendix I. All materials and methods, as well as an introduction to the project, are included in the thesis.

## SECTION 4.0

### RESULTS AND DISCUSSION

#### 4.1. BALD EAGLES

##### 4.1.1 *Vitamin Levels*

No significant correlations were observed between plasma Vitamin A (retinol, retinyl palmitate, or beta-carotene levels) or Vitamin E (alpha tocopherol) and total PCB concentrations. Significant differences were observed between retinyl palmitate, alpha tocopherol (Vitamin E) and *p,p'*-DDE concentrations. Retinyl palmitate was correlated with 4,4'-DDE concentration with the non transformed data ( $p=0.0058$ ) (Figure 4.1.1.1.). Both log transformed and ranked data showed significant positive correlations between *p,p'*-DDE concentrations and alpha tocopherol (Vitamin E) levels ( $p=0.0136$  and  $p=0.0020$  respectively) (Figure 4.1.1.2). No correlations were observed between *p,p'*-DDE concentrations and plasma retinol or beta-carotene levels.

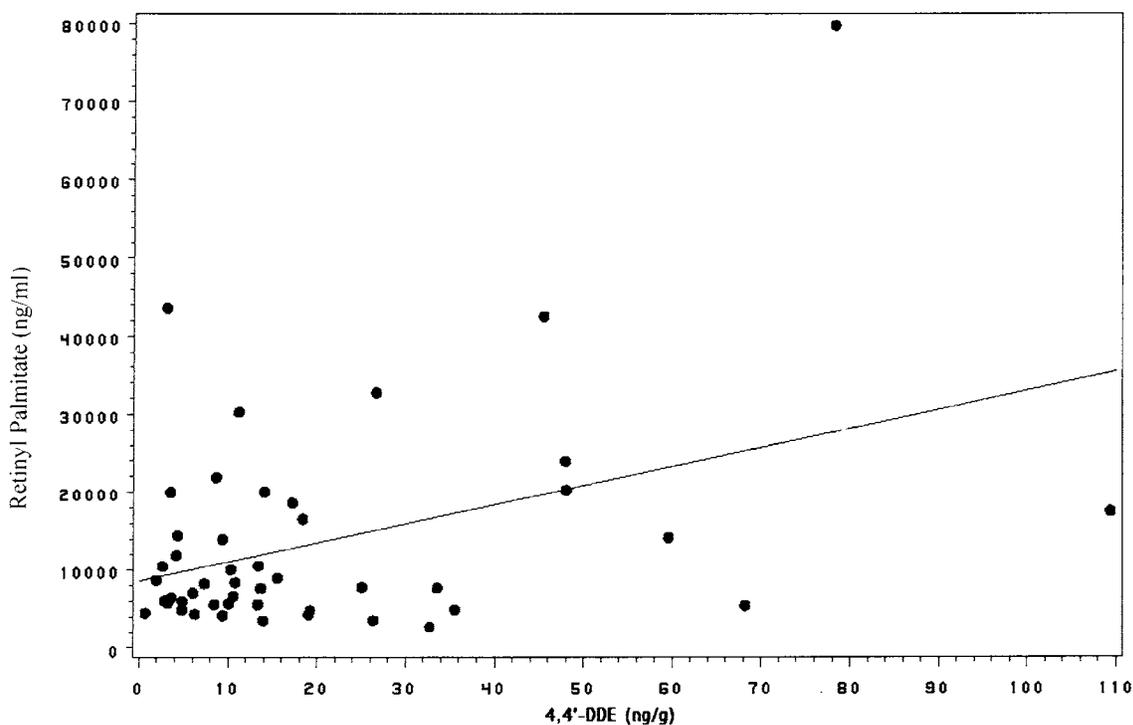


Figure 4.1.1.1. Correlation between *p,p'*-DDE and plasma retinyl palmitate levels.

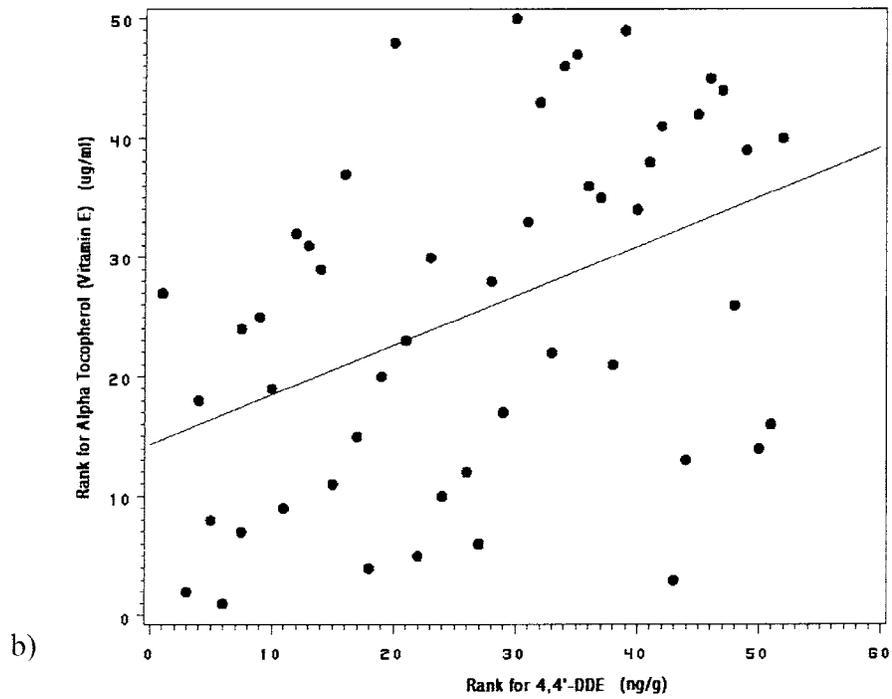
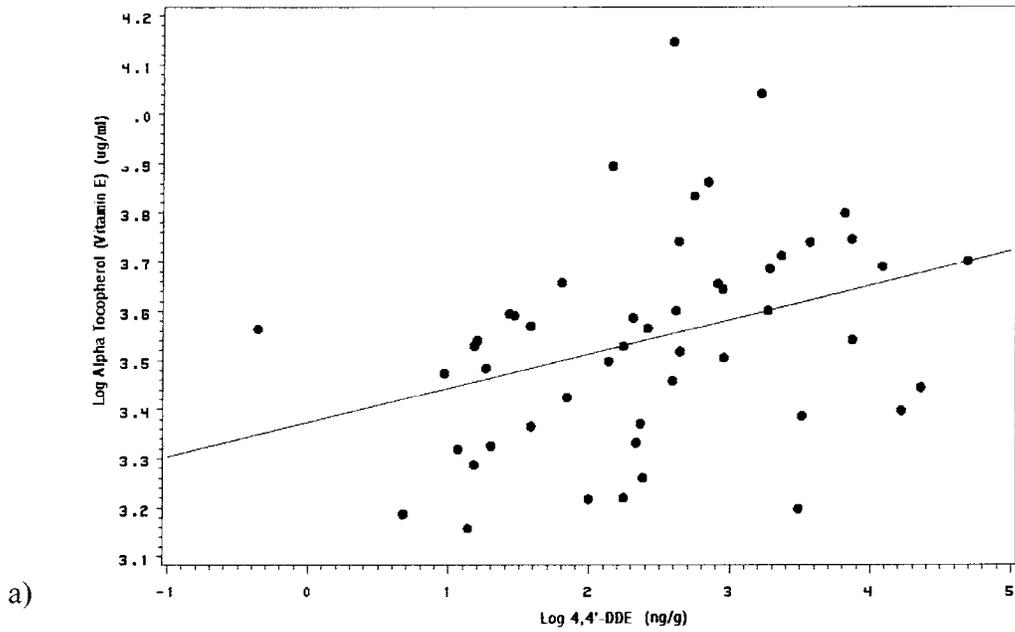


Figure 4.1.1.2. Correlations between  $p,p'$ -DDE concentrations and plasma alpha tocopherol (Vitamin E) levels, a) log transformed data, b) ranked data.

#### 4.1.2. *Thyroid Hormone Levels*

No significant correlations were seen between total PCB or 4,4'-DDE concentrations and circulating thyroid hormone levels. However, with increasing contaminant concentrations, total T4 (TT4) levels tended to generally increase and free T4 (FT4) levels tended to decrease. There were significant correlations between the ratio of TT4/FT4 and both total PCBs and 4,4'-DDE. The TT4/FT4 ratio increased with increasing total PCB concentration for both log transformed data ( $p=0.0219$ ) and ranked data ( $p=0.0345$ ) (Figure 4.1.2.1). The TT4/FT4 ratio also increased with increasing 4,4'-DDE concentration for log transformed ( $p=0.0478$ ) and ranked data ( $p=0.0126$ ) (Figure 4.1.2.2).

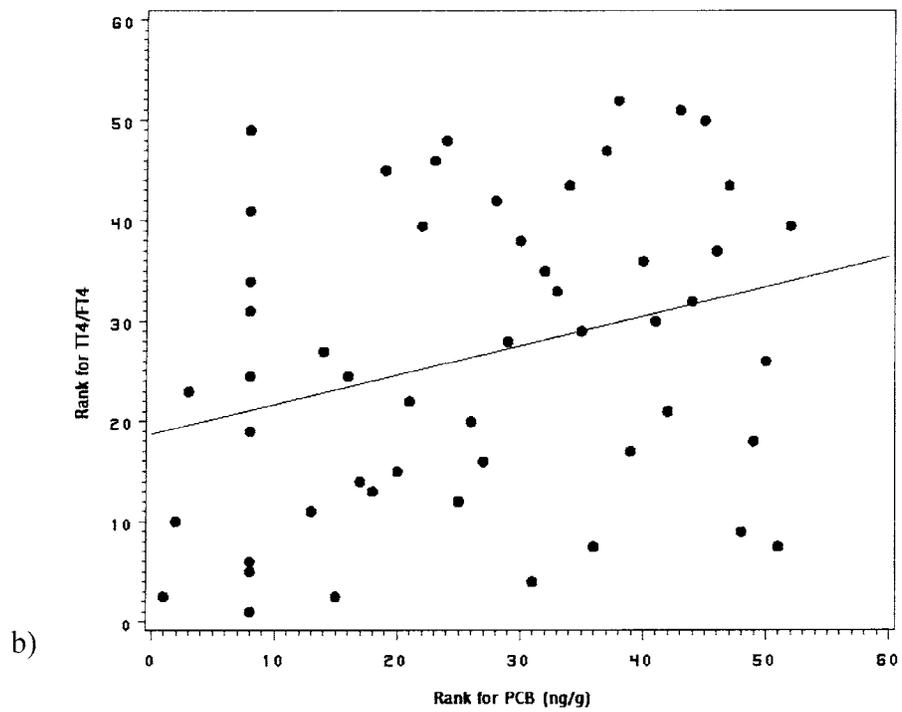
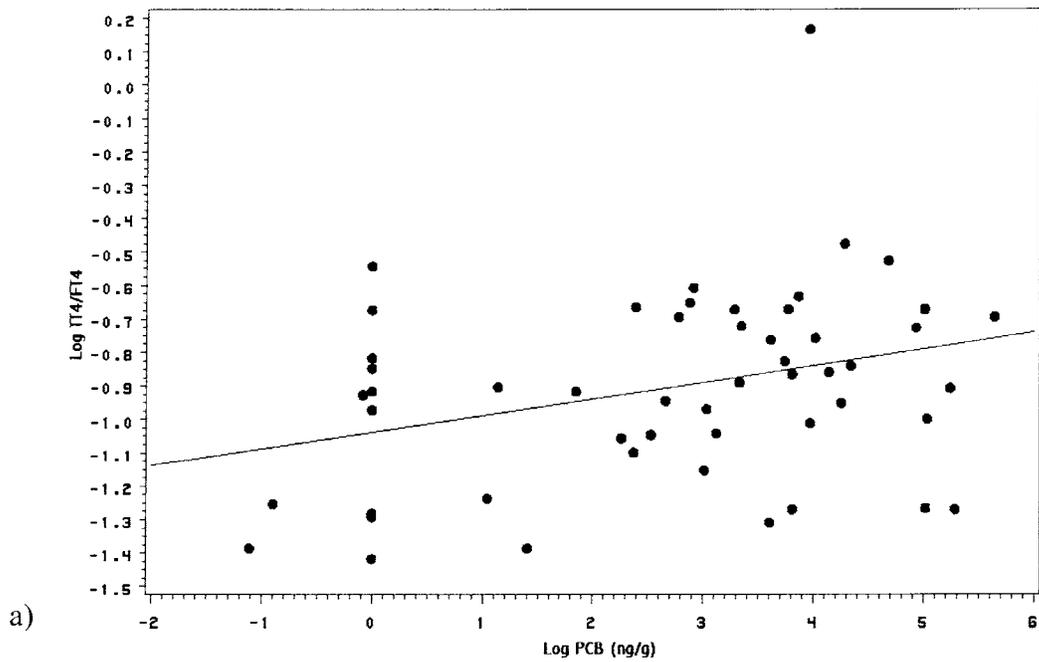


Figure 4.1.2.1. Correlation between total PCB concentration and ratio of total T4/free T4, a) log transformed data, b) ranked data.

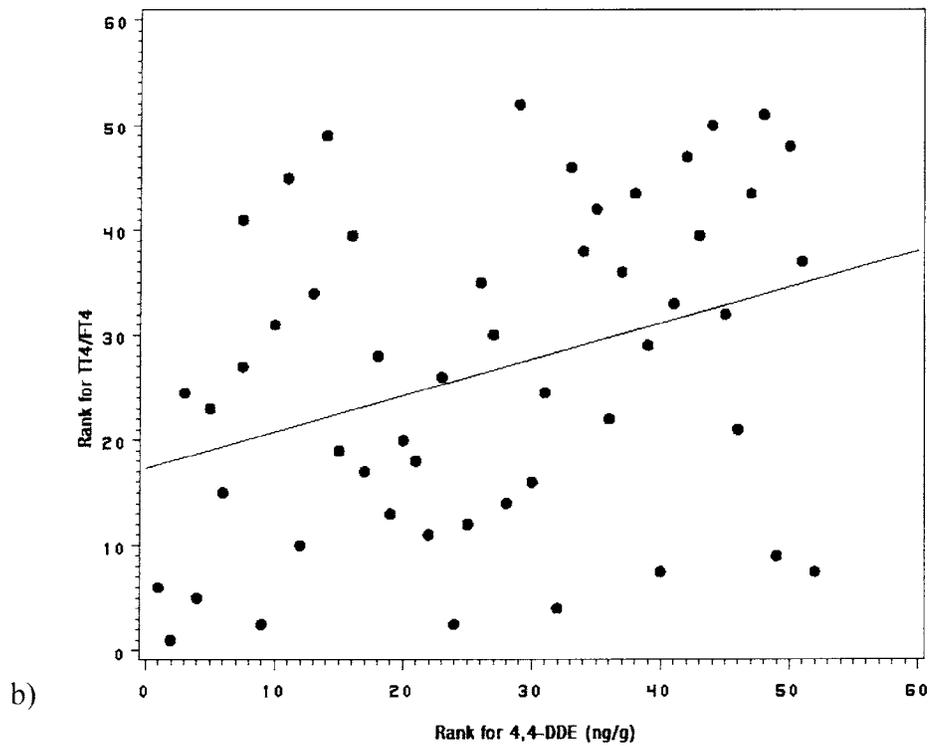
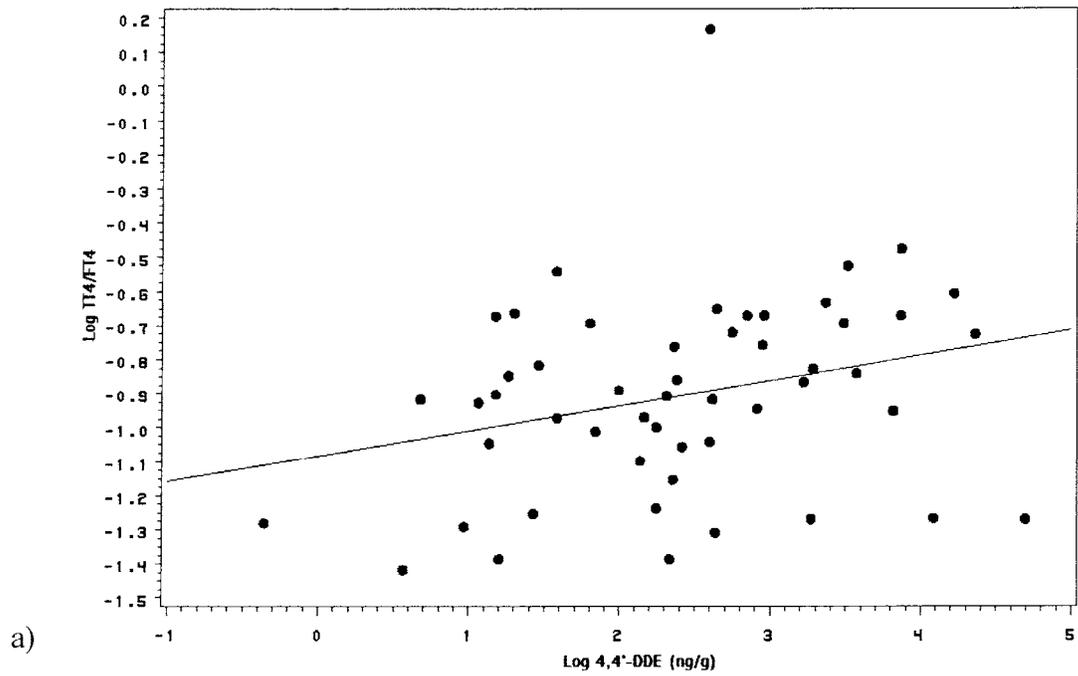


Figure 4.1.2.2. Correlations between  $p,p'$ -DDE and ratio of total T4/free T4, a) log transformed data, b) ranked data.

#### 4.1.3. *Corticosterone Levels*

Significant correlations were observed between ACTH response and *p,p'*-DDE concentration. Both non-transformed and ranked data correlations showed an increasing ACTH response with increasing *p,p'*-DDE concentration ( $p=0.0441$  and  $p=0.0344$ , respectively) (Figure 4.1.3.1). Correlations with total PCB concentrations demonstrated the same trends, although they were not significant.

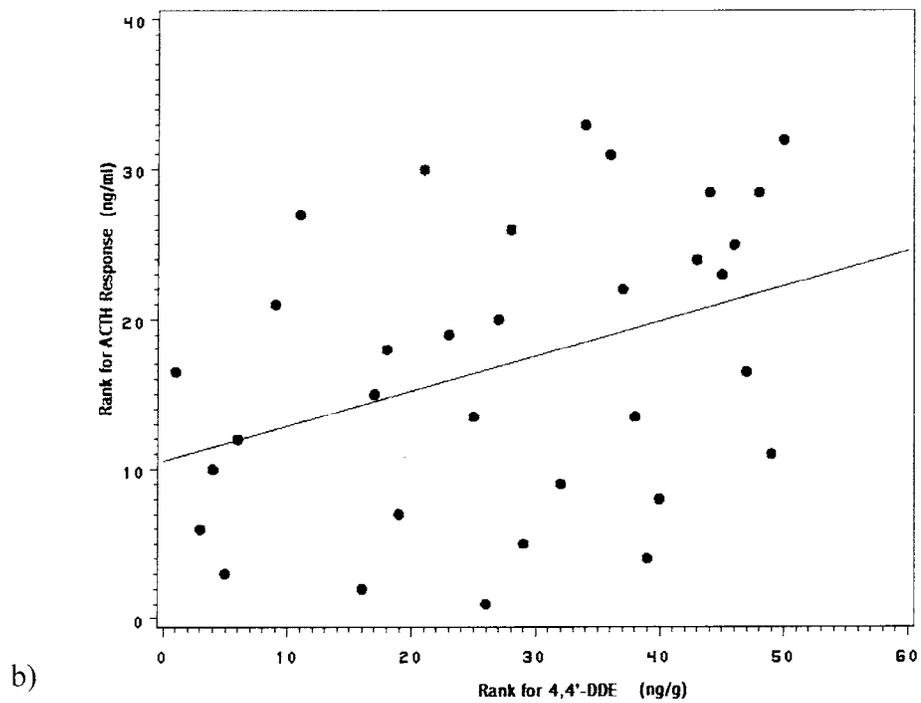
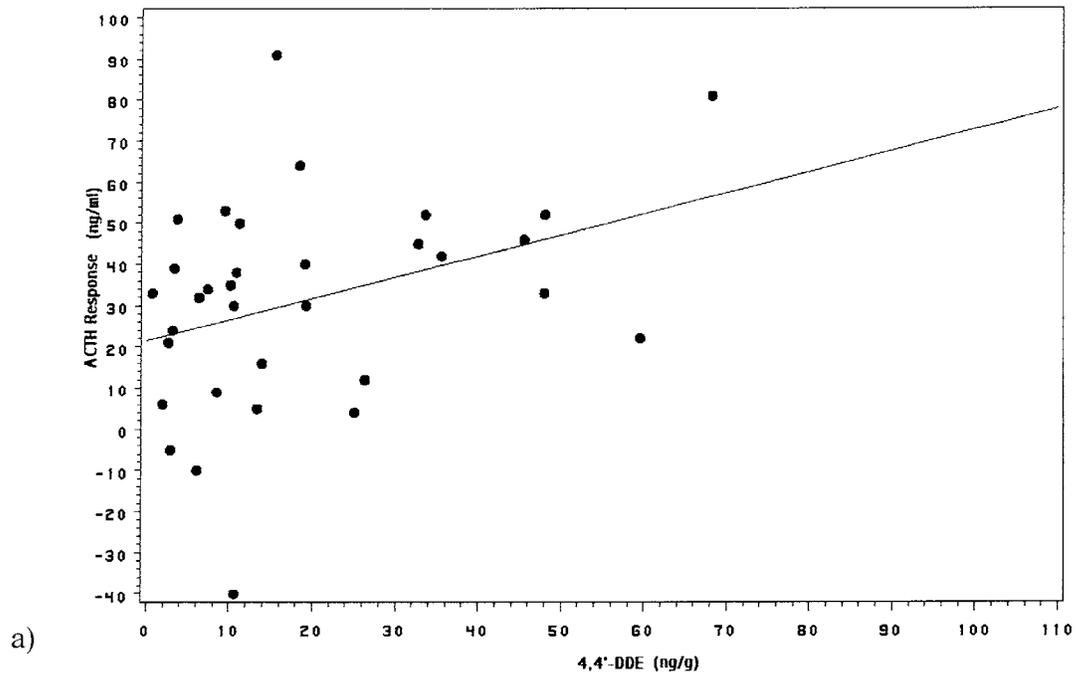


Figure 4.1.3.1. Correlations between 4,4'-DDE concentration and ACTH response, a) non transformed data, b) ranked data.

## 4.2. CHICKENS – DOUBLE-CRESTED CORMORANT EGG EXTRACT

### 4.2.1 *Dosing, Hatch, and Colony Care*

Significant differences were seen in observed versus expected hatchability for several dose groups. Numbers of observed and expected birds and percent mortality are shown in Table 4.2.1.1.

Table 4.2.1.1. Observed versus expected hatchability by dose group with significance determined by Z-statistic.

Dose Group	Alive Observed	Dead Observed	Alive Expected	Dead Expected	Z-obs	% Mortality
Control	34	20	30	24	1.095	37.0
Vehicle Control	22	20	20	22	0.618	47.6
0.0625 egg-EQ	29	18	20	27	2.655*	38.3
0.125 egg-EQ	35	49	20	64	3.843*	58.3
0.1875 egg-EQ	27	75	20	82	1.746	73.5
0.250 egg-EQ	30	86	20	96	2.458*	74.1

\*Indicates significant differences in observed versus expected values with Z-critical value of  $\pm 1.96$  ( $\alpha = 0.05$ ).

Several deformities were noted in the egg break-out analysis. Types of deformities included edema, gastroschisis, crossbill, limb and eye malformations, and small body size. Incidence of deformity increased with higher dose concentrations, as shown in Table 4.1.2.

Table 4.2.1.2. Total number of deformities per dose group.

Dose Groups	# of Birds with Deformity	% of total
Control	2	3.70
Vehicle Control	1	2.38
0.0625 egg-EQ	2	4.26
0.125 egg-EQ	6	7.14
0.1875 egg-EQ	13	12.75
0.250 egg-EQ	17	14.66
Total	41	9.21

Both percent mortality and percent of birds with deformities increased with the concentration of the extract. The 0.125, 0.1875, and 0.250 egg-EQ groups all had significantly higher mortality than the control group and the 0.1875 and 0.250 egg-EQ groups had significantly higher mortality than vehicle control. The 0.250 egg-EQ group had a significantly higher percentage of birds with deformities than both the control and vehicle control groups.

#### 4.2.2 Growth and Organ Weights

##### Growth Weights

No significant differences were observed among dose groups for weight at hatch ( $\chi^2 = 4.1976$ ,  $df=5$ ,  $p=0.5213$ ), which is consistent with results obtained in the PCB 126 dosing study. Based on these experiments, hatch weight does not seem to be affected by contaminant burden, at least at the concentrations used in these studies. Significant differences were observed among groups at week one ( $\chi^2 = 12.7827$ ,  $df=5$ ,  $p=0.0255$ ), week two ( $\chi^2 = 13.7284$ ,  $df=5$ ,  $p=0.0174$ ), week three ( $\chi^2=21.7090$ ,  $df=5$ ,  $p=0.0006$ ), week four ( $\chi^2 = 23.1319$ ,  $df=5$ ,  $p=0.0003$ ), and week five ( $\chi^2 = 17.0872$ ,  $df=5$ ,  $p=0.0043$ ). At week one, control birds had significantly higher weights than birds in the vehicle control, 0.0625, 0.125, and 0.1875 egg-EQ dose groups. At week two, birds in the control and 0.250 egg-EQ dose groups weighed significantly more than those in the 0.125 and 0.1875 egg-EQ groups. At weeks three, four, and five, birds in the control, vehicle control, and 0.250 egg-EQ groups weighed significantly more than those in the 0.125 and 0.1875 egg-EQ groups. Mean growth weights are shown in Table 4.2.2.1.

Table 4.2.2.1. Mean growth weights per dose group (g).

Dose Group	Weight 1/ Hatch	Weight 2/ 1 Week	Weight 3/ 2 Weeks	Weight 4/ 3 Weeks	Weight 5/ 4 Weeks	Weight 6/ 5 Weeks
Control	30.36 ± 2.71	57.89 ± 6.08	98.37 ± 12.48	161.88 ± 22.10	234.11 ± 31.51	303.25 ± 30.30
Vehicle	29.41 ± 2.15	53.80 ± 6.89	93.79 ± 16.42	165.40 ± 18.73	239.30 ± 26.18	303.12 ± 30.52
0.0625 egg-EQ	31.04 ± 3.02	53.30 ± 6.84	94.06 ± 13.88	158.29 ± 17.45	227.98 ± 26.16	280.90 ± 27.34
0.125 egg-EQ	30.78 ± 2.38	52.69 ± 6.30	91.44 ± 11.44	143.05 ± 18.29	199.95 ± 23.58	243.96 ± 38.83
0.1875 egg-EQ	30.41 ± 2.93	51.26 ± 6.68	86.09 ± 13.88	145.42 ± 21.43	206.32 ± 35.68	271.30 ± 41.25
0.250 egg-EQ	30.78 ± 2.79	55.04 ± 6.25	98.30 ± 15.71	166.60 ± 14.71	238.77 ± 22.99	305.51 ± 37.14

The growth weights tended to decrease with dose concentration with the exception of the highest dose group, in which weights were often as high or higher than controls. These results may be partially explained by the sex ratios in this dose group. Eight out of ten birds in this group were males. Since males tend to start growing faster than females after a certain age this may have skewed the results.

The effect of sex on growth weight was examined for those birds euthanized at five weeks of age. (No sex data was obtained on the other birds). Differences in weight between males and females were examined within each dose group for each weekly weight. For all dose groups, males were larger than females starting at 3 weeks of age (weight 4). For the vehicle control and extract dosed groups, male weights were larger starting at one week of age (weight 2). Therefore, it does appear that uneven sex ratios in the dose groups may be obscuring the effects of the contaminants on growth.

Bird growth was also analyzed by looking at the weight gain (percent of body weight at hatch) for each week of the experiment. Results were similar to the analysis of growth weights. Significant differences were observed among dose groups for weight gain at week one ( $\chi^2 = 17.5652$ ,  $df=5$ ,  $p=0.0035$ ), week two ( $\chi^2 = 15.3577$ ,  $df=5$ ,  $p=0.0089$ ), week three ( $\chi^2 = 26.5513$ ,

df=5,  $p < 0.0001$ ), week four ( $\chi^2 = 30.9127$ , df=5,  $p < 0.0001$ ), and week five ( $\chi^2 = 17.6518$ , df=5,  $p = 0.0034$ ). For all weeks except week five, birds in the control, vehicle control, and 0.250 egg-EQ groups had a significantly higher weight gain than those in the 0.125 and 0.1875 egg-EQ dose groups. At week five, birds in the control and vehicle control groups had significantly higher weight gain than those in the 0.125 and 0.1875 egg-EQ groups. Figure 4.2.2.1 illustrates the trends in weight gain among the dose groups. As in the analysis of growth weights, skewed sex ratios may account for the high weight gain observed in the highest dose group.

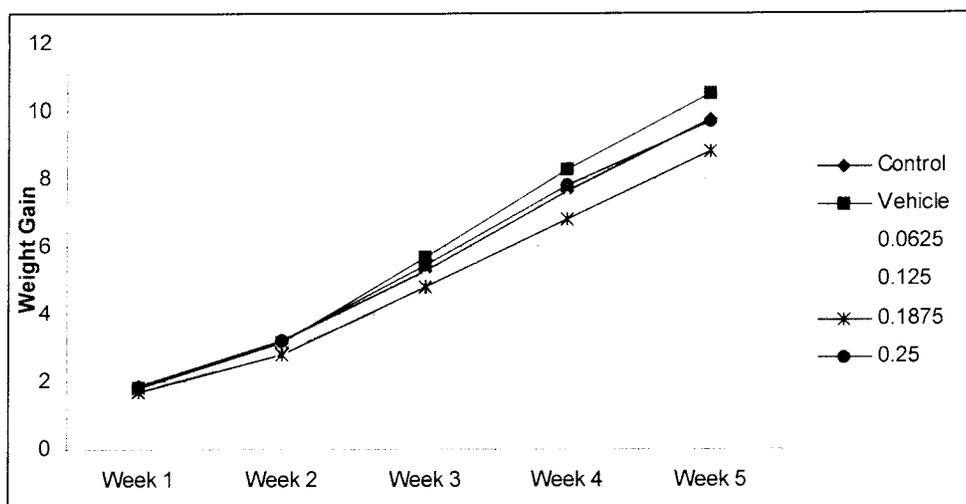


Figure 4.2.2.1. Mean weight gain (weekly weight divided by body weight at hatch) by dose group for each week of the study.

Overall, the extract does appear to negatively impact weight gain. Results from the PCB 126 study showed that this compound had a negative effect on weight gain in the highest dose groups (0.250 ng/g and 0.325 ng/g PCB 126) when birds were one week of age. The older birds, however, did not display significant differences in weight gain. The doses of extract used in the DCC study were more toxic than those in the PCB 126 study (see Table 3.2.1.1). The increased toxicity and possibly additive effects of the extract mixture may have resulted in the lower weight gains observed throughout the experiment during the DCC study.

### Organ Weights

At two weeks of age, a significant difference was observed among dose groups for relative bursa weight ( $\chi^2 = 12.6697$ , df=5,  $p = 0.0267$ ). Control birds exhibited the largest relative weights, while birds in the vehicle control and 0.1875 egg-EQ dose groups had the smallest weights. There was no dose-response relationship apparent, with the highest dose group exhibiting the highest relative weights among the extract-dosed groups. No significant differences were observed among dose groups for left thymus ( $\chi^2 = 6.1915$ , df=5,  $p = 0.2880$ ), right thymus ( $\chi^2 = 3.8001$ ,

df=5, p=0.5785), heart ( $\chi^2 = 3.0726$ , df=5, p=0.6888), liver ( $\chi^2 = 3.3159$ , df=5, p=0.6514), or brain ( $\chi^2 = 6.0897$ , df=5, p=0.2976).

At five weeks of age, a significant difference was observed among dose groups for relative weight of the left thymus ( $\chi^2 = 18.3528$ , df=5, p=0.0025). Control birds had the highest relative weights with significantly larger left thymi than birds of the vehicle control, 0.125, 0.1875, and 0.250 egg-EQ groups. There was not a clear dose response relationship among the extract-dosed birds, with the smallest thymi occurring in birds of the 0.1875 egg-EQ group. There was also a significant difference among dose groups for relative brain weights at five weeks of age ( $\chi^2 = 12.8315$ , df=5, p=0.0250). No dose response patterns were evident however; the 0.125 egg-EQ group has the largest relative brain weights and differed significantly from the control, vehicle control, 0.0625 and 0.250 egg-EQ dose groups. No significant differences were noted among dose groups for relative weights of bursa ( $\chi^2 = 1.7725$ , df=5, p=0.8796), right thymus ( $\chi^2 = 5.6216$ , df=5, p=0.3448), heart ( $\chi^2 = 6.6872$ , df=5, p=0.2450), or liver ( $\chi^2 = 0.7292$ , df=5, p=0.9813).

The DCC extract did not appear to alter heart, liver, or brain weights at either age. These results are consistent with the PCB 126 study. Some effect on immune organs was seen with the DCC extract, but it did not appear to significantly alter these organ weights. In contrast, both bursa and thymi weights were significantly lower than controls in the highest doses of the PCB 126 study at two weeks of age. Again, the interactions among the compounds in the extract are probably the cause of this discrepancy.

#### 4.2.3 Vitamins

Significant differences were observed for plasma vitamin D concentrations at both two ( $\chi^2 = 15.0155$ , df=5, p=0.0103) and five ( $\chi^2 = 13.2455$ , df=5, p=0.0212) weeks of age. Mean plasma vitamin D concentrations are shown in Table 4.2.3.1.

Table 4.2.3.1. Mean plasma vitamin D concentrations at two and five weeks post-hatch (nmol/l).

Dose Group	Two week Vitamin D	Five week Vitamin D
Control	20.92 ± 6.35	16.00 ± 3.92
Vehicle Control	16.88 ± 6.20	16.90 ± 2.56
0.0625 egg-EQ	17.11 ± 4.59	10.88 ± 2.32
0.125 egg-EQ	14.44 ± 5.41	16.10 ± 3.54
0.1875 egg-EQ	11.56 ± 3.47	13.50 ± 3.79
0.250 egg-EQ	13.50 ± 5.70	13.33 ± 2.87

At two weeks of age, plasma concentrations of vitamin D in the 0.125, 0.1875, and 0.250 egg-EQ dose groups were significantly less than controls. A general decreasing trend in vitamin D concentration occurred, which can be seen in Figure 4.3.1. In addition, the 0.1875 egg-EQ dose

group concentration was significantly less than both the vehicle control and 0.0625 egg-EQ dose groups. At five weeks of age, birds in the 0.0625 egg-EQ dose group had significantly lower plasma concentrations of vitamin D than the remaining groups. The 0.1875 and 0.250 egg-EQ dose groups contained significantly lower concentrations than vehicle control birds.

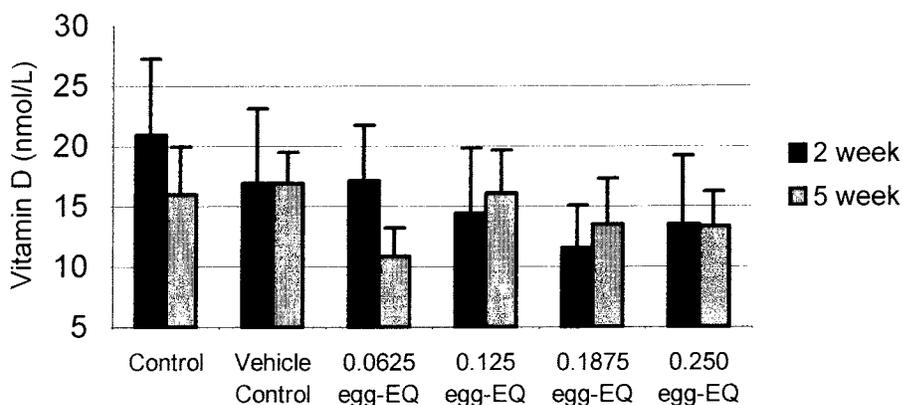


Figure 4.2.3.1. Mean plasma concentrations and standard deviations of Vitamin D (nmol/L) per dose group at two and five weeks of age.

#### 4.2.4 Thyroid Hormones

No significant differences were observed at two weeks of age for total T3 ( $\chi^2 = 1.5589$ ,  $df=5$ ,  $p=0.9062$ ), total T4 ( $\chi^2 = 2.7761$ ,  $df=5$ ,  $p=0.7345$ ), or free T4 ( $\chi^2 = 2.2062$ ,  $df=5$ ,  $p=0.8199$ ). No significant differences were observed at five weeks for total T3 ( $\chi^2 = 5.5167$ ,  $df=5$ ,  $p=0.3561$ ), total T4 ( $\chi^2 = 6.8186$ ,  $df=5$ ,  $p=0.2345$ ), or free T4 ( $\chi^2 = 7.4409$ ,  $df=5$ ,  $p=0.1899$ ). However, at five weeks of age, control birds experienced higher plasma concentrations of all thyroid hormones than the remaining groups, with significant differences between groups occurring in some cases. For total T4 concentrations, control birds had significantly higher concentrations than the 0.0625 and 0.250 egg-EQ groups. Total T3 concentrations in control birds were significantly higher than concentrations of birds in the 0.1875 egg-EQ dose group. Finally, free T4 concentrations in control birds were significantly higher than those of the vehicle control and 0.0250 egg-EQ dose groups. Mean plasma thyroid hormone concentrations are shown in Tables 4.2.4.1 and 4.2.4.2.

Table 4.2.4.1. Mean levels of Total T4, Total T3, and Free T4 in chicken plasma at two weeks of age.

Dose Group	Total T4 (nmol/l)	Total T3 (nmol/l)	Free T4 (pmol/l)
Control	19.08 ± 6.08	4.34 ± 0.32	10.92 ± 6.99
Vehicle Control	18.60 ± 6.60	4.26 ± 0.53	10.40 ± 6.50
0.0625 egg-EQ	18.60 ± 4.48	4.30 ± 0.63	11.80 ± 4.21
0.125 egg-EQ	22.11 ± 8.81	4.06 ± 0.76	10.56 ± 4.50
0.1875 egg-EQ	19.40 ± 9.08	4.22 ± 0.59	10.70 ± 6.55
0.250 egg-EQ	22.20 ± 6.53	3.82 ± 0.92	13.60 ± 7.03

Table 4.2.4.2. Mean levels of Total T4, Total T3, and Free T4 in chicken plasma at five weeks of age.

Dose Group	Total T4 (nmol/l)	Total T3 (nmol/l)	Free T4 (pmol/l)
Control	24.60 ± 3.92	3.52 ± 0.64	13.20 ± 4.78
Vehicle Control	20.40 ± 4.55	3.14 ± 0.83	7.80 ± 3.49
0.0625 egg-EQ	19.90 ± 4.82	3.05 ± 0.65	9.60 ± 2.80
0.125 egg-EQ	20.90 ± 6.47	3.12 ± 0.37	9.90 ± 4.95
0.1875 egg-EQ	21.10 ± 6.95	2.93 ± 0.50	9.30 ± 4.22
0.250 egg-EQ	18.90 ± 4.72	3.23 ± 0.74	9.10 ± 5.57

The extract appears to cause a decrease in circulating thyroid hormones in birds five weeks of age, although the loss of plasma concentration of these hormones is was not significant and not dose-dependent.

#### 4.2.5 Corticosterone

No significant differences were seen among dose groups for ACTH response ( $\chi^2 = 4.9821$ ,  $df=5$ ,  $p=0.4181$ ). Although ACTH response values varied among dose groups, no obvious trend is apparent and no individual dose groups were significantly different from the others. Mean values for ACTH response are shown in Table 4.2.5.1.

Table 4.2.5.1. Mean ACTH response by dose, measured as the difference in corticosterone levels between T<sub>0</sub> and T<sub>30</sub>.

Dose Groups	ACTH response (ng/ml)
Control	80.00 ± 24.05
Vehicle Control	68.80 ± 23.97
0.0625 egg-EQ	87.60 ± 28.09
0.125 egg-EQ	96.60 ± 13.79
0.1875 egg-EQ	79.00 ± 46.53
0.250 egg-EQ	67.40 ± 38.41

#### 4.2.6 Immunotoxicology

##### *Immune Function Tests*

The extract did not cause significant changes in total anti-SRBC titers (ANOVA p=0.75) but mean titers in 0.0625 and 0.125 egg-EQ groups were 1.5 units (~3 fold) higher than controls or the two higher treatment groups (Figure 4.2.6.1). The IgM titer for the birds from the 0.250 egg-EQ group was significantly lower than the 0.1875 egg-EQ group and marginally lower than both controls (Tukey's p=0.01 and 0.09 respectively). However, the mean IgM titer for the 0.250 egg-EQ group was negligible (mean = -0.2) and thus the mean IgG titer appeared to account for the entire mean total titer. IgG titers were not significantly different between groups (ANOVA p=0.66). Mean IgG titers in 0.0625, 0.125, and 0.250 egg-EQ groups were 2-4 fold higher than controls; the 0.1875 egg-EQ titer was similar to controls. The magnitude of these increases in anti-SRBC titer are likely to be biologically significant but low sample sizes per group (n=6 or 8) yielded little statistical power. PHA skin response in 11 day old chicks was not affected by DCCO egg extract (ANOVA p=0.30, Table 4.2.6.1).

##### *Immune Organs*

The DCC extract did not cause any significant alterations in thymus or bursa mass or cellularity in chicks aged 14 (Figure 4.2.6.2 and 4.2.6.3) or 35 days (Table 2). There were significant effects of age on thymus cellularity and density (2-way ANOVA p<0.05) and marginal effects on bursa cellularity (2-way ANOVA p=0.08). Thymocyte cellularity was significantly greater in 14 day old chicks compared to 12 and 13 day old chicks (64 and 52% greater respectively, Tukey's p<0.05). Thymocyte cellularity was significantly greater in 14 day old chicks compared to 12 and 13 day old chicks (35% and 64% greater respectively, Tukey's p<0.05). Bursal cellularity was marginally greater in 14 day old chicks compared to 12 and 13 day old chicks (88 and 16% greater respectively, Tukey's p=0.056). Dissections were made in 12-14 day old birds instead of only 14 day old birds because the large number of birds (60) to be euthanized could not be accomplished in one day. The intention was not to detect differences in immune organ structure between different age birds therefore there were low samples sizes for each day of dissection.

The egg extract did not affect the thymus or bursa mass at day 35 (Table 4.2.6.2). The mean thymus index for the 0.1875 egg-EQ group was significantly less than the control but not significantly less than the vehicle control (Tukey's  $p=0.005$  and  $p=0.47$  respectively). However, in regression analysis using TEQ/g egg, dose did explain 9% of the data ( $R=-0.30$ ,  $R^2=0.09$ ,  $p=0.02$ ).

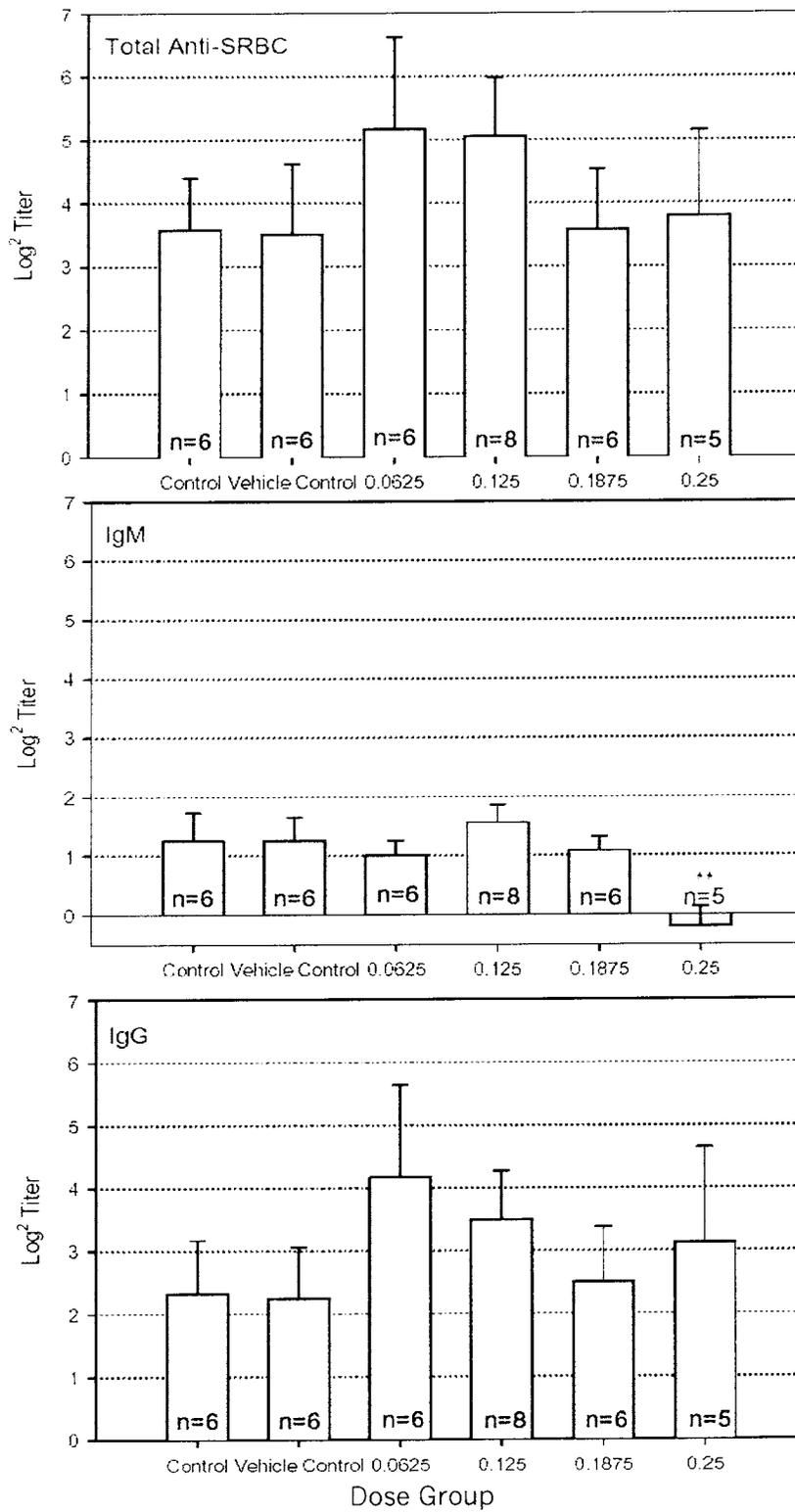


Figure 4.2.6.1. Anti-SRBC total, IgM and IgG titers of 28 day old chicks after *in ovo* exposure to organochlorines extracted from double crested cormorants. \*\* Denotes significant decrease from control and vehicle control.

Table 4.2.6.1. PHA skin test response (interdigital) of 11 day old chicks and thymus and bursa index in 35 day old chicks after *in ovo* exposure to organochlorines extracted from double crested cormorant eggs.

Dose Group	PHA Skin Response		Thymus Index		Bursa Index	
	n	Mean Response (mm) (standard error)	n	Mean (standard error)	n	Mean (standard error)
Control	15	0.56 (0.04)	10	0.532 (0.083)	10	0.458 (0.08)
Vehicle Control	15	0.57 (0.04)	10	0.455 (0.063)	10	0.445 (0.07)
0.0625 egg-EQ	15	0.53 (0.03)	10	0.459 (0.1)	10	0.448 (0.1)
0.125 egg-EQ	15	0.58 (0.05)	10	0.462 (0.091)	10	0.431 (0.094)
0.1875 egg-EQ	15	0.65 (0.05)	10	0.385 (0.1)	10	0.44 (0.13)
0.250 egg-EQ	16	0.52 (0.04)	10	0.441 (0.075)	10	0.49 (0.12)

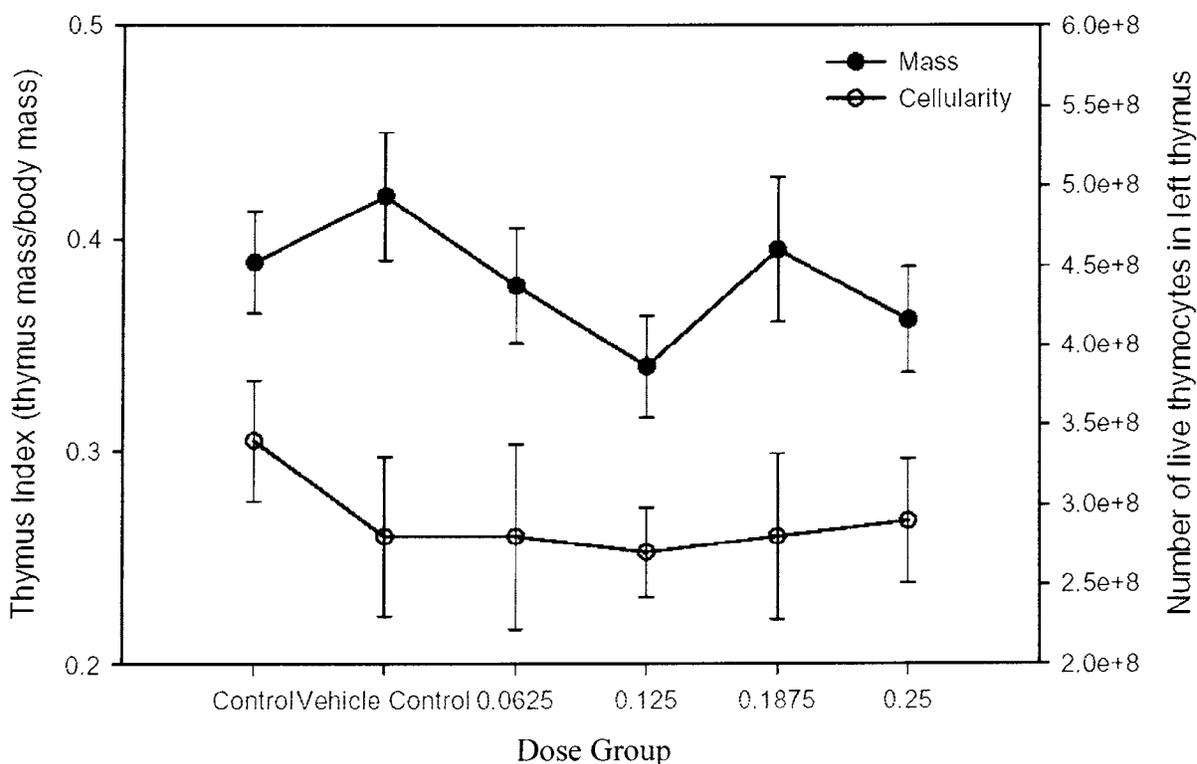


Figure 4.2.6.2. Thymus index and cellularity in chicks aged 13-15 days exposed *in ovo* to organochlorines extracted from double crested cormorant eggs. Plots represent mean +/- S.E.

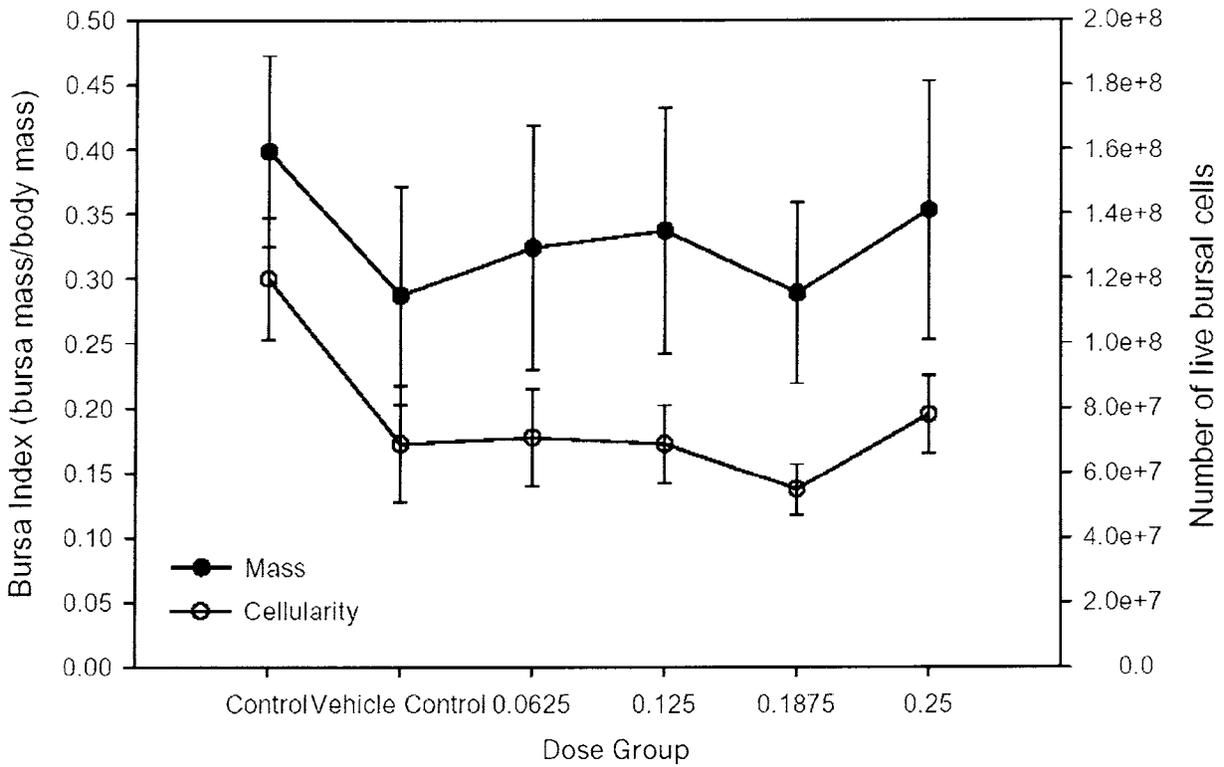


Figure 4.2.6.3. Bursa index and cellularity in 13-15 day old chicks exposed *in ovo* to organochlorines extracted from double crested cormorant eggs. Plots represent mean +/- S.E.

Table 4.2.6.2. Day 35 thymus and bursa masses of chicks exposed *in ovo* to organochlorines extracted from double crested cormorant eggs.

Dose Group	Thymus Index		Bursa Index	
	n	Mean (standard error)	n	Mean (standard error)
Control	10	0.532 (0.083)	10	0.458 (0.08)
Vehicle Control	10	0.455 (0.063)	10	0.445 (0.07)
0.0625 egg-EQ	10	0.459 (0.1)	10	0.448 (0.1)
0.125 egg-EQ	10	0.462 (0.091)	10	0.431 (0.094)
0.1875 egg-EQ	10	0.385 (0.1)	10	0.44 (0.13)
0.250 egg-EQ	10	0.441 (0.075)	10	0.49 (0.12)

### Discussion

*In ovo* exposure to the DCC extract at the dose range studied did not cause any significant immunotoxicity to juvenile chickens at the ages tested. In other egg injection studies with chickens, 0.1 to 0.5 ng/g PCB 126 decreased antibody responses but no decreasing trends were noticed in this data set. In fact, 0.0625 and 0.125 egg-EQ doses of DCC extract caused non-significant two-three fold increases in mean anti-SRBC response. This elevation in antibody response may have been significantly different from controls given higher sample sizes and is comparable to increased antibody titers observed in wild pre-fledgling Caspian terns. Caspian terns from Saginaw Bay had non-significantly elevated (0.5 – 2 titer units) antibody responses in 1992-1994, and in 1997-1999 had significantly elevated (1.3 titer units or 2.5 fold) antibody responses compared to terns from the less contaminated colony on Elm Island in the North Channel of Lake Huron (Grasman et al. 1996, Grasman and Fox 2001). An elevation in antibody response could denote an inappropriate stimulation of immune function or an imbalance in the immune system. Although studies of rodents exposed to organochlorines usually results in decreased antibody titers, juvenile chickens exposed to 50 parts per million DDT in feed for two weeks had elevated antibody titers after immunization with heat-killed *Salmonella pullorum*. Tissue concentrations of DDT were 3.38 +/- 1.02 parts per million in liver of the birds (Latimer and Siegel 1974).

Neither thymus nor bursa mass was significantly altered by DCC extract. However, age of birds affected thymocyte and bursal cellularity with 14 day old birds having higher numbers of cells than 12 and 13 day old birds suggesting that a day to day growth of the chicks influences thymus cellularity or a significant proliferation event or influx occurs at day 14. There appeared to be an effect of vehicle (sunflower oil) on immune organ parameters and male chick's weight gain. This has not been noted in previous studies using sunflower oil in air-cell injections of chicken eggs (Fox and Grasman 1999, Grasman and Whitacre 2001). One egg equivalent of DCC extract caused a significant decrease in bursa mass in 3 week old birds but 0.1 egg-EQ and lower did not (Powell et al. 1997). Therefore, the lack of bursal atrophy in this study in two-week-old birds agrees with Powell's study. In studies of *in ovo* exposure to PCB 126 only, immune organ atrophy occurs at 0.128 ng/g and higher in day 20 embryos and eventually recovers to control levels in six week old chicks (Fox and Grasman 1999).

The TEQ doses and toxic effects can be compared to doses of PCB 126 studied in earlier experiments and doses of TCDD used in other avian toxicity studies to compare and contrast effects on immune function, mortality, deformities and growth. Comparing approximate concentrations of PCB 126 in the DCC extract to doses of PCB 126 known to be immunotoxic in juvenile chickens, the 0.0625 egg-EQ dose contains an average of 0.19 ng/g PCB 126 and 0.26 ng/g PCB 126 TEQs per egg and was therefore most similar to the 0.175 or 0.25 ng/g doses of PCB 126 studied previously. The 0.175 and 0.25 ng/g doses of PCB 126 caused significant decreases in antibody response. Thus, we predicted to see decreased antibody responses even at the lower TEQ doses tested, but this was not the case. The presumption is, therefore, that non-ortho substituted PCBs (e.g. PCB 153) in the mixture acted antagonistically with the PCB 126 (Harper et al. 1995). Adjusting the doses to actual per gram egg doses of extract (rather than TEQ per egg) and using regression analysis did not reveal any significant associations between extract dose and immune function except in thymus mass in 35 day old birds, mentioned

previously. Therefore the variability in egg mass did not contribute to the lack of significance in antibody response.

In conclusion, the dose range of DCC extract studied here did not significantly alter immune function even though Great Lakes birds from colonies of high organochlorine contamination have consistently suppressed T-cell mediated immune responses and Caspian terns have elevated antibody responses. Nonetheless, the elevated antibody response in Caspian terns was similar to the non-significant elevation in antibody response in chickens in this experiment. The combined exposure to these contaminants *in ovo* and through food post-hatch may be driving immune suppression in Great Lakes birds rather than only developmental (*in ovo*) exposure which was modeled in this study.

#### 4.2.7 Cytochrome P450

The EROD data show there was no significant difference between the controls and the vehicle controls (oil) for both the two week and five week sample series. The two week data show a significant increase with increasing egg-equivalent doses. This increase appears to be linear, with a  $R^2$  of 0.75, and a  $p < 0.0001$ . After five weeks, there are still significant differences between treatments ( $p < 0.03$ ), and, even though the correlation is small ( $R^2 = 0.33$ ), the linear increase is still significant ( $p = 0.005$ ). Data from two and five week birds is shown in Figure 4.2.7.1. From these data it is clear that the DCC egg extract contains compounds that are strong cytochrome P450-1A (CYP1A) inducers. The 10-20 fold induction in the highest dose group in the week two samples is probably not a maximal induction. In other species, induction of two orders of magnitude has been observed upon dosage of good inducers. The decline of the CYP1A activity after five weeks may be attributed to excretion, biotransformation or sequestering in adipose tissue of the inducing agents.

## EROD-CCextract

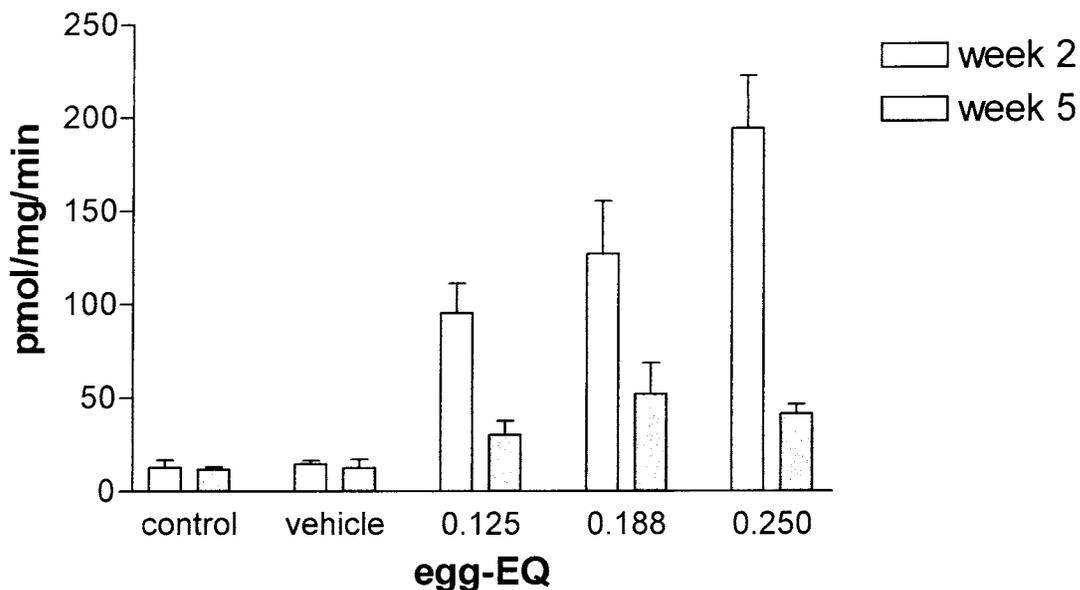


Figure 4.2.7.1. EROD activity per dose group as measured in the livers of two and five week old chickens.

Compared to the first laboratory experiment using PCB 126, the DCC egg extract doses were stronger inducers of CYP1A. It is unclear whether this is due to the higher concentrations of PCB 126 in the extract (see Table 3.2.1.1) or whether the other components of the mixture may have had an influence. It may be a combination of the higher PCB 126 and the other compounds that caused the increase in activity.

### 4.2.8 Intestinal Bacteria

See Appendix I

## SECTION 5.0

### FINAL CONCLUSIONS AND RECOMMENDATIONS

#### *Vitamin Levels*

We found that vitamin A, measured as plasma retinyl palmitate, was significantly positively correlated with *p,p'*-DDE in the bald eagle samples. Similar correlations were seen with total PCBs, although these results were not significant. Elliot et al. (2001) and Murroll et al. (1999) observed positive correlations between plasma retinol and PCB concentrations in osprey and shag chicks, respectively. Although plasma retinol was not significantly correlated to contaminant concentrations in this study, plasma retinyl palmitate may have some promise as a potential biomarker for eagles. Unfortunately, retinyl palmitate was undetectable in the chicken samples and thus these results could not be confirmed with the laboratory experiments.

We also found that plasma vitamin E (alpha tocopherol) levels were significantly positively correlated with *p,p'*-DDE in the nestling eagles. However, no dose-related patterns were observed with the laboratory experiments. Other studies have shown an increase in alpha tocopherol levels in the liver and serum of rats (Katayama et al. 1991, Twaroski et al. 2001), but further study need to be done in avian species.

#### *Thyroid Hormones*

No significant correlations were observed between individual plasma thyroid hormone concentrations and contaminant levels in bald eagles or domestic chickens. Plasma thyroxine (T4) levels have been shown to be reduced in several species, including terns, cormorants, and American kestrels, when exposed to PCBs (van den Berg et al. 1994, Bosveld et al. 2000, Quinn et al. 2002). However, levels of plasma T4 do not seem to be a good biomarker for bald eagles.

We did find significant correlations between the ratio of total T4 to free T4 and total PCB and *p,p'*-DDE concentrations in bald eagles. No such correlations were seen in the laboratory experiments. This ratio may be a good predictor of contaminant concentration in the eagles, but need to be investigated further before being considered as a biomarker.

#### *Corticosterone Levels/ACTH Response*

We found that ACTH response was positively correlated with both total PCBs and *p,p'*-DDE in nestling bald eagles. These results agree with a previous eagle study by Bowerman et al. (2002). No dose-response pattern was noted for ACTH response in the double-crested cormorant (DCC) laboratory study, but a significant decrease in ACTH response was noted for the highest dose group in the PCB 126 study. Despite the inconclusive laboratory results, ACTH response has been shown to be positively correlated with contaminant levels in bald eagles in two separate studies, and should therefore be considered for use as a biomarker for this species.

### *Immunotoxicology*

Peripheral blood lymphocyte (PBL) mitogenesis was examined as a biomarker of immune function in bald eagles and was reported in the Phase I report. The Con-A assay was found to be a good method of analysis and it was concluded that this assay has potential for measuring immune function in the nestling eagles.

### *Intestinal Bacteria*

Significant differences were found among eagle and chicken intestinal bacterial populations with contaminant concentration. However, these differences were not dose-related or predictable, and the mechanism behind the alterations seen could not be determined. Overall, contaminant concentrations had little effect on the intestinal bacteria of eagles, and this technique would not contribute to a successful monitoring program.

### *Other Results*

*Vitamin D:* Vitamin D was not measured in eagle samples, but significant results were observed in the DCC laboratory experiment. In that study, plasma vitamin D concentrations decreased with dose in two-week-old chickens. This indicator may be worth studying in eagles in the future.

*Cytochrome P450:* Induction of cytochrome P450-1A in the livers of chickens proved to be an excellent measure of contaminant exposure in the laboratory experiments. Although it is not possible to perform this test on eagles since liver tissue is needed, this indicator may be a good biomarker for other species.

*Embryo Study:* The effects of the DCC extract on organ mass and femur length were examined in 20 day-old embryos. A general decrease in both parameters was observed with increasing dose. See Appendix II for a full report.

## SECTION 6.0

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## SECTION 7.0

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**Appendix I**  
**Intestinal Bacteria Study**

AN INVESTIGATION OF INTESTINAL FLORA AS A POTENTIAL BIOMARKER  
OF CONTAMINANT EXPOSURE IN AVIAN SPECIES

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A Thesis

Presented to  
the Graduate School of  
Clemson University

---

In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science  
Environmental Toxicology

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by

Faith Elizabeth Wiley

August 2003

Advisor: Dr. William W. Bowerman

## ABSTRACT

The effects of bioaccumulative contaminants on the intestinal flora of bald eagles (*Haliaeetus leucocephalus*) and domestic chickens (*Gallus domesticus*) were examined in a field study and two laboratory dosing experiments. The objective of these studies was to determine if any changes observed in the intestinal flora could be used as a new biomarker of exposure to bioaccumulative contaminants. The first laboratory study involved dosing white leghorn chicken eggs with four concentrations of polychlorinated biphenyl (PCB) 126, 0.100 ng/g, 0.175 ng/g, 0.250 ng/g, and 0.325 ng/g, via air cell injection. Cloacal swabs were obtained to observe the microbial flora at two weeks and five weeks post-hatch. Significant differences were observed among dose groups for both microbial composition and antibiotic susceptibility of bacterial isolates. A second laboratory dosing study was conducted in which chickens were exposed *in ovo* to an extract obtained from double-crested cormorant (*Phalacrocorax auritus*) eggs collected from Green Bay, Wisconsin. Methods of bacterial analysis were the same as in the first study. The concentrations of the extract, 0.0625 egg-equivalents (egg-EQ), 0.125 egg-EQ, 0.1875 egg-EQ, and 0.250 egg-EQ, also produced significant differences in bacterial populations. In both laboratory studies, however, the differences seen were not clearly dose-dependent and the mechanisms involved in the changes were not evident. Effects of bioaccumulative contaminants on bald eagles were examined by obtaining cloacal swabs from nestling eagles in Michigan and Minnesota. Bacterial populations were compared to blood plasma contaminant levels. Few significant correlations were observed between contaminant concentrations and microbial flora and contaminant levels did not seem to

affect the composition of eagle flora or the antibiotic susceptibility of the bacteria present. It does appear that bioaccumulative contaminants may exert some effect on avian intestinal flora, but the mechanism of those effects is unknown. Due to the uncertainty in the mechanism and the lack of dose-response relationship for the field samples, it was determined that alterations in microbial flora do not hold much promise as a potential biomarker of exposure to bioaccumulative contaminants.

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## TABLE OF CONTENTS

	Page
TITLE PAGE .....	i
ABSTRACT.....	ii
DEDICATION .....	iv
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES .....	vii
LIST OF FIGURES .....	ix
PREFACE.....	1
INTRODUCTION .....	2
EFFECTS OF BIOACCUMULATIVE CONTAMINANTS ON THE INTESTINAL FLORA OF DOMESTIC CHICKENS.....	16
Introduction.....	16
Materials and Methods.....	18
Results.....	23
Discussion.....	54
References.....	66
EFFECTS OF BIOACCUMULATIVE CONTAMINANTS ON THE INTESTINAL FLORA OF NESTLING BALD EAGLES .....	70
Introduction.....	70
Materials and Methods.....	73
Results.....	76
Discussion.....	83
References.....	90
CONCLUSIONS.....	93

## LIST OF TABLES

Table	Page
1. Doses of double-crested cormorant egg extract, expressed as cormorant egg-equivalents, Toxic Equivalents, and ng of PCB 126 .....	22
2. Observed versus expected hatchability by dose group for PCB 126 experiment with significance determined by Z statistic .....	23
3. Samples sizes per dose group for PCB 126 experiment .....	23
4. Correlations between biochemical reactions and antibiotic susceptibility of <i>Enterococcus gallinarum</i> isolates from chicks in PCB 126 study .....	33
5. Correlations between biochemical reactions and antibiotic susceptibility of <i>Escherichia coli</i> isolates from two-week-old chicks in PCB 126 study .....	34
6. Observed versus expected hatchability by dose group for double-crested cormorant experiment with significance determined by Z statistic .....	34
7. Sample sizes per dose group for double-crested cormorant extract experiment .....	35
8. Type and prevalence of bacteria identified in chicks at two-weeks post-hatch in double-crested cormorant extract experiment .....	36
9. Type and prevalence of bacteria identified in chicks at five-weeks post-hatch in the double-crested cormorant extract experiment .....	39
10. Correlations between biochemical reactions and antibiotic susceptibility profiles of <i>Enterococcus faecalis</i> isolates from chicks in the double-crested cormorant extract experiment .....	46
11. Correlations between biochemical reactions and antibiotic susceptibility profiles of <i>Enterococcus faecium</i> isolates from two-week-old chicks in the double-crested cormorant extract experiment .....	46

List of Tables (Continued)

Table	Page
12. Correlations between biochemical reactions and antibiotic susceptibility of <i>Enterococcus gallinarum</i> isolates from chicks in the double-crested cormorant extract experiment .....	47
13. Correlations between biochemical reactions and antibiotic susceptibility of <i>Klebsiella pneumoniae</i> isolates from two-week-old chicks in the double-crested cormorant extract experiment .....	47
14. Correlations between biochemical reactions and antibiotic susceptibility of <i>Escherichia coli</i> isolates from chicks in the double-crested cormorant extract experiment .....	48
15. Blood plasma concentrations of PCBs and organochlorine pesticides from nestling bald eagle samples, reported in ng/g .....	77
16. Type and prevalence of bacteria identified in nestling bald eagles .....	78
17. Correlations between biochemical reactions and antibiotic susceptibility of <i>Escherichia coli</i> isolates from nestling bald eagles .....	82
18. Correlations between biochemical reactions and antibiotic susceptibility of <i>Proteus mirabilis</i> isolates from nestling bald eagles .....	82
19. Correlations between biochemical reactions and antibiotic susceptibility of <i>Staphylococcus intermedius</i> isolates from nestling bald eagles .....	83

## LIST OF FIGURES

Figure	Page
1. Basic structure of a PCB .....	3
2. Basic structure of a polychlorinated dioxin .....	5
3. Basic structure of a polychlorinated dibenzofuran .....	5
4. Presence of <i>Klebsiella pneumoniae</i> by dose group in two-week-old chicks in PCB 126 study .....	25
5. Presence of <i>Enterococcus gallinarum</i> by dose group in two-week-old chicks in PCB 126 study .....	25
6. Percent of <i>Enterococcus gallinarum</i> isolates positive for alkaline phosphatase production and ribose fermentation in two-week-old chicks in PCB 126 study .....	27
7. Percent of <i>Klebsiella pneumoniae</i> isolates positive for raffinose fermentation in two-week-old chicks in PCB 126 study .....	27
8. Percent of <i>Enterococcus gallinarum</i> isolates positive for growth in 40% bile and esculin hydrolysis in five-week-old chicks in PCB 126 study .....	28
9. Antibiotic susceptibility profiles of <i>Escherichia coli</i> isolates in two-week-old chicks in PCB 126 study .....	29
10. Antibiotic susceptibility profiles of <i>Enterococcus gallinarum</i> isolates in two-week-old chicks in PCB 126 study .....	32
11. Presence of <i>Enterococcus faecium</i> by dose group in two-week-old chicks in double-crested cormorant extract study .....	37
12. Percent of <i>Escherichia coli</i> isolates positive for sucrose and adonitol fermentation in two-week-old chicks in double-crested cormorant extract study .....	37

List of Figures (Continued)

Figure	Page
13. Percent of <i>Klebsiella pneumoniae</i> isolates positive for raffinose and arabinose fermentation in two-week-old chicks in double-crested cormorant extract study .....	38
14. Presence of <i>Enterococcus faecalis</i> by dose group in five-week-old chicks in double-crested cormorant extract study .....	40
15. Presence of <i>Enterococcus gallinarum</i> by dose group in five-week-old chicks in double-crested cormorant extract study .....	40
16. Percent of <i>Escherichia coli</i> isolates positive for sucrose and adonitol fermentation in five-week-old chicks in double-crested cormorant extract study .....	41
17. Antibiotic susceptibility profiles of <i>Escherichia coli</i> isolates in two-week-old chicks in double-crested cormorant extract study .....	44
18. Antibiotic susceptibility of <i>Enterococcus faecalis</i> isolates to erythromycin in two-week-old chicks in double-crested cormorant extract study .....	45
19. Antibiotic susceptibility profiles of <i>Escherichia coli</i> isolates in five-week-old chicks in double-crested cormorant extract study .....	51
20. Antibiotic susceptibility profiles of <i>Enterococcus faecalis</i> isolates to erythromycin in five-week-old chicks in double-crested cormorant extract study .....	53
21. Antibiotic susceptibility profiles of <i>Enterococcus gallinarum</i> isolates to erythromycin in five-week-old chicks in double-crested cormorant extract study .....	53
22. Mean number of bacterial species present per bird by age (in weeks) in nestling bald eagles .....	79

## PREFACE

This thesis is organized into two major research components, a laboratory and a field study. These studies have been written up separately in journal style with the intent of submitting manuscripts for each section. Therefore, some of the information included in this work may be repeated as it was important to include it in both manuscripts.

## INTRODUCTION

The use of biomarkers to understand subtle effects of bioaccumulative compounds of concern (BCCs) in avian species is a relatively new and emerging field. A number of biomarkers have been studied for their applicability as measures of BCC exposure, including cytochrome P450 monooxygenases, thyroid function, retinoid homeostasis, porphyrin profiles, and immune function (14, 16). Several of these measures have proven to be effective biomarkers, and new biomarkers are being developed as the scientific community continues to better understand the effects of BCCs on avian physiology.

The Michigan Bald Eagle Biosentinel Project, developed by the Michigan Department of Environmental Quality (MDEQ), is designed to monitor bald eagle (*Haliaeetus leucocephalus*) contaminant levels in the Great Lakes region as an indicator of environmental health. The bald eagle makes an excellent sentinel species for a number of reasons. The bald eagle has been extensively studied, resulting in the availability of a great literature on life history. In addition, the eagle is a tertiary predator and is therefore exposed to BCCs at higher levels than animals lower in the food chain. As our national symbol, the eagle also has societal value (8).

The Bald Eagle Biosentinel Project, which began in 1999, monitors concentrations of organochlorine pesticides, polychlorinated biphenyls, and mercury in bald eagles through sampling and analysis of juvenile blood and feathers (8). To further enhance this program, biomarkers are being developed that can be applied to the field so

that we can better understand the subtle effects on environmental toxicants on this species and have a more accurate view of environmental contamination impacts.

Bioaccumulative contaminants of concern include polychlorinated biphenyls (PCBs), dioxins, furans, and organochlorine pesticides. These compounds are all anthropogenic in origin, persistent in the environment, and are known to bioaccumulate in organisms. Seemingly insignificant quantities of contaminant in the soil, water, or air can enter the food chain and increase exponentially, resulting in significant and dangerous quantities in animals of high trophic status.

PCBs are a group of synthetic compounds consisting of a biphenyl ring with a number of substituted chlorine atoms. There are 209 congeners that vary by number and position of chlorines. (Figure 1). PCBs were manufactured in the United States as Aroclor mixtures that contained between 60 and 90 congeners (37). These mixtures were used as electrical insulators, high temperature lubricants and hydraulic fluids. Their thermal and chemical stability and low flammability made them extremely useful in such applications, but these properties also make them very stable in the environment (1).

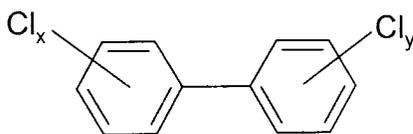


Figure 1. Basic structure of a PCB.

PCBs were first identified as environmental contaminants by Jensen in 1966 and were banned by the United States in the late 1970s (18). Since then they have been explored extensively for their toxic effects on organisms as well as their movement in the

environment. It has been reported that one-third of the total US PCB production has been released into the environment (37). Since PCBs have properties such as low water solubility and low vapor pressure they have remained in the environment and continue to be available to organisms. Their lipophilic properties and high octanol/water partitioning coefficient make them able to move readily into biota. Once in the food chain they bioaccumulate in the higher trophic levels, resulting in toxic amounts in higher organisms (26).

PCB toxicity has been examined for many different species of aquatic organisms, birds, and mammals. These compounds exhibit many toxic effects, including reproductive and behavioral effects. Avian studies have shown PCBs to have detrimental effects on fertility, egg production and hatch success, growth weight, impaired courtship behavior, decreased parental attention, edema, and beak and limb deformities in chicks. Acutely toxic doses can result in tremors, lethargy, and paralysis (4).

Dioxins and furans are similar to PCBs in both structure and toxicity. (Figures 2 and 3) These compounds are introduced into the environment as byproducts of industrial processes and combustion. The toxicity of these compounds is similar to PCBs because they act through the same mechanism.

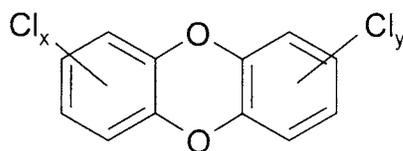


Figure 2. Basic structure of a polychlorinated dioxin.

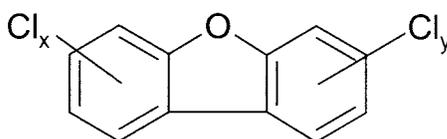


Figure 3. Basic structure of a polychlorinated dibenzofuran.

The mechanism of toxicity for PCBs, dioxins, and furans is mediated through the aryl hydrocarbon (Ah) receptor. The compounds bind to the Ah receptor and are taken into the cell nucleus where they bind to specific DNA fragments. The end result is an alteration of gene expression which leads to the variety of toxic effects (27). 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) is considered to be the most toxic of the AhR mediated compounds because it has such a strong affinity for the receptor. The other compounds mediated through the Ah receptor are compared to TCDD in terms of relative toxicity. The toxicity of other dioxins, furans, and of PCB congeners is related to the position of chlorine atoms. Those compounds with chlorines in the *para* and *meta* positions, rather than *ortho* are more flat or planar. Planar compounds are more similar in structure to TCDD and therefore more likely to fit in the AhR. PCB congeners vary greatly in their toxicity due to their structural differences, and only 12 of the 209

congeners are reported to have 'dioxin-like' activity. The congener whose toxicity is most similar to TCDD is PCB 126 (13).

Organochlorine pesticides include such compounds as DDT, chlordane, dieldrin, and mirex, among others. DDT is probably the most infamous of these compounds because of its role in the mass reproductive failures of bald eagles and other birds during the mid 1900s. As with PCBs, dioxins, and furans, these compounds persist in the environment and bioaccumulate in food chains, exposing tertiary predators to large concentrations (11). In addition to reproductive impairment, these compounds can induce a variety of neurological disorders, and due to their toxicity, most uses have been banned by the United States (12).

The exposure of an organism to bioaccumulative compounds of concern is measured using biomarkers. Biomarkers are physiological, morphological, or biochemical measures of exposure to or effects of chemical contaminants. A number of different biomarkers, or bioindicators, have been tested and are in use today. Many more measures are being currently examined for their potential usefulness as biomarkers. The continued development of biomarkers is important for several reasons. New biomarkers may be more accurate, easier to perform, or less stressful for the organism. In addition, the more biomarkers that are available, the more accurate the picture of contaminant exposure becomes. "A monitoring program using multiple biomarkers or a suite of measurements would better enable identification of stressed populations" (20).

Many factors must be taken into consideration when determining whether a specific measure will make an effective biomarker. Biomarkers range from simple measures of growth and symmetry to more complex molecular tests, but the criteria put

forth to evaluate these measures is the same. As outlined by Huggett (20), criteria for evaluating field applicable biomarkers include ease of measurement and sensitivity to the pollutant of concern, whether or not the biomarker responds to the pollutant in a dose or time dependent manner, understanding of the natural variability associated with the biomarker, and whether or not physiological changes observed can be linked to an interpretable biological process.

Avian intestinal flora have been proposed as a potential biomarker for several reasons. First, samples are easy to collect in the field and the procedure is quick and noninvasive. Because the procedure does not require the animal to be euthanized, it can be performed on threatened and endangered species, causing only minimal stress to the animal. Secondly, procedures for identifying bacteria and testing antibiotic susceptibility have been in place for many years and are relatively easy to perform. In addition, both sample collection and identification procedures are relatively inexpensive.

The avian intestinal flora may potentially be affected by chemical contaminants by direct effects of the toxic compound, as a side effect of the compounds' action on the host organism, or some combination of these factors. Studies concerning direct effects of chemical contaminants on bacteria are somewhat limited. The main focus of microorganisms in toxicology has been on their use as a remediation tool for contaminant cleanup. Several species of microbes have been utilized in this manner and have shown great potential for metabolizing chemical contaminants, particularly persistent chemicals such as PCBs (1, 37). The species utilized in such ways are typically soil microorganisms, such as *Pseudomonas* species, not enteric bacteria.

The few studies that have been conducted on the effects of environmental contaminants on bacteria have shown that these compounds can have either a beneficial or a toxic effect, depending on what type of bacterium is involved. Bourquin and Cassidy (7) reported growth inhibition of estuarine bacteria from seven different genera in the presence of PCBs. The mechanism of the inhibition was not known, although it was thought to be bacteriostatic. Another study demonstrated a growth inhibitory effect of the organochlorine pesticide chlordane on gram-positive bacteria, although gram-negative strains were not affected (36). Even among closely related bacteria, effects of PCBs can be quite different. Blakemore (6) reported a dose dependent decrease in growth rate and cell yield in a marine pseudomonad when exposed to Aroclor 1254. In a different study, PCBs were found to stimulate both growth and oxygen uptake in a *Pseudomonas* species. (32).

Few studies have examined the effects of BCCs on intestinal organisms. Keil et al. (22) demonstrated stimulation of *in vitro* growth of *Escherichia coli* by Aroclor 1242 and DDT. Chlordane has been shown to inhibit growth of *Enterococcus faecalis* (36). However, *E. faecalis* was shown to be much less sensitive to this compound than other gram-positive organisms, with inhibition only occurring at very high concentrations. Rybosova *et al.* (28) examined the effects of the commercial PCB mixture Delor 105 on the ruminal bacteria of sheep. Virtually no effects were observed, although there was a slight decrease in the volatile fatty acid concentrations of the exposed bacteria.

Intestinal bacterial populations may also be affected by contaminants indirectly, by means of immune system suppression of the host organism. PCBs and other bioaccumulative compounds are known to be immunosuppressive, weakening the

immune system of the exposed organism and making it more susceptible to disease (16). The colonization of the avian intestine by bacteria begins to occur immediately after hatch and is regulated in part by immunoglobulins. Immunoglobulin A (IgA) is particularly important in immunosurveillance of the gastrointestinal tract and plays a major role in the selective colonization of bacteria (23). If the immune system is weakened by contaminants, this immunosurveillance may not be as effective as it should, allowing abnormal and possibly pathogenic strains of bacteria to colonize.

Environmental contaminants may not only affect the type of bacteria allowed to colonize the avian gut, but may also affect the antibiotic susceptibility of those bacteria. Several studies have shown that exposure to chemical pollutants and, in particular heavy metals, is associated with increased antibiotic resistance of bacterial species, due to a genetic linkage of these traits (5, 35, 38). This means that, for these species, the presence of heavy metals in the environment will select for antibiotic resistant bacteria. In addition to heavy metals, organic solvents, biocides, and detergents have been shown to select for antibiotic resistant bacterial strains (2).

Antibiotic susceptibility may also be indirectly affected by contaminants if more pathogenic strains of bacteria are allowed to colonize the gut due to immune suppression. Just as genetic linkages have been discovered for drug and/or heavy metal resistance and antibiotic susceptibility, there have been several studies showing a genetic linkage between antibiotic resistance and virulence determinants. Virulence determinants can be any characteristic that enables a bacterium to invade a host or survive the host's immune system. With bacteria such as *Escherichia coli* and *Salmonella* spp., strains have been

discovered that contain linked genetic material which confers both antibiotic resistance and certain virulence determinants (19, 31, 33).

This study is composed of both laboratory and field components. The laboratory portion was conducted using domestic chickens (*Gallus domesticus*) exposed to contaminants through egg dosing procedures. In the field, juvenile bald eagles exposed to contaminants predominantly through diet were examined. Two laboratory egg dosing experiments were conducted, the first using PCB 126 and the second, a double-crested cormorant (*Phalacrocorax auritus*) egg extract containing a mixture of environmental contaminants, the principle contaminant present being PCB 126. Other biomarkers, including immune system effects, growth weights, thyroid hormones, vitamins, and Phase II enzymes, were studied during the same experiments and results are reported elsewhere.

The domestic chicken is an ideal subject for a laboratory model, in that its physiology has been well studied, it is easy to care for and manage, and its intestinal flora has been well studied (3, 10, 21, 29, 30, 34). The chicken is also well established as a good laboratory model for avian toxicity tests, being used in a number of previous experiments (15, 17, 24, 25). Egg dosing experiments with PCB 126 have been performed previously with this species as well, meaning that the mortality at specific concentrations is known, making it easier to choose appropriate dose concentrations to inject.

Juvenile bald eagles were sampled in Michigan and Minnesota during normal banding and sampling (8). In contrast to chickens, not much is known about the normal intestinal flora of bald eagles. The normal gut flora of wild species has not been well documented, with most reports coming from songbirds (9). It is known that intestinal

flora in general varies with age, diet, and environmental conditions. From this it is expected that the flora of eaglets may be quite different than that of chickens and this factor must be considered in any extrapolations from the laboratory to the field.

To determine whether intestinal flora is affected by contaminant burden, cultures collected from cloacal swabs were used to both identify the microorganisms present as well as test their antibiotic susceptibility. Identification was accomplished with biochemical testing and the microbes were identified to the species level when possible. Antibiotic susceptibility was tested using differing concentrations of a number of antibiotics to obtain a Minimum Inhibitory Concentration (MIC). The MIC is defined as the lowest concentration of an antibiotic compound that completely inhibits growth of the bacteria.

The objectives of this study were to 1: examine the effects of bioaccumulative compounds on the intestinal flora of avian species, and 2: determine the potential for those effects to act as biomarkers of exposure to bioaccumulative contaminants.

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## EFFECTS OF BIOACCUMULATIVE CONTAMINANTS ON THE INTESTINAL FLORA OF DOMESTIC CHICKENS

### Introduction

The use of biomarkers to understand subtle effects of bioaccumulative compounds of concern (BCCs) in avian species is a relatively new and emerging field. A number of biomarkers have been studied for their applicability as measures of BCC exposure, including cytochrome P450 monooxygenases, thyroid function, retinoid homeostasis, porphyrin profiles, and immune function (13, 16). Several of these measures have proven to be effective biomarkers, and new biomarkers are being developed as the scientific community continues to better understand the effects of BCCs on avian physiology.

The effect of contaminants on microorganisms in the intestinal tract of birds has been proposed as a potential new nondestructive biomarker of exposure to bioaccumulative compounds of concern (BCCs). These compounds, which include polychlorinated biphenyls (PCBs), dioxins, furans, and organochlorine pesticides, accumulate and concentrate in the fatty tissue of organisms feeding at high trophic levels. They have been of particular concern to fish-eating birds and mammals. These compounds produce a variety of detrimental effects, even at small concentrations, including reproductive and developmental abnormalities, and immune and nervous system effects (6).

Alteration of intestinal flora by BCCs may occur directly by pollutant effects on the microbes or indirectly by suppressing the immune system of the host organism.

Studies concerning direct effects of contaminants on microbes is limited. The primary interest for microorganisms in toxicology is on their ability to breakdown or transform chemical compounds and aid in bioremediation. Studies concerning the effects of BCCs on bacteria have shown them to have both beneficial and detrimental effects, depending on the type of bacteria affected (7, 9, 30, 34).

Immune system suppression may alter normal intestinal flora populations by allowing abnormal and/or more pathogenic strains to colonize the intestinal tract. The colonization and the immune surveillance of the intestinal tract are largely regulated by immunoglobulins (22). Absence or suppression of immunoglobulins could result in the formation of an abnormal flora population.

In addition to affecting the types of bacteria present in the avian gut, BCCs may also influence the antibiotic susceptibility of those compounds. Several studies have shown that genes encoding for antibiotic resistance may be selected for in the presence of heavy metals, organic solvents, and biocides (2). There have also been studies showing genetic linkage between virulence determinants and antibiotic resistance (20, 33, 35). Therefore, with the colonization of more pathogenic species, there is the potential for a change in antibiotic resistance as well.

The purpose of this study was to examine the effects of bioaccumulative pollutants on avian intestinal flora by utilizing controlled laboratory studies. Domestic chickens (*Gallus domesticus*) were used as the avian model and were exposed to contaminants in two separate laboratory experiments, the first using PCB 126 and the second an extract taken from double-crested cormorant (*Phalacrocorax auritis*) eggs containing a mixture of environmental contaminants including PCBs, dioxins, furans, and

organochlorine pesticides. The domestic chicken has been used as an avian model for wild species in several other biomarker studies (14, 17, 28, 29). In addition, intestinal flora development in this species has been well studied and characterized (5, 12, 21, 31, 32, 36).

The objectives of this study were to examine the effects of environmental contaminants on: 1. the composition of microorganisms allowed to colonize and persist in the intestinal tract of domestic chickens; and 2. the antibiotic susceptibility of those organisms. The overall goal of this research is to apply the results of these studies to a field situation and examine the possibility of using this technique as a new nondestructive biomarker.

### Materials and Methods

#### Egg Dosing and Colony Management

Dosing procedures were performed at the Clemson Institute of Environmental Toxicology (CIET). White leghorn chicken eggs were obtained from the Clemson University Morgan Poultry Center prior to incubation and transported to CIET. Prior to dosing, eggs were randomized, weighed, and candled to locate the air cell. After wiping the air cell region with 70% ethanol, a small hole was made in the shell above the air cell with a sterile dissecting needle. The appropriate dosing solution was injected into the air cell with a 10 $\mu$ l Hamilton® syringe. The hole was then sealed with a thin layer of paraffin wax. After all injections were complete, the eggs were transported to the Morgan Poultry Center and placed in a commercial incubator set at 37.6°C and 58% humidity. Eggs were candled each week to check for mortality. Nonviable eggs were removed and their contents were examined for stage of development and structural and developmental

deformities. On Day 18 of incubation eggs were transferred from the incubator to a hatcher set at 37.2°C and 50% humidity.

At hatch, chicks were weighed, banded with wing tags for identification, and placed in a brooder. Nonmedicated food and water were given *ad libitum* and weights were recorded weekly. Chickens were euthanized in two groups, at either two weeks or five weeks post-hatch. These ages were chosen because they correspond to six and ten weeks of age for bald eagle nestlings based on developmental stages. Birds were euthanized by a precharged CO<sub>2</sub> chamber (3). A sample size of ten birds was desired per dose group for each age group. When sample size was <20 for any dose group, 10 birds were kept for the 5 week analysis. All procedures were conducted under an Animal Use Protocol approved by the Clemson University Animal Research Committee.

#### Bacterial Analysis

To reduce the numbers of bacteria analyzed, only aerobic organisms were examined. A group of 10 non-injected eggs was tested at day 0 to obtain baseline data. Both the egg contents and the egg surface were examined for presence of microbes. Cloacal swabs were performed immediately following euthanasia for both two week and five week old birds. The cloacal region was disinfected with a 70% isopropanol wipe and a sterile, cotton-tipped swab was inserted into the cloaca to obtain some fecal material. The swab was then used to inoculate a Levine EMB agar plate and a Columbia CNA agar plate. The plates were streaked for colony isolation and stored at room temperature until transport to an incubator. Plates were incubated for 20-24 h at 37°C and observed for colony formation. Microbial colonies present were identified and tested for antibiotic susceptibility using MicroScan® Dried Gram Negative Panel 22 and Dried Gram

Positive Panel 14 (Dade Behring Inc., Deerfield, IL). The Dried Gram Negative Panel 22 included the following antibiotics: penicillin, kanamycin, nitrofurantoin, colistin, cephalothin, ampicillin/sulbactam, aztreonam, ceftazidime, trimethoprim/sulfamethoxazole, ciprofloxacin, cefpodoxime, ampicillin, piperacillin, cefazolin, ceftazidime, cefuroxime, ceftriaxone, cefotaxime, gentamicin, tobramycin, amikacin, levofloxacin, meropenem, imipenem. The Dried Gram Positive Panel 14 included the following antibiotics: streptomycin (synergy screen), gentamicin (synergy screen), penicillin, tetracycline, amoxicillin/clavulanate, trimethoprim/sulfamethoxazole, ampicillin, erythromycin, clindamycin, gentamicin, oxacillin, vancomycin, cefazolin, cefotaxime, ciprofloxacin, levofloxacin, norfloxacin, imipenem, cephalothin, nitrofurantoin, trovafloxacin, rifampin. Quality Control organisms *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were analyzed in conjunction with the sample organisms. Identification to species level and antibiotic susceptibility profiles were determined following MicroScan® Procedural Manual guidelines. For several *Enterococcus* species, additional tests (motility and colony pigment) were needed to obtain positive identification. For comparing antibiotic susceptibility, Minimum Inhibitory Concentrations (MICs) were obtained from National Committee for Clinical Laboratory Standards (NCCLS) Interpretive Breakpoints as provided by the MicroScan® manual (23, 24).

#### PCB 126

This study took place during the winter of 2001-2002. A stock solution of PCB 126 was obtained from ULTRA Scientific, Inc. (North Kingstown, RI, USA). Four dosing solutions of differing concentration were prepared at Clemson University by

dilution of the stock solution with pure sunflower oil. The four dose concentrations, 0.100 ng/g, 0.175 ng/g, 0.250 ng/g, and 0.325 ng/g, were determined by examining previous egg dosing studies using PCB 126 (14, 17). Control (no injection) and vehicle control (sunflower oil carrier) groups were also included. Dosing solutions were injected at 0.1  $\mu$ l/g of egg. The number of eggs dosed per concentration was determined by examining mortality from previous experiments and was designed to produce 20 live chicks for each dose group. At hatch, birds were placed in the brooder and separated by treatment group. For bacterial analysis, only the three predominant aerobic organisms seen consistently across samples were analyzed.

#### Double-crested Cormorant Extract

This study took place during the fall of 2002. White-leghorn chicken eggs were dosed on Day 0 of incubation with a double-crested cormorant egg extract. This extract, obtained from Dr. Donald Tillitt of the United States Geological Survey, was taken from unincubated double-crested cormorant eggs collected near Green Bay, Wisconsin in 1988 and has been characterized in a previous study (28). Four concentrations of the extract were used in dosing (Table 1). Control (no injection) and vehicle control (sunflower oil carrier) groups were also included. Dosing solutions were injected at 8  $\mu$ l per egg. The number of eggs dosed per concentration was determined by examining mortality from previous experiments (28) and was designed to produce 20 live chicks for each dose group. For this experiment, cloacal swabs were also performed after euthanasia on 10 control birds immediately following hatch. At hatch, birds were placed randomly in the brooder. For bacterial analysis, all aerobic colonies observed on the culture plates were analyzed.

Table 1. Doses of double-crested cormorant egg extract, expressed as cormorant egg-equivalents, Toxic Equivalents, and ng of PCB 126.

Dose	Egg-EQ <sup>1</sup>	TEQs (pg/g) <sup>2</sup>	ng PCB 126 <sup>3</sup>
Control	0	0	0
Vehicle Control	0	0	0
Dose 1	0.0625	20.13	0.210
Dose 2	0.125	40.25	0.421
Dose 3	0.1875	60.38	0.631
Dose 4	0.250	80.50	0.842

<sup>1</sup>Doses expressed in units of cormorant egg-equivalent/egg. (The concentration contained in one cormorant egg would be 1 egg-EQ).

<sup>2</sup>Dose expressed in Toxic Equivalents, relative to 2,3,7,8-TCDD (in pg/g of cormorant egg)

<sup>3</sup>Doses expressed in ng PCB 126.

### Statistical Analysis

Microbial composition, biochemical profiles, and antibiotic susceptibility were compared among dose groups. Results were initially analyzed by a chi-square test.

Because sample sizes were small, results were also analyzed by a Fisher's Exact Test.

All p values reported here are results of Fisher's Exact test. Biochemical profiles were also compared directly to antibiotic susceptibility profiles using Fisher's Exact test.

## Results

### PCB 126

#### Hatchability

There were significant differences in observed versus expected hatchability for several dose groups (Table 2). Due to unexpected hatch size, sample sizes differed among dose groups (Table 3).

Table 2. Observed versus expected hatchability by dose group for PCB 126 experiment with significance determined by Z statistic.

Dose Group	Alive Observed	Dead Observed	Alive Expected	Dead Expected	Z-obs	% Mortality
Control	18	8	20	6	-4.75*	30.8
Vehicle Control	14	12	20	6	-14.24*	46.2
0.100 ng/g	20	10	20	10	0	33.3
0.175 ng/g	20	10	20	10	0	33.3
0.250 ng/g	43	14	20	38	50.49*	24.6
0.325 ng/g	39	40	20	60	46.19*	50.0

\*Indicates significant difference in observed versus expected values with Z-critical value of  $\pm 1.96$  ( $\alpha = 0.05$ ).

Table 3. Sample sizes per dose group for PCB 126 experiment at necropsy.

Dose Group	2 week	5 week
Control	8	10
Vehicle Control	4	10
0.100 ng/g	9	9
0.175 ng/g	10	10
0.250 ng/g	10	11
0.325 ng/g	10	10
Total	51	60

## Microbial Identification

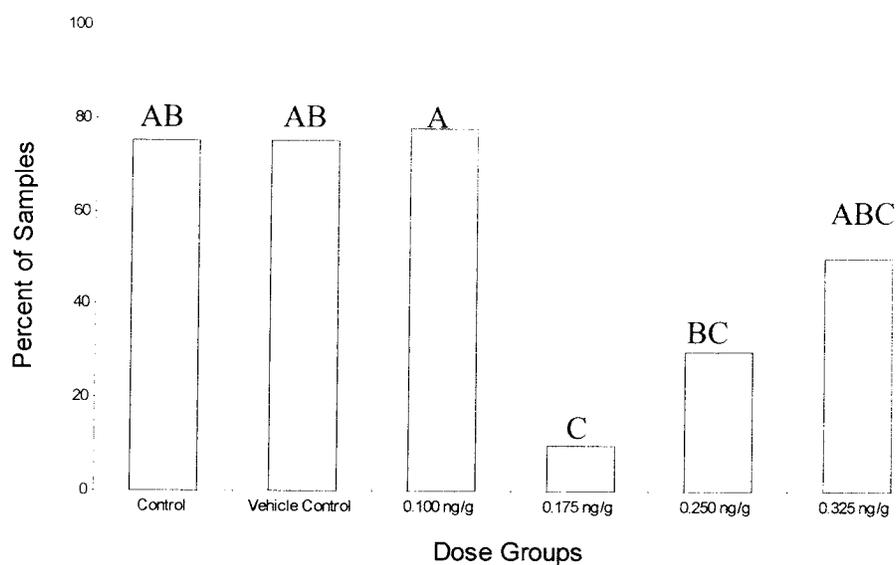
### Baseline

Of the ten eggs tested at dosing, only one contained bacteria on the egg surface, identified as *Staphylococcus capitis* subspecies *urealyticus*. No bacteria were cultured from any egg contents tested.

### Samples from Two-week-old Chicks

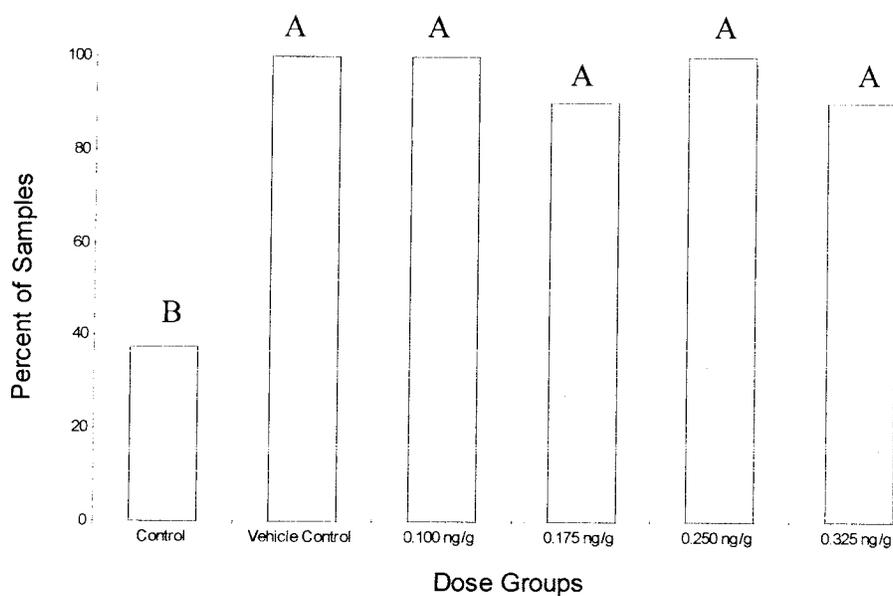
The predominant bacteria identified in the chickens analyzed at two-weeks post-hatch were *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus gallinarum*. Several other colony types were identified randomly among the dose groups, including *Enterobacter cloacae*, *Enterobacter sakazakii*, *Enterobacter taylorae*, *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus casseliflavus*. Each bird sampled contained *Escherichia coli*; however, a significant difference was noted among the dose groups for presence of *K. pneumoniae* ( $p=0.0140$ ) and *Enterococcus gallinarum* ( $p=0.0021$ ) (Figures 4 and 5).

Different strains of each bacterial species were found among and within dose groups, as shown by different biotype results from the MicroScan® analysis. Four strains of *Escherichia coli* were identified among the samples taken at two-weeks. There were significant differences among the predominant strains of bacteria per dose group ( $p<0.0001$ ). The predominant strain of bacteria in the control group differed significantly from the predominant strains of the remaining groups. Five strains of *K. pneumoniae* were identified among the samples, but there were no significant differences for strain presence among dose groups. Six strains of *Enterococcus gallinarum* were



\*Groups with the same letter are not significantly different.

Figure 4. Presence of *Klebsiella pneumoniae* by dose group in two-week-old chicks in PCB 126 study.



\*Groups with the same letter are not significantly different.

Figure 5. Presence of *Enterococcus gallinarum* by dose group in two-week-old chicks in PCB 126 study.

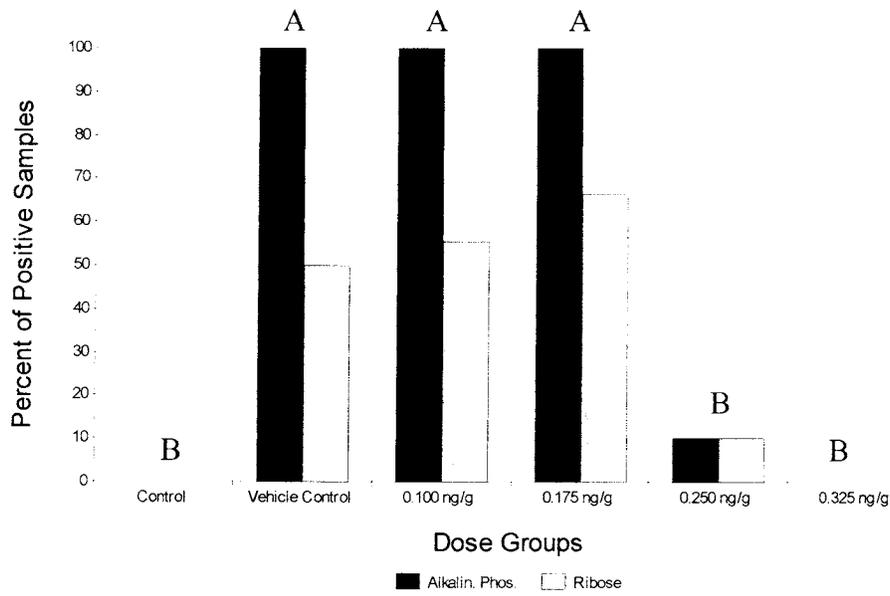
identified in the samples at two-weeks post-hatch, with significant differences occurring among dose groups for strain occurrence ( $p < 0.0001$ ). The control, 0.250 ng/g, and 0.325 ng/g groups were statistically similar, while differing in strain presence from the vehicle control, 0.100 ng/g, and 0.175 ng/g groups.

Dose groups were also examined for differences in various biochemical results. Alkaline phosphatase activity and ribose fermentation differed significantly among dose groups for *Enterococcus gallinarum* (Figure 6). Raffinose fermentation by *K. pneumoniae* also differed significantly among doses (Figure 7).

#### Samples from Five-week-old Chicks

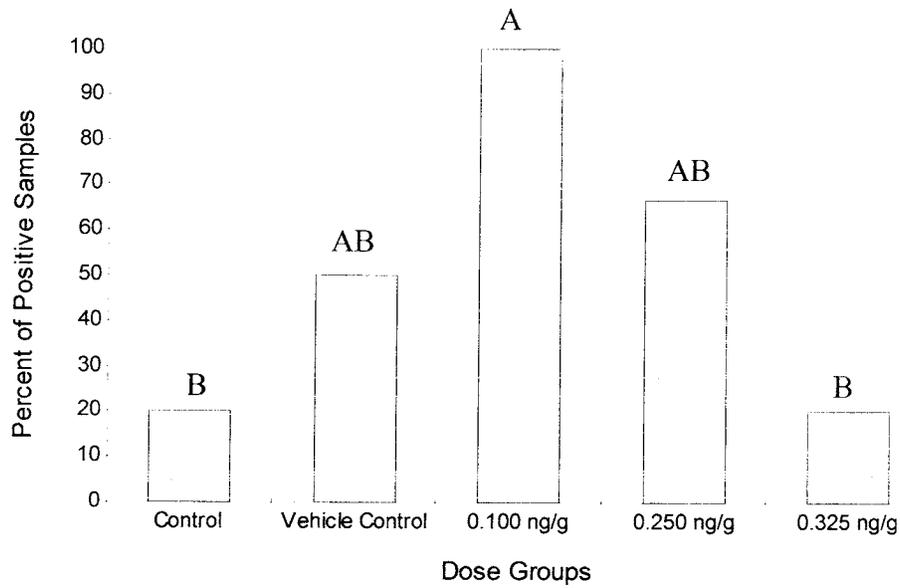
As in the samples taken at two weeks of age, the predominant bacteria identified in chicks sampled at five weeks were *Escherichia coli*, *K. pneumoniae*, and *Enterococcus gallinarum*. Again, all of the birds contained *Escherichia coli*. Unlike the two-week samples however, no significant differences were noted among the dose groups for presence of *K. pneumoniae* ( $p = 0.5724$ ) or *Enterococcus gallinarum* ( $p = 0.7513$ ).

Four strains of *Escherichia coli*, four strains of *K. pneumoniae*, and seven strains of *Enterococcus gallinarum* were identified among the samples at five weeks of age. No significant differences were found among the dose groups for strain presence. However, one significant difference was noted in comparing biochemical results among doses. Growth in 40% bile and esculin hydrolysis by *Enterococcus gallinarum* was lower among control and vehicle controls than the remaining groups ( $p = 0.0412$ ) (Figure 8).



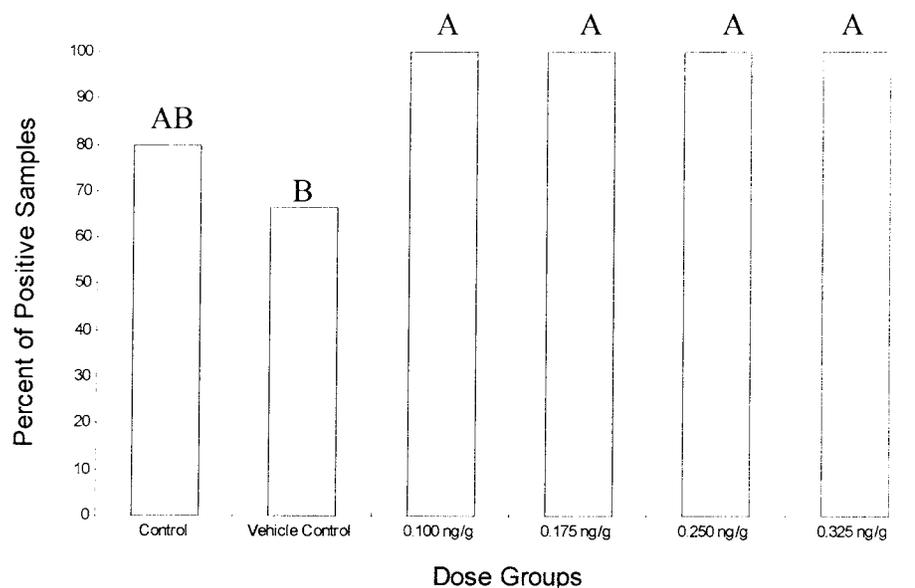
\*Groups with the same letter are not significantly different.

Figure 6. Percent of *Enterococcus gallinarum* isolates positive for alkaline phosphatase production and ribose fermentation in two-week-old chicks in PCB 126 study.



\*Groups with the same letter are not significantly different.

Figure 7. Percent of *Klebsiella pneumoniae* isolates positive for raffinose fermentation in two-week-old chicks in PCB 126 study.



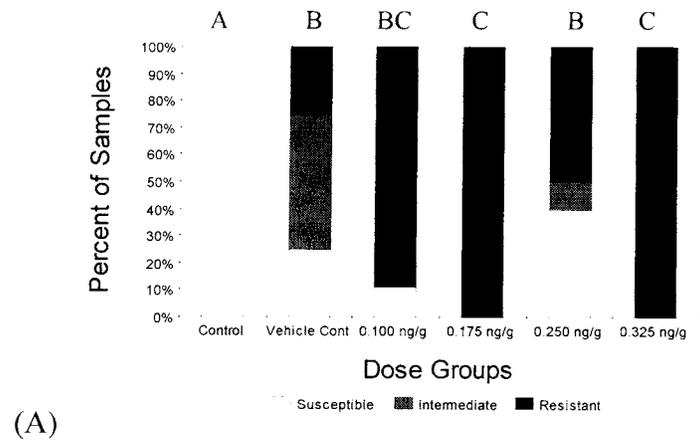
\*Groups with the same letter are not significantly different.

Figure 8. Percent of *Enterococcus gallinarum* isolates positive for growth in 40% bile and esculin hydrolysis in five-week-old chicks in PCB 126 study.

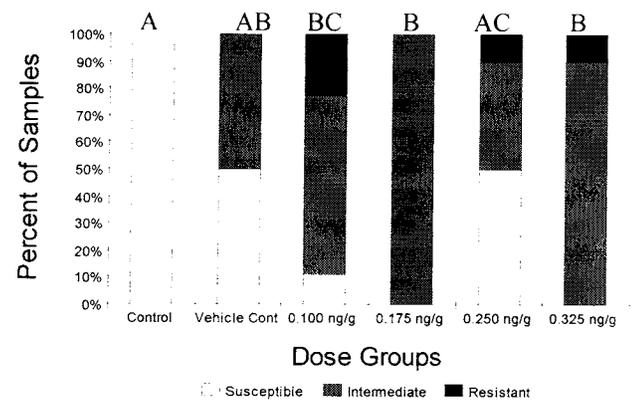
### Antibiotic Susceptibility Profiles

In addition to prevalence of bacterial species, significant differences were noted among dose groups for antibiotic susceptibility profiles of the bacteria. Differences were noted only with samples taken from two-week-old birds, not at five weeks of age.

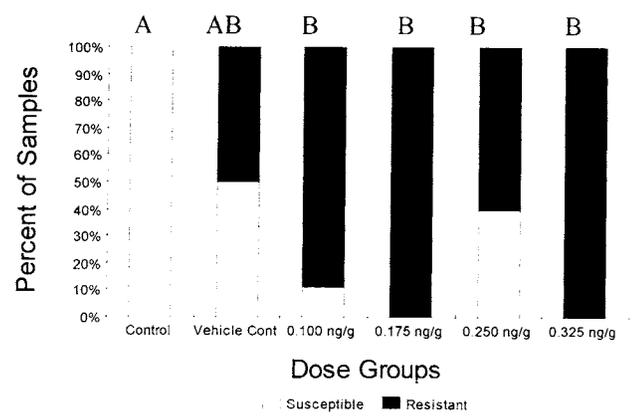
Significant differences were noted among dose groups for susceptibility of *Escherichia coli* to several antibiotics, including cephalothin ( $p < 0.0001$ ), ampicillin/sulbactam ( $p < 0.0001$ ), ampicillin ( $p < 0.0001$ ), piperacillin ( $p < 0.0001$ ), gentamicin ( $p < 0.0001$ ), and tobramycin ( $p = 0.0070$ ). For each of these antibiotics, the control samples experienced significantly more susceptibility than the PCB 126 dosed groups (Figure 9).



(A)



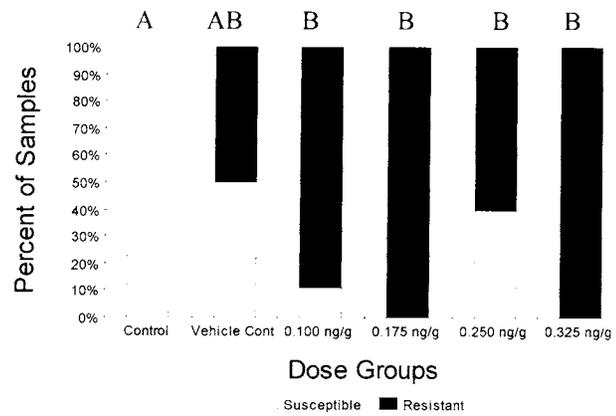
(B)



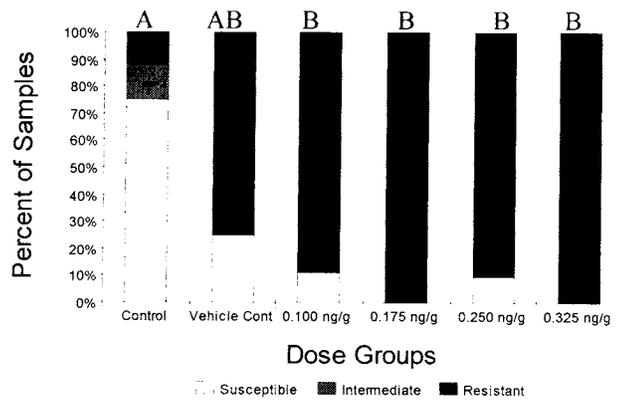
(C)

\*Groups with the same letter are not significantly different.

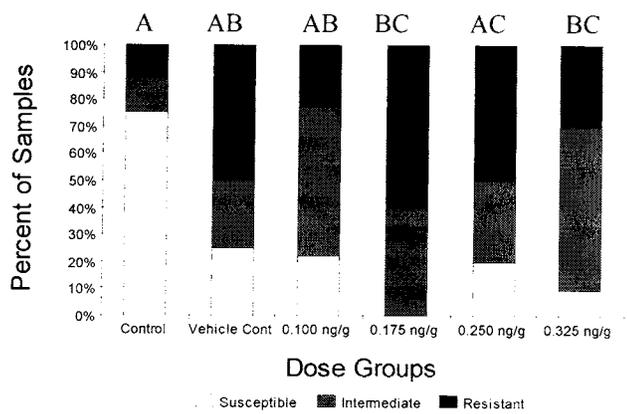
Figure 9. Antibiotic susceptibility profiles of *Escherichia coli* isolates in two-week-old chicks in PCB 126 study, (A) cephalothin, (B) ampicillin/sulbactam, (C) ampicillin, (D) piperacillin, (E) gentamicin, (F) tobramycin.



(D)



(E)



(F)

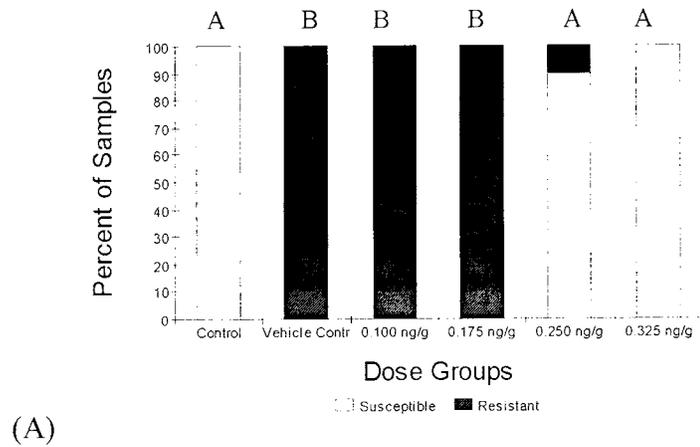
\*Groups with the same letter are not significantly different.

Figure 9. Antibiotic susceptibility profiles of *Escherichia coli* isolates in two-week-old chicks in PCB 126 study, (A) cephalothin, (B) ampicillin/sulbactam, (C) ampicillin, (D) piperacillin, (E) gentamicin, (F) tobramycin (Continued).

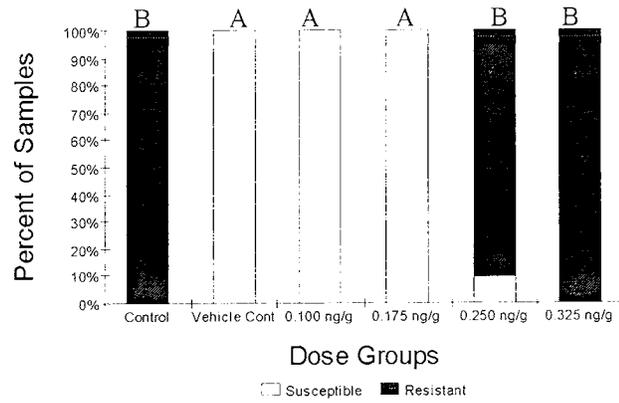
Significant differences were noted among the dose groups for *Enterococcus gallinarum* susceptibility to gentamicin, ciprofloxacin, norfloxacin, and rifampin ( $p < 0.0001$  for each) at two weeks of age. However, these differences were not dose-related (Figure 10).

Antibiotic susceptibilities were compared among the different strains of bacteria by analyzing for differences in antibiotic susceptibility profiles within a specific biotype. For the majority of bacterial species, differences in susceptibility did not occur within specific strains. That is, samples having the same strain type had the same antibiotic susceptibility profiles as well. However, two exceptions to this trend did occur. Antibiotic susceptibility profiles did differ among samples identified as *Escherichia coli* biotype 77115012 and *K. pneumoniae* biotype 72744370. These differences occurred with Ampicillin/Sulbactam susceptibilities in *Escherichia coli* and *K. pneumoniae*, as well as Tobramycin susceptibilities in *Escherichia coli*.

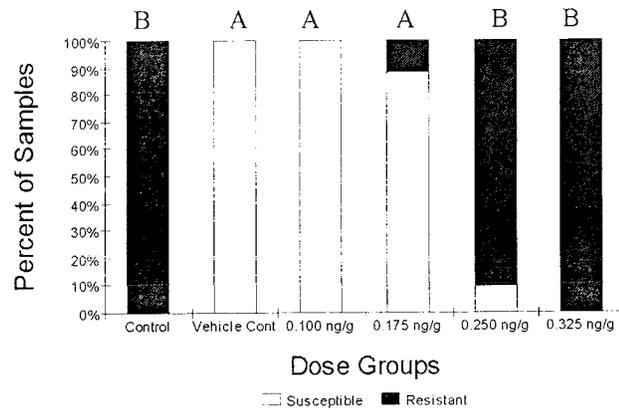
Antibiotic susceptibility profiles were examined for correlations with biochemical results. Several significant correlations were noted, for both *Enterococcus gallinarum* and *Escherichia coli* species. With *Enterococcus gallinarum* samples, alkaline phosphatase activity and ribose fermentation were found to be significantly correlated with antibiotic susceptibilities to gentamicin (synergy screen), ciprofloxacin, norfloxacin, and rifampin at 2 weeks (Table 4). At five weeks, similar correlations were seen with alkaline phosphatase activity and gentamin (synergy screen), ciprofloxacin, and norfloxacin (Table 4). For *Escherichia coli*, significant differences were noted between sucrose fermentation and susceptibility to cephalothin, ampicillin/sulbactam, ampicillin, piperacillin, and tobramycin (Table 5).



(A)



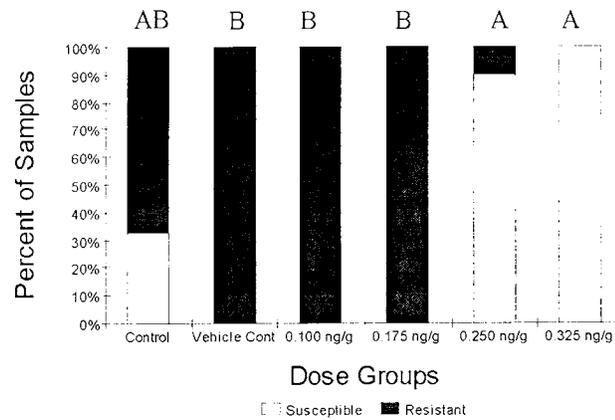
(B)



(C)

\*Groups with the same letter are not significantly different.

Figure 10. Antibiotic susceptibility profiles of *Enterococcus gallinarum* isolates in two-week-old chicks in PCB 126 study, (A) gentamicin (synergy screen), (B) ciprofloxacin, (C) norfloxacin, (D) rifampin.



(D)

\*Groups with the same letter are not significantly different.

Figure 10. Antibiotic susceptibility profiles of *Enterococcus gallinarum* isolates in two-week-old chicks in PCB 126 study, (A) gentamicin (synergy screen), (B) ciprofloxacin, (C) norfloxacin, (D) rifampin (Continued).

Table 4. Correlations between biochemical reactions and antibiotic susceptibility of *Enterococcus gallinarum* isolates from chicks in PCB 126 study.

Antibiotics	2 weeks				5 weeks	
	Alkaline Phosphatase		Ribose Fermentation		Alkaline Phosphatase	
	+	-	+	-	+	-
Gentamicin (synergy screen)	R	S	R	V	R	V
Ciprofloxacin	S	I	S	V	S	V
Norfloxacin	S	I	S	V	S	V
Rifampin	R	I	R	V		

\*S =  $\geq 75\%$  of samples are susceptible, I =  $\geq 75\%$  of samples are intermediate, R =  $\geq 75\%$  of samples are resistant, V = resistance phenotype is variable (i.e. no correlation).

Table 5. Correlations between biochemical reactions and antibiotic susceptibility of *Escherichia coli* isolates from two-week-old chicks in PCB 126 study.

Antibiotics	Sucrose Fermentation	
	+	-
Cephalothin	V	S
Ampicillin/Sulbactam	V	S
Ampicillin	R	S
Piperacillin	R	S
Tobramycin	V	R

\*S =  $\geq 75\%$  of samples are susceptible, R =  $\geq 75\%$  of samples are resistant, V = resistance phenotype is variable (i.e. no correlation).

#### Double-crested Cormorant Extract

##### Hatch

Significant differences were seen in observed versus expected hatchability for several dose groups (Table 6). Sample sizes are shown in Table 7.

Table 6. Observed versus expected hatchability by dose group for double-crested cormorant experiment with significance determined by Z statistic.

Dose Group	Alive Observed	Dead Observed	Alive Expected	Dead Expected	Z-obs	% Mortality
Control	34	20	30	24	1.095	37.0
Vehicle Control	22	20	20	22	0.618	47.6
0.0625 egg-EQ	29	18	20	27	2.655*	31.6
0.125 egg-EQ	35	49	20	64	3.843*	58.3
0.1875 egg-EQ	27	75	20	82	1.746	73.5
0.250 egg-EQ	30	86	20	96	2.458*	74.1

\*Indicates significant differences in observed versus expected values with Z-critical value of  $\pm 1.96$  ( $\alpha = 0.05$ ).

Table 7. Sample sizes per dose group for double-crested cormorant extract experiment.

Dose Group	2 week	5 week
Control	12	10
Vehicle Control	10	10
0.0625 egg-EQ	10	10
0.125 egg-EQ	10	10
0.1875 egg-EQ	10	10
0.250 egg-EQ	10	10
Total	62	60

### Microbial Identification

#### Baseline

Eight of the ten eggs tested at dosing contained bacteria on the surface. Six of these eggs contained *Staphylococcus xylosus*. The other bacteria identified were *Staphylococcus hominis* subspecies *hominis*, *Pseudomonas aerogenes*, *Acinetobacter lwoffii*, and *Aerococcus viridans*. Each of these bacteria was identified only once. None of the tested egg contents contained bacteria. No bacteria were found in any of the ten control birds tested at hatch.

#### Samples from Two-week-old Chicks

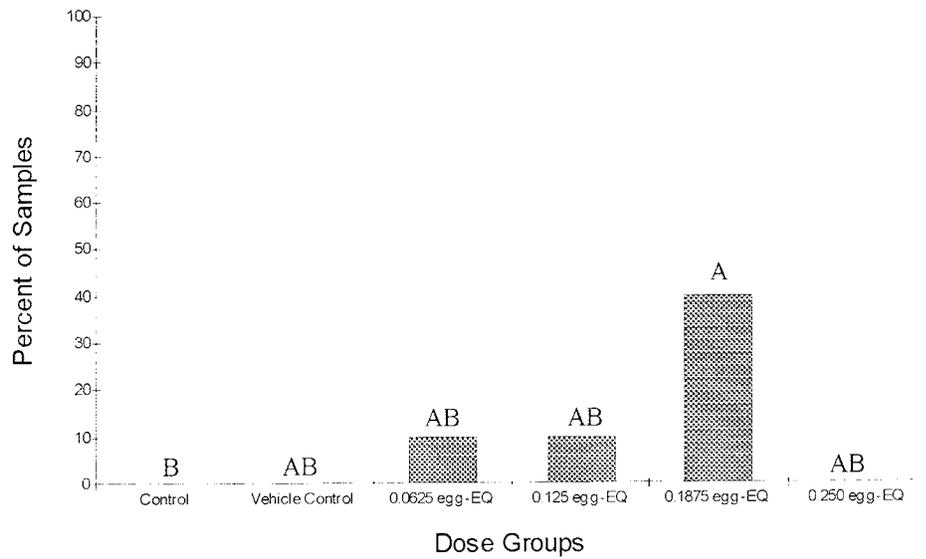
For birds sampled at two-weeks post-hatch, 18 bacterial species were identified from seven genera (Table 8). The predominant species identified were *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*. No significant differences were noted for prevalence of these three species per dose group. However, there were significant differences among dose groups for prevalence of *Enterococcus faecium* ( $p=0.0181$ ) (Figure 11). Several different strains of the bacterial species were identified among the

samples, but no significant differences were noted among dose groups for prevalence of particular strains. Biochemical results were also examined to determine any significant difference by dose group. No differences were observed for gram positive bacteria.

*Escherichia coli* samples showed significant differences by dose group for fermentation of sucrose ( $p=0.0072$ ) and adonitol ( $p=0.0422$ ) (Figure 12). Raffinose and arabinose fermentation differed among dose groups for *K. pneumoniae* species ( $p=0.0060$  and  $p=0.0382$  respectively) (Figure 13).

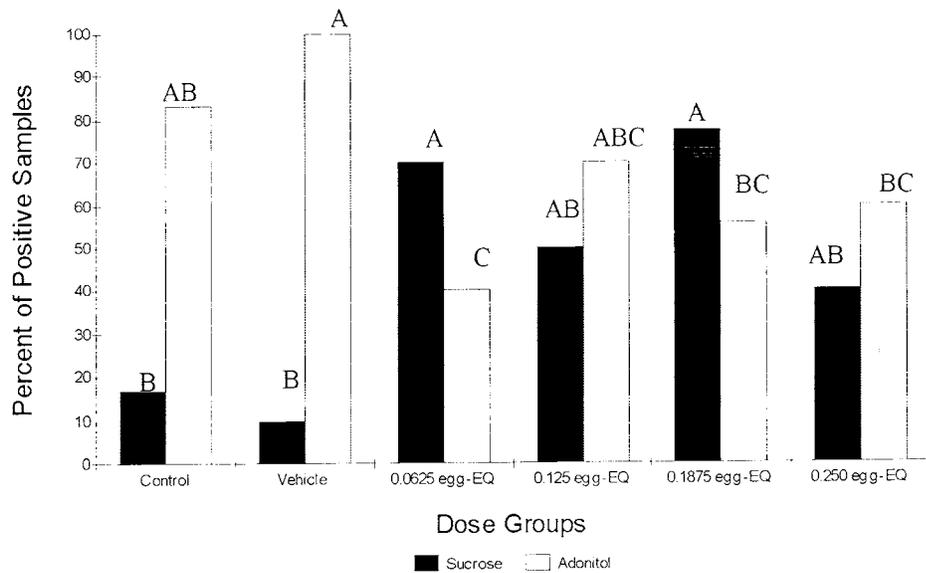
Table 8. Type and prevalence of bacteria identified in chicks at two-weeks post-hatch in double-crested cormorant extract experiment.

Bacteria	Number of positive swabs
<i>Cedecea lapagei</i>	1
<i>Enterobacter</i> spp.	14
<i>E. aerogenes</i>	1
<i>E. cloacae</i>	3
<i>E. sakazakii</i>	9
<i>E. taylorae</i>	1
<i>Enterococcus</i> spp.	96
<i>E. avium</i>	7
<i>E. casseliflavus</i>	3
<i>E. durans-hirae</i>	15
<i>E. faecalis</i>	47
<i>E. faecium</i>	7
<i>E. gallinarum</i>	16
<i>E. raffinosus</i>	1
<i>Escherichia coli</i>	60
<i>Klebsiella pneumoniae</i>	49
<i>Proteus mirabilis</i>	1
<i>Staphylococcus</i> spp.	6
<i>S. cohnii</i> subspecies <i>urealyticum</i>	4
<i>S. haemolyticus</i>	1
<i>S. hyicus</i>	1



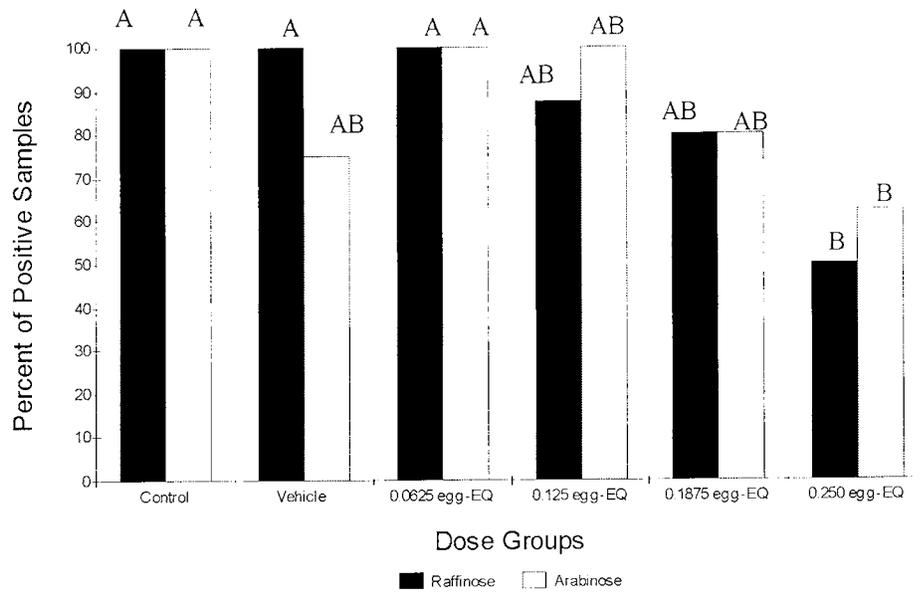
\*Groups with the same letter are not significantly different.

Figure 11. Presence of *Enterococcus faecium* by dose group in two-week-old chicks in double-crested cormorant extract study.



\*Groups with the same letter are not significantly different.

Figure 12. Percent of *Escherichia coli* isolates positive for sucrose and adonitol fermentation in two-week-old chicks in double-crested cormorant extract study.



\*Groups with the same letter are not significantly different.

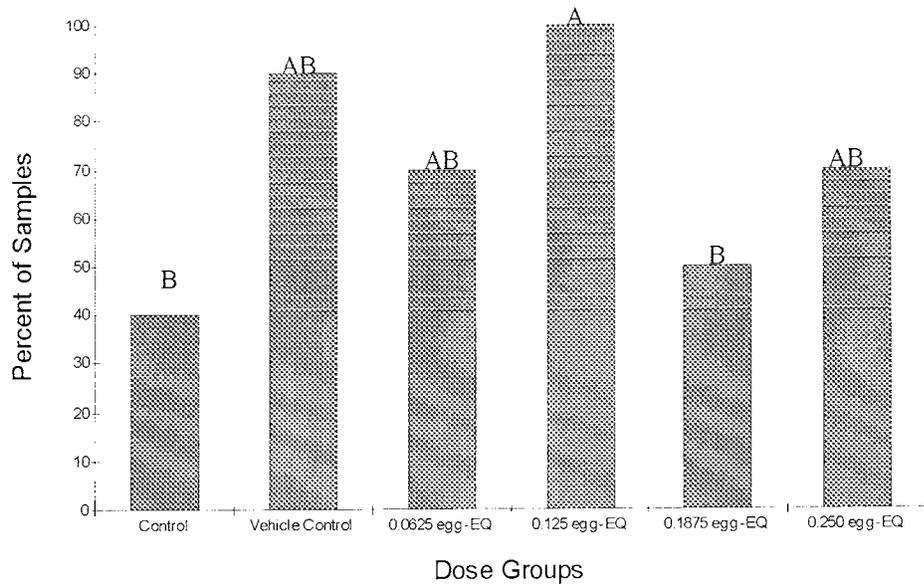
Figure 13. Percent of *Klebsiella pneumoniae* isolates positive for raffinose and arabinose fermentation in two-week-old chicks in double-crested cormorant extract study.

#### Samples from Five-week-old Chicks

The samples taken at five-weeks of age were similar in bacterial composition to the two-week samples, with the same predominant species. Fifteen species were identified from 7 genera (Table 9). As for samples from two-week-old chicks, no significant differences were noted among dose groups for prevalence of *Escherichia coli* or *K. pneumoniae*. Significant differences were noted among dose groups for prevalence of *Enterococcus faecalis* ( $p=0.0183$ ) and *Enterococcus gallinarum* ( $p=0.0072$ ) (Figures 14 and 15). No significant differences were noted among dose groups for prevalence of particular strains. Sucrose and adonitol fermentation differed significantly among dose groups for *Escherichia coli* species ( $p=0.0111$  and  $p=0.0305$  respectively) (Figure 16), but no other differences were noted with the biochemical results.

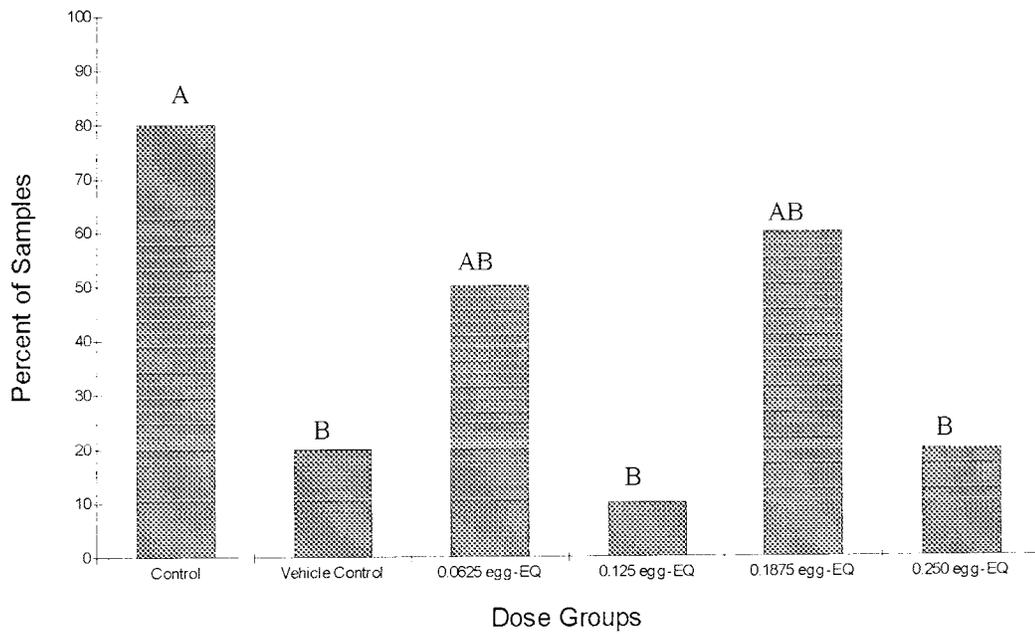
Table 9. Type and prevalence of bacteria identified in chicks five-weeks post-hatch in the double-crested cormorant extract experiment.

Bacteria	Number of positive swabs
<i>Acinetobacter lwoffii</i>	1
<i>Enterobacter</i> spp.	3
<i>E. cloacae</i>	1
<i>E. sakazakii</i>	2
<i>Enterococcus</i> spp.	81
<i>E. avium</i>	1
<i>E. casseliflavus</i>	4
<i>E. durans hirae</i>	1
<i>E. faecalis</i>	44
<i>E. faecium</i>	6
<i>E. gallinarum</i>	25
<i>Escherichia coli</i>	60
<i>Klebsiella pneumoniae</i>	51
<i>Pseudomonas</i> spp.	1
<i>Staphylococcus</i> spp.	36
<i>S. cohnii</i> subspecies <i>urealyticum</i>	1
<i>S. epidermis</i>	13
<i>S. xylosus</i>	22



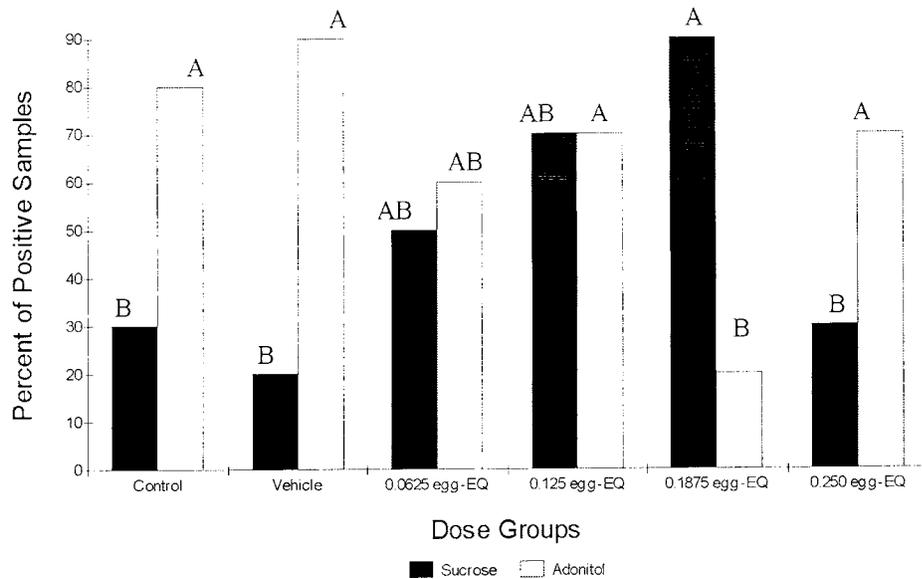
\*Groups with the same letter are not significantly different.

Figure 14. Presence of *Enterococcus faecalis* by dose group in five-week-old chicks in double-crested cormorant extract study.



\*Groups with the same letter are not significantly different.

Figure 15. Presence of *Enterococcus gallinarum* by dose group in five-week-old chicks in double-crested cormorant extract study.



\*Groups with the same letter are not significantly different.

Figure 16. Percent of *Escherichia coli* isolates positive for sucrose and adonitol fermentation in five-week-old chicks in double-crested cormorant extract study.

### Antibiotic Susceptibility Profiles

#### Samples from Two-week-old Chicks

For the samples taken from two-week-old chicks, significant differences were noted among dose groups for susceptibility of *Escherichia coli* to cephalothin ( $p=0.0497$ ), ampicillin/sulbactam ( $p=0.0228$ ), cefpodoxime ( $p=0.0497$ ), and ampicillin ( $p=0.0497$ ). (Figure 17). No differences were observed among dose groups for *K. pneumoniae* susceptibility to any antibiotic. A significant difference was observed for *Enterococcus faecalis* susceptibility to erythromycin ( $p=0.0115$ ) (Figure 18), but no other antibiotic.

Antibiotic susceptibility profiles were also examined for correlations with strain type and biochemical profiles for each bacteria examined. For most bacterial species,

antibiotic susceptibility did not differ within specific strains. However, samples within a few strains did produce different antibiotic susceptibility profiles. Significant differences were seen in samples taken at two-weeks of age identified as *E. coli* 77315012 to ampicillin/sulbactam.

Several significant correlations were seen between biochemical reactions and antibiotic susceptibility profiles. For *Enterococcus faecalis* species, lactose and raffinose fermentations were significantly correlated with susceptibility to gentamicin (synergy screen) ( $p=0.0012$  and  $p<0.0001$  respectively) (Table 10). With *Enterococcus faecium* isolates, growth in crystal violet and glycosidase production was correlated with both gentamicin (synergy screen) ( $p=0.0286$  for both) and tetracycline ( $p=0.0286$  for both) (Table 11). Glycosidase production and indoxyl phosphatase production in *Enterococcus gallinarum* isolates were correlated with gentamicin (synergy screen) ( $p=0.0037$ ,  $p=0.0110$ ), tetracycline ( $p=0.0220$ ,  $p=0.0440$ ), and vancomycin ( $p=0.0037$ ,  $p=0.0110$ ) (Table 12). As for the gram-negative bacteria, raffinose and arabinose fermentation was correlated with colistin susceptibility in *Klebsiella pneumoniae* isolates ( $p=0.0361$  for both) (Table 13). Numerous correlations were observed with *Escherichia coli* isolates, as shown in Table 14. For this species sucrose fermentation was correlated with kanamycin ( $p<0.0001$ ), cephalothin ( $p<0.0001$ ), ampicillin/sulbactam ( $p<0.0001$ ), cefpodoxime ( $p<0.0001$ ), ampicillin ( $p<0.0001$ ), piperacillin ( $p=0.0083$ ), cefazolin ( $p<0.0001$ ), ceftiofur ( $p<0.0001$ ), cefuroxime ( $p<0.0001$ ), and gentamicin ( $p=0.0111$ ). Adonitol fermentation was correlated with kanamycin ( $p=0.0074$ ), cephalothin ( $p<0.0001$ ), ampicillin/sulbactam ( $p<0.0001$ ), cefpodoxime ( $p<0.0001$ ), ampicillin ( $p<0.0001$ ), piperacillin ( $p=0.0087$ ), cefazolin ( $p<0.0001$ ), ceftiofur ( $p<0.0001$ ),

cefuroxime ( $p < 0.0001$ ), and gentamicin ( $p = 0.0020$ ). Finally, sorbitol fermentation was correlated with susceptibility to ampicillin/sulbactam ( $p < 0.0001$ ), piperacillin ( $p < 0.0001$ ), and cefuroxime ( $p < 0.0001$ ) in *Escherichia coli* isolates.

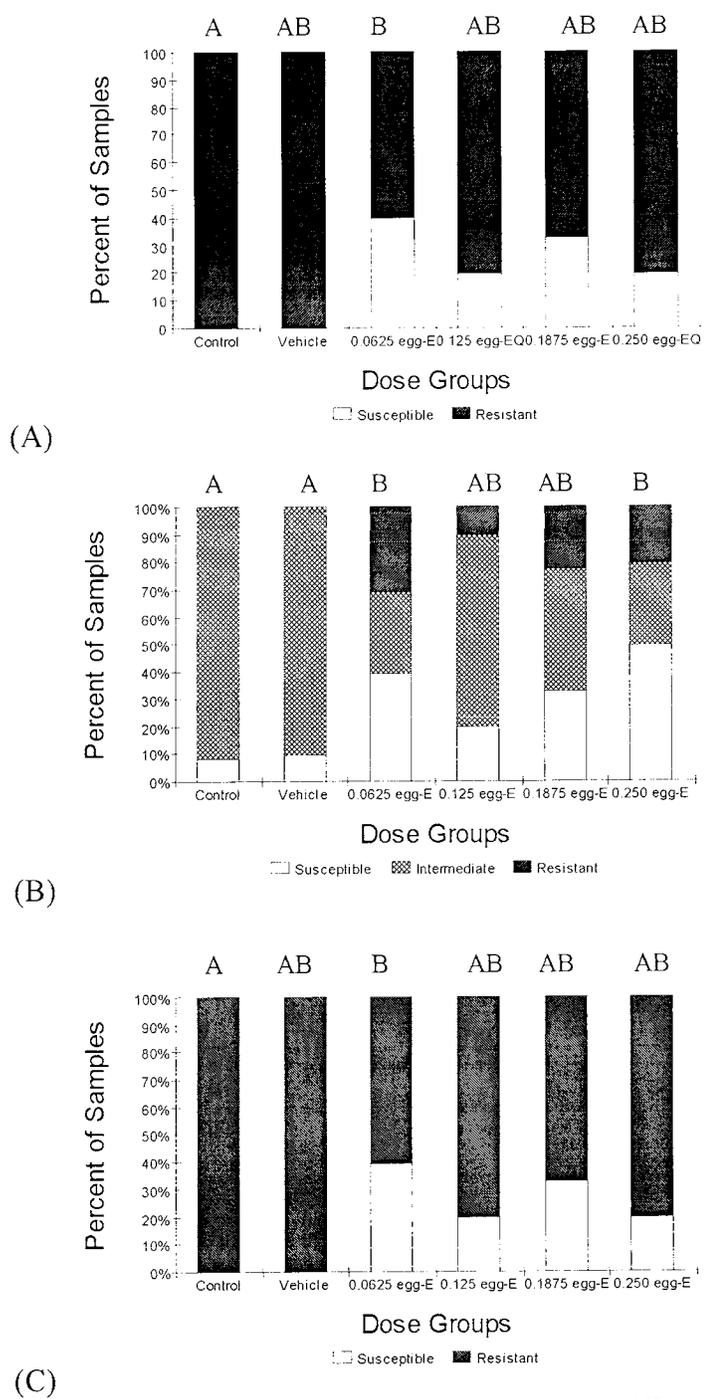
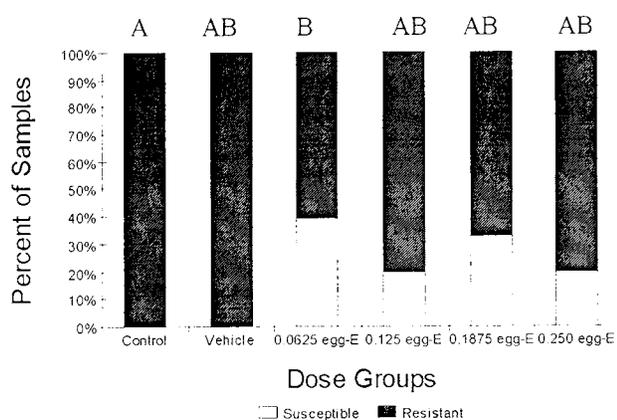


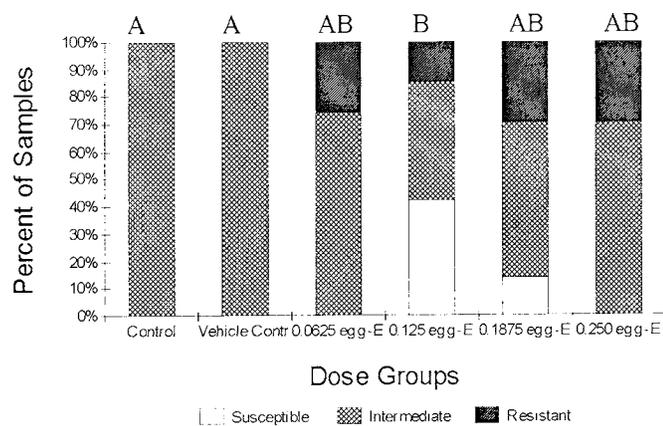
Figure 17. Antibiotic susceptibility profiles of *Escherichia coli* isolates in two-week-old chicks in double-crested cormorant extract study, (A) cephalothin, (B) ampicillin/sulbactam, (C) cefpodoxime, (D) ampicillin.



(D)

\*Groups with the same letter are not significantly different.

Figure 17. Antibiotic susceptibility profiles of *Escherichia coli* isolates in two-week-old chicks in double-crested cormorant extract study, (A) cephalothin, (B) ampicillin/sulbactam, (C) cefpodoxime, (D) ampicillin (Continued).



\*Groups with the same letter are not significantly different.

Figure 18. Antibiotic susceptibility of *Enterococcus faecalis* isolates to erythromycin in two-week-old chicks in double-crested cormorant extract study.

Table 10. Correlations between biochemical reactions and antibiotic susceptibility profiles of *Enterococcus faecalis* isolates from chicks in the double-crested cormorant extract experiment.

Antibiotics	2 weeks				5 weeks			
	Lactose		Raffinose		Lactose		Raffinose	
	+	-	+	-	+	-	+	-
Gentamicin (synergy screen)	V	R	R	V	S	V	R	S

\*S =  $\geq 75\%$  of samples are susceptible, R =  $\geq 75\%$  of samples are resistant. V = resistance phenotype is variable (i.e. no correlation).

Table 11. Correlations between biochemical reactions and antibiotic susceptibility of *Enterococcus faecium* isolates from two-week-old chicks in double-crested cormorant extract experiment.

Antibiotics	Growth in Crystal Violet		Glycosidase Production	
	+	-	+	-
Gentamicin (synergy screen)	S	R	R	S
Tetracycline	V	R	R	V

\*S =  $\geq 75\%$  of samples are susceptible, R =  $\geq 75\%$  of samples are resistant. V = resistance phenotype is variable (i.e. no correlation).

Table 12. Correlation between biochemical reactions and antibiotic susceptibility of *Enterococcus gallinarum* isolates from chicks in double-crested cormorant extract experiment.

Antibiotic	2 weeks				5 weeks										
	Glycosidase Production		Indoxyl Phosphatase		Glycosidase Production		Indoxyl Phosphatase		Alkaline Phosphatase		Bile Esculin		Crystal Violet		
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	
Gentamicin (synergy screen)	R	S	R	V	R	S	R	V							
Tetracycline	R	V	R	V	R	S	R	V							
Erythromycin					V	V	V	V	V	S	V	R	V	R	
Vancomycin	S	I	S	V	S	V									
Rifampin														R	I

\*S =  $\geq 75\%$  of samples are susceptible, I =  $\geq 75\%$  of samples are intermediate, R =  $\geq 75\%$  of samples are resistant, V = resistance phenotype is variable (i.e. no correlation).

Table 13. Correlations between biochemical reactions and antibiotic susceptibility of *Klebsiella pneumoniae* isolates from two-week-old chicks in double-crested cormorant extract experiment.

Antibiotics	Raffinose Fermentation		Arabinose Fermentation	
	+	-	+	-
Colistin	S	V	S	V

\*S =  $\geq 75\%$  of samples are susceptible, V = resistance phenotype is variable (i.e. no correlation).

Table 14. Correlations between biochemical reactions and antibiotic susceptibility of *Escherichia coli* isolates from chicks in double-crested cormorant extract experiment.

Antibiotic	2 week			5 week				
	Sucrose	Sorbitol	Adonitol	Sucrose	Adonitol	Ornithine Decarboxylation	Raffinose	Lysine Decarboxylation
	+	-	+	+	-	+	+	-
Kanamycin	R		R	R	V			
Cephalothin	V		R	R	V			
Ampicillin/Sulbactam	V	V	R	I	S	V	V	
Cefpodoxime	V		R	R	V	V	R	
Ampicillin	V		R	R	V	V	V	V
Piperacillin	V	S	S	R	V	V		
Cefazolin	V	R	R	R	V	R		
Cefoxitin	V		R	R	I		V	S
Cefuroxime	V	V	V	I	V			
Gentamicin	R		R	R	V		S	V
Tobramycin			R	V	V			

\*S =  $\geq 75\%$  of samples are susceptible, I =  $\geq 75\%$  of samples are intermediate, R =  $\geq 75\%$  of samples are resistant. V = resistance phenotype is variable (i.e. no correlation).

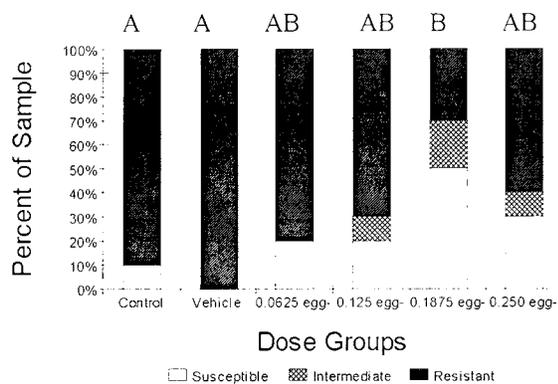
### Samples from Five-week-old Chicks

Significant differences in antibiotic susceptibility in samples taken at five-weeks post-hatch were noted for *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus gallinarum*. Significant differences in *Escherichia coli* susceptibility were noted to cephalothin ( $p=0.0454$ ), ampicillin/sulbactam ( $p=0.0257$ ), cefpodoxime ( $p=0.0098$ ), ampicillin ( $p=0.0106$ ), cefazolin ( $p=0.0098$ ), and cefuroxime ( $p=0.0161$ ) (Figure 19). *K. pneumoniae* showed no significant differences in susceptibility per dose group for any antibiotic. Significant differences were noted for *Enterococcus faecalis* susceptibility to erythromycin ( $p=0.0385$ ) (Figure 20). Susceptibility of *Enterococcus gallinarum* samples to erythromycin were also significantly different among dose groups ( $p=0.0038$ ) (Figure 21). No other differences were noted in susceptibility for any other bacterial species.

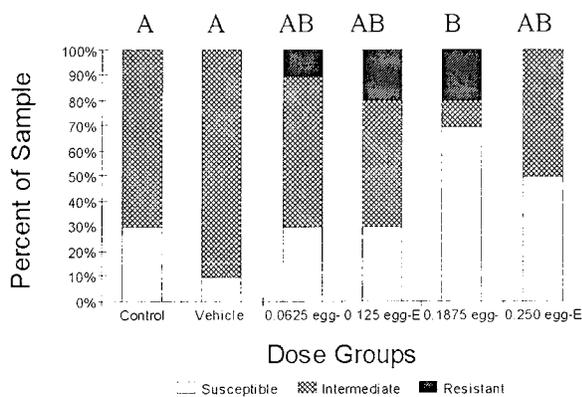
As in the samples taken at two-weeks of age most bacterial strains did not differ among themselves in their antibiotic susceptibility profiles, with a few exceptions. Significant differences were seen in antibiotic susceptibility of samples taken at five-weeks of age identified as *Escherichia coli* 77115012 to cephalothin, cefpodoxime, ampicillin, cefazolin, and cefuroxime and in five-week *K. pneumoniae* 77744370 samples to ampicillin.

As in the samples taken at two-weeks of age, lactose and raffinose fermentation was significantly correlated with susceptibility to gentamicin (synergy screen) in *Enterococcus faecalis* isolates ( $p=0.0032$  and  $p<0.0001$  respectively). For *Enterococcus gallinarum* isolates, glycosidase production and indoxyl phosphatase production were correlated with susceptibility to gentamicin (synergy screen) ( $p<0.0001$ ,  $p=0.0311$ ), tetracycline ( $p<0.0001$ ,  $p=0.0311$ ), and erythromycin ( $p=0.0104$ ,  $p=0.0325$ ). In addition,

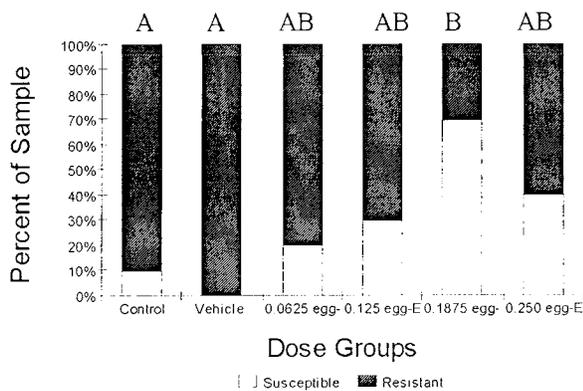
glycosidase production was correlated to vancomycin susceptibility ( $p=0.0217$ ), erythromycin susceptibility was correlated to growth in crystal violet ( $p=0.0031$ ), alkaline phosphatase production ( $p=0.0122$ ), and esculin hydrolysis ( $p=0.0415$ ), and rifampin susceptibility was correlated to growth in crystal violet ( $p=0.0069$ ) (Table 12). As in the samples taken at two-weeks, several correlations were observed for *Escherichia coli* isolates, as shown in Table 14. Sucrose fermentation was correlated with susceptibility to kanamycin ( $p=0.0040$ ), cephalothin ( $p=0.0018$ ), ampicillin/sulbactam ( $p<0.0001$ ), cefpodoxime ( $p=0.0013$ ), ampicillin ( $p=0.0013$ ), cefazolin ( $p=0.0013$ ), cefoxitin ( $p=0.0015$ ), and cefuroxime ( $p=0.0097$ ). Adonitol fermentation was correlated with susceptibility to kanamycin ( $p<0.0001$ ), cephalothin ( $p<0.0001$ ), ampicillin/sulbactam ( $p<0.0001$ ), cefpodoxime ( $p<0.0001$ ), ampicillin ( $p<0.0001$ ), cefazolin ( $p<0.0001$ ), cefoxitin ( $p<0.0001$ ), cefuroxime ( $p<0.0001$ ), gentamicin ( $p=0.0019$ ), and tobramycin ( $p<0.0001$ ). In addition, ornithine decarboxylation was correlated with susceptibility to ampicillin/sulbactam ( $p=0.0410$ ), cefpodoxime ( $p=0.0399$ ), ampicillin ( $0.0297$ ), and cefazolin ( $p=0.0399$ ). Finally, raffinose fermentation and lysine decarboxylation was correlation to ampicillin susceptibility ( $p=0.0040$  for both), and cefoxitin ( $p=0.0483$  for both).



(A)



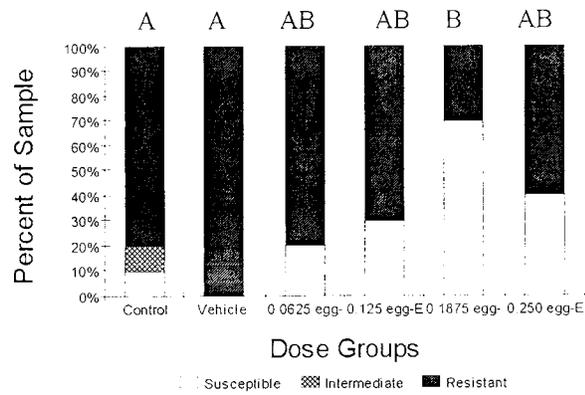
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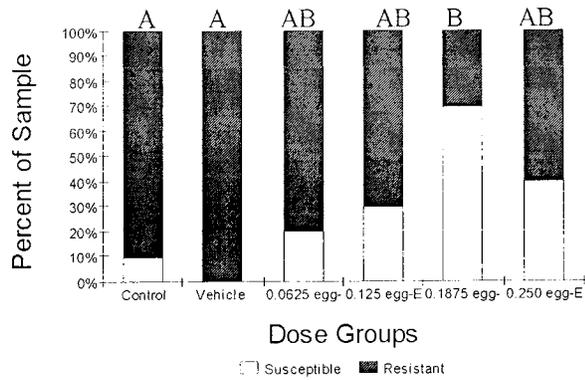
(C)

\*Groups with the same letter are not significantly different.

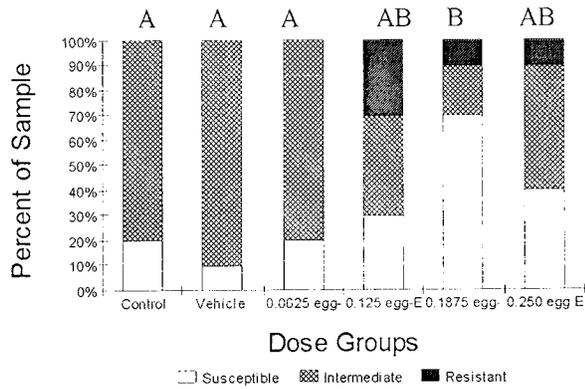
Figure 19. Antibiotic susceptibility profiles of *Escherichia coli* isolates in five-week-old chicks in double-crested cormorant extract study, (A) cephalothin, (B) ampicillin/sulbactam, (C) cefpodoxime, (D) ampicillin, (E) cefazolin, (F) cefuroxime.



(D)



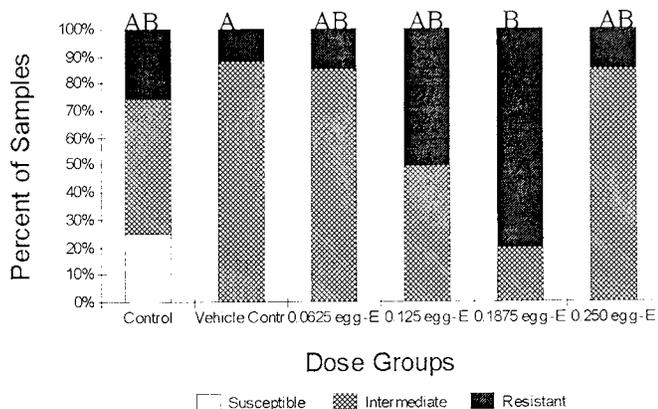
(E)



(F)

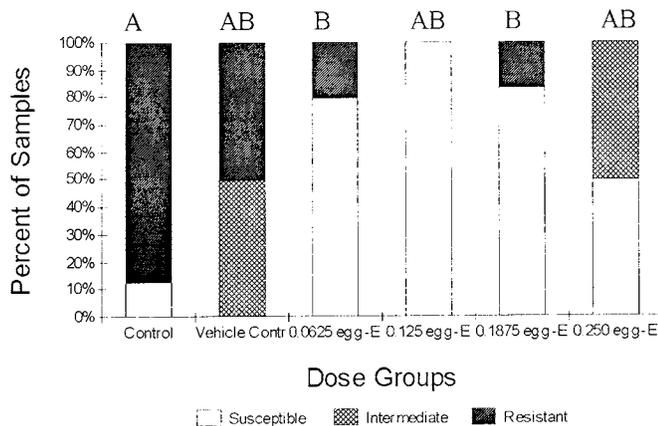
\*Groups with the same letter are not significantly different.

Figure 19. Antibiotic susceptibility profiles of *Escherichia coli* isolates in five-week-old chicks in double-crested cormorant extract study, (A) cephalothin, (B) ampicillin/sulbactam, (C) cefpodoxime, (D) ampicillin, (E) cefazolin, (F) cefuroxime (Continued).



\*Groups with the same letter are not significantly different.

Figure 20. Antibiotic susceptibility profiles of *Enterococcus faecalis* isolates to erythromycin in five-week-old chicks in double-crested cormorant extract study.



\*Groups with the same letter are not significantly different.

Figure 21. Antibiotic susceptibility profiles of *Enterococcus gallinarum* isolates to erythromycin in five-week-old chicks in double-crested cormorant extract study.

## Discussion

### PCB 126

#### Hatch

Mortality in several dose groups was different than what was expected for PCB 126 as compared to other experiments. Studies by Fox and Grasman (14) and Grasman and Whitacre (17), in which similar dose concentrations of pure PCB 126 were used and delivered in a sunflower oil carrier, experienced significantly less mortality for all doses. Those studies also had significantly less mortality in the control group than in our experiment, with control mortality being 7% or less for their studies as compared to 30.8% for our study. However, the authors in those studies did not count infertiles as part of their mortality and we included these eggs in our percentages. Infertiles were included in our total mortality because of the difficulty in distinguishing infertile eggs from embryos that died during very early in incubation. This study had significantly less mortality than a study by Powell et al. (29). In that study, 50% mortality was observed at 0.6 ng/g PCB 126, while we observed 50% mortality at 0.325 ng/g. However, that study involved yolk injections, which has been shown to result in higher mortality than air cell injection (14).

The increased mortality in our experiment as compared to the previous studies involving air cell injection may be the result of differences in methods or equipment. The incubator used in our experiment may have resulted in less hatchability or the fertility of the eggs used may have been less in our study. Also, the transportation of the eggs to and from CIET for dosing may have resulted in an increased mortality. Since the control

groups as well the dosed groups experienced greater mortality than the other studies, it is likely that differences in methods and not in the dosing solution is the cause.

### Microbial Identification

Intestinal bacteria identified in this study were all examples of normal gut flora associated with the domestic chicken (21, 31, 36). No obvious pathogens, such as *Salmonella*, were identified among the birds, and the Morgan Poultry Center has no problems with pathogenic bacteria. Absence of bacteria in the egg contents tested was expected, as this environment should be sterile (22). The presence of bacteria on the egg surface is most likely due to egg handling. No external disinfectant was applied to the eggs after laying.

Results for microbial identification show dose related differences in bacterial types present at two weeks of age. The presence or absence of *K. pneumoniae* and *Enterococcus gallinarum* varied among the dose groups. The presence of *K. pneumoniae* was significantly more likely in the control, vehicle control, and lowest PCB group (0.100 ng/g) than in the higher PCB-dosed groups. These results were not clearly dose-related though, with the lowest incidence of *K. pneumoniae* in the second dose concentration (0.175 ng/g) instead of the highest dose. PCB 126 may have a negative effect on the growth or survival of this species. There is no literature on the effects of PCBs or related compounds to *K. pneumoniae* so it is difficult to interpret these results. It may also be possible that in the presence of PCB 126, another species in the microbial flora may experience increased fitness and out compete *K. pneumoniae* in the intestinal environment.

As for the differences in gram-positive bacteria, the control group had significantly less occurrence of *Enterococcus gallinarum* than the remaining groups. Samples in the control groups contained a relatively even mixture of four bacterial types, while samples in the remaining dose groups contained predominantly *Enterococcus gallinarum*. These results could suggest some PCB 126-related alteration of intestinal flora populations, but they point to a vehicle effect as well. The results appear to suggest that the microbial population in the control birds was more species diverse than birds in the dosed groups. However, since the entire microbial population was not examined in this experiment, no such conclusions can be drawn. It is also difficult to determine whether the control and vehicle control populations were indeed significantly different since the vehicle control group had a samples size of only four.

Perhaps more interesting than the differences in presence of these species among doses are the differences in presence of specific strains among the dose groups. Due to different biochemical profiles, different strains of each predominant bacterial species were observed. In the case of *Escherichia coli* and *Enterococcus gallinarum*, there were significant differences among dose groups for the strain type present. These differences, which are reflected in the varying biochemical profiles, are most pronounced for *Enterococcus gallinarum*. Alkaline phosphatase activity and ribose fermentation are elevated in the vehicle control, 0.100 ng/g, and 0.175 ng/g groups. Again, these differences are not correlated with dose groups and the control and vehicle control appear to be significantly different. If PCB 126 is the cause of difference for these reactions, one possible explanation could be a hormesis effect. In some cases, low concentrations of contaminants can result in an increased fitness for the affected organism. This effect

fades away as contaminant concentrations increase and produce more of a toxic effect. Graphically this phenomenon resembles a bell curve, much like the graphical representation of these results.

It is unclear how PCB 126 may affect the production of alkaline phosphatase or ribose fermentation in these bacteria. A study with *Burkholderia cepacia* demonstrated a disulfide bond oxidoreductase system that was linked to both alkaline phosphatase production and resistance to several metals and antibiotics (19). Another study with *Pseudomonas marina* demonstrated an increase in alkaline phosphatase activity and resistance to antibiotics in a cadmium resistant strain (11). The authors of that study believed that the cadmium may reduce the activity of the phosphatase and therefore more enzyme was synthesized to take its place. The results of these studies do not offer an explanation for what is seen in our study, but they do give some evidence that alkaline phosphatase production is related to antibiotic resistance in some bacterial species. Since the alkaline phosphatase activity in our study was also shown to be significantly correlated to antibiotic susceptibility, perhaps the significances seen by dose group are related to an impact of the PCB 126 on antibiotic susceptibility. The correlations with ribose fermentation are also difficult to explain. Since the patterns of positive reactions are not dose-dependent it is even harder to hypothesize what effect the PCB 126 may be having on this species.

The absence of significant differences in microbial composition in samples taken at five-weeks of age may suggest that if the PCB 126 was indeed the cause of differences seen at two weeks, the effects dissipated as the animals grew older. The contaminant may have been sufficiently metabolized in the five week old birds to prevent any effects.

Another biomarker being tested during this experiment seems to support this idea. Several of the immune function tests that were performed on the same birds demonstrated a decrease in the immune functions of the treated birds at two weeks of age, but this effect was no longer apparent at five weeks (10).

#### Antibiotic Susceptibility Profiles

The frequency of antibiotic resistance seen in this study was similar to that seen in other studies involving the domestic chicken (8, 37). Significant differences were noted among dose groups for antibiotic susceptibility of *Escherichia coli* and *Enterococcus gallinarum*. With *Escherichia coli* isolates, the number of susceptible bacteria in the vehicle and PCB-dosed groups were greatly reduced compared with controls. These results suggest a dose effect, as well as a vehicle effect, but again are not clearly dose-related. The bacteria showed varying levels of resistance to four  $\beta$ -lactam antibiotics and two aminoglycosides. The patterns of resistance were the same for each antibiotic, in that bacteria demonstrating resistance to one class of antibiotic were also resistant to the other. The multiple resistance patterns suggest the presence of plasmids or drug efflux pumps as the mechanism of resistance. Bacterial plasmids are known to be able to confer resistance to several classes of antibiotics (1, 15). Drug efflux pumps are also recognized as a mechanism of multiple antibiotic resistance (25). However, aminoglycosides are not thought to be excreted through these pumps due to their hydrophilic properties (25). Therefore, presence of bacterial plasmids may be more likely in this case.

The antibiotics to which resistance was seen showed variability with sucrose fermentation. *Escherichia coli* strains that were negative for sucrose fermentation were

always susceptible to the  $\beta$ -lactams, and always resistant to the aminoglycosides. However, strains that were positive for sucrose fermentation varied in their resistance phenotypes. It may be that PCB 126 exerts an effect on sucrose fermentation and that in turn affects antibiotic susceptibility. However, no significant differences were observed among dose groups for sucrose fermentation so this seems unlikely. PCB 126 may also select for a certain resistance pattern and that in turn may be correlated with sucrose fermentation. In either case, it appears that sucrose fermentation is correlated with susceptibility to these antibiotics. Perhaps these bacteria contain plasmids which encode for both the resistance phenotype as well as the sucrose phenotype. Several studies have shown the existence of plasmid-mediated sucrose fermentation (4, 18, 26). It is also possible that the genes encoding the sucrose phenotype are chromosomally located and that correlations with antibiotic resistance are due to some other physiological reason. A study by Petti et al. (27) found that strains of *Streptococcus mutans* experienced greater sensitivity to cephalosporins when in the presence of sucrose. The mechanism was unknown but thought to be due to the production of insoluble glucan which increases interbacterial distance and therefore may encourage diffusion of the antibiotic into the cells.

With the *Enterococcus gallinarum* isolates, significant differences in several antibiotics susceptibilities occurred among dose groups in chickens at two weeks. These differences, which include susceptibility to gentamicin (synergy screen), ciprofloxacin, norfloxacin, and rifampin, were not dose related. As with the differences in biochemical profiles for *Enterococcus gallinarum*, the vehicle control, 0.100 ng/g, and 0.175 ng/g groups display similar profiles, while differing significantly from the other groups.

Interestingly, each of these antibiotics were also found to be significantly correlated with alkaline phosphatase activity and ribose fermentation, the two biochemical results that were found to be significantly different among dose groups. As discussed earlier, PCB 126 may have an effect on the biochemical profiles of the bacteria, and this in turn may influence antibiotic susceptibilities. The opposite situation may also occur, with PCB 126 affecting the antibiotic susceptibility which in turn influences the biochemical reactions.

The absence of significant differences in antibiotic susceptibility in the 5 week birds mirrors the results from the microbial identification. As mentioned earlier, if the effects are caused by PCB 126, the compound may be sufficiently metabolized in the older birds and therefore no longer producing an effect.

One weakness of this study is the small sample sizes present in the two-week age group. Due to less than expected hatchability, samples sizes for control and vehicle control birds were smaller than planned. This small sample size, especially in the vehicle control group (n=4), may be a factor in the statistical differences noted between the control and vehicle control groups.

Another factor to consider is that the chicks were placed in a single brooder but separated according to dose group in different levels. This was done in an effort to lower handling time and stress levels when taking birds out to be weighed and perform various tests. The slight difference in the chicks' environment could have been a factor in the differences observed between dose groups for both the microbes identified and the susceptibility profiles. Birds were placed in larger floor pens at four weeks of age and

dose groups were housed together, which could explain the absence of significant differences in the five-week samples.

### Double-crested Cormorant Extract

#### Hatch

Mortality observed in this experiment was slightly different than expected and also different than a previous dosing study using the same extract. In a study by Powell et al. (28), 76.7% mortality was observed in chicken embryos dosed with 1 egg-equivalent of double-crested cormorant extract. We experienced similar mortality at a much lower dose concentration, with 74.1% mortality at 0.25 egg-equivalents. However, the Powell study observed much lower mortality in control and vehicle control groups than in our study. Therefore, it is likely that the increased mortality seen in our experiment is due to differences in methods or equipment used and not as a result of the extract.

#### Microbial Identification

As in the PCB 126 experiment, all bacteria identified were members of the normal avian intestinal flora. The species identified in the two experiments differs somewhat but this was to be expected, as the experiments took place at different time periods and chickens were housed in separate areas. As with the first experiment, no bacteria were found in the egg contents tested at dosing. Several species were cultured from the egg surfaces, but this is to be expected from handling and exposure to the environment. No

bacteria were cultured from the cloacal swabs of newly hatched chicks. This result was also expected, as bacteria do not begin colonizing the intestine until after hatch (32, 36).

For the bacteria isolated at two weeks of age, only one species showed any significant differences for presence among dose groups. This species, *Enterococcus faecium*, had a greater likelihood of being present in the treated groups, although correlations were not exact. The small sample size (n=7) hinders the formation of any sound conclusion.

As for the bacteria observed at five weeks, significant differences were seen among dose groups for presence of *Enterococcus faecalis* and *Enterococcus gallinarum*. For both of these species the differences seen among the dose groups do not appear to have a clear pattern. It is not apparent whether the extract may be influencing the presence of the species or if some other factor may be involved.

Differences in biochemical profiles among dose groups are more interesting. *Escherichia coli* isolates with the ability to ferment sucrose but not adonitol were significantly more likely in the treated groups at both two and five weeks of age. In addition, *K. pneumoniae* isolates at two weeks of age were less likely to possess the ability to ferment raffinose and arabinose in the treated groups. As with the results from the first experiment, it is not clear what mechanism may be involved in these alterations.

#### Antibiotic Susceptibility Profiles

Susceptibility of *Escherichia coli* to several of the  $\beta$ -lactams was significantly correlated with dose group. However, the same trend of susceptibility observed in the PCB 126 study did not occur with this experiment. The control and vehicle control groups exhibited almost complete resistance, while the treated groups were

predominantly resistant but also contained many susceptible isolates. This trend occurred at both two and five weeks of age. Since only one class of antibiotics was involved in the resistance phenotypes, the mechanism of resistance may be a chromosomal  $\beta$ -lactamase, although the presence of a plasmid may be involved as well.

Significant differences in *Enterococcus faecalis* susceptibility also occurred at both two and five weeks. At two weeks, the control and vehicle control isolates were all intermediate in resistance to erythromycin, while the treated groups displayed a mix of susceptible, intermediate, and resistant bacteria. In contrast, at five weeks of age *Enterococcus faecalis* isolates from control birds displayed a mix of susceptible, intermediate, and resistant phenotypes, while the remaining groups were a mix of intermediate and resistant phenotypes. It is important to remember that the samples obtained at five weeks did not come from the same birds as in the two week samples. It is not possible to determine if the microbial flora changed over time or if the differences are due to individual variation in the birds.

Antibiotic susceptibility of *Enterococcus gallinarum* isolates to erythromycin was also significantly different among dose groups. For these bacteria, the percent of susceptible bacteria was higher in the treated groups, although, as in the other differences seen, the correlations were not clearly dose-related. Erythromycin resistance has been shown to be associated with multi-drug resistance efflux systems (1). With no clear pattern of association with the extract concentrations it is difficult to speculate on what, if any, effect the extract may be having on the susceptibility.

This experiment also demonstrated several correlations between antibiotic susceptibility profiles and biochemical profiles. For *Escherichia coli*, sucrose

fermentation was again correlated with several antibiotics, although not in the same way as in the PCB 126 experiment. Because the two experiments revealed different patterns of correlation, it is safe to assume that the ability to ferment sucrose does not necessarily predict antibiotic susceptibility phenotypes. However, since the two phenotypes are correlated with one another in both experiments, some sort of association appears to exist.

For *Enterococcus faecalis*, gentamicin (synergy screen) susceptibility is correlated to both lactose and raffinose fermentation at two and five weeks of age. *Enterococcus gallinarum* isolates also exhibited correlations between biochemical profiles and susceptibility to several antibiotics. These correlations were not the same as in the PCB 126 experiment. As with the *Escherichia coli* correlations, the two experiments do seem to show the existence of some association between the phenotypes. However, no biochemical profile was shown to be predictive of a particular antibiotic susceptibility phenotype.

### General Conclusions

Drawing comparisons between the two laboratory experiments is difficult and probably not appropriate. The two experiments were conducted at different times during the year, a different flock of hens produced the eggs, the chicks were reared in different facilities, and perhaps most importantly, the dosing solutions were different. The first experiment involved the use of a pure compound, while the second experiment used a mixture. Compounds in the mixture could have acted synergistically, additively, or in an antagonistic fashion. In addition, the potency of the doses of cormorant extract were greater than those in the PCB 126 doses.

Two essential findings that have resulted from these studies are the significant correlations between biochemical profiles and antibiotic susceptibility, and the significant differences seen among the dose groups for microbial composition, biochemical profiles, and antibiotic susceptibility profiles. Although it is not clear what mechanism may be involved in these differences, it does appear that the bacterial populations have been affected by the contaminant concentrations to which the chickens were exposed.

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## EFFECTS OF BIOACCUMULATIVE CONTAMINANTS ON THE INTESTINAL FLORA OF NESTLING BALD EAGLES

### Introduction

The use of biomarkers to understand the more subtle effects of environmental contaminants on wildlife is a growing field. Numerous biomarkers, which include various physiological, morphological, and biochemical measures, have already been studied and many have proven to be effective in predicting toxicant exposure (12, 15). New biomarkers are continually being developed as the scientific community continues to better understand the physiological effects of contaminants.

The Michigan Bald Eagle Sentinel Project, created by the Michigan Department of Environmental Quality (MDEQ), is a program that uses the bald eagle (*Haliaeetus leucocephalus*) as an indicator of Great Lakes contaminant levels and regional environmental health. The bald eagle is being utilized in this manner for several reasons. The bald eagle has been extensively studied, resulting in the availability of large amounts of life history information and census data. In addition, the eagle is a tertiary predator and is therefore exposed to bioaccumulative compounds at higher levels than animals lower in the food chain. The bald eagle also has the societal value necessary to attract public attention and support (9).

The Bald Eagle Sentinel Project, which began in 1999, monitors concentrations of organochlorine pesticides, polychlorinated biphenyls, and mercury in bald eagles through sampling and analysis of juvenile blood and feathers (9). To further enhance this program, biomarkers are being developed that can be applied to the field so that we can

better understand the pollutant load of this species and thus have a more accurate picture of environmental contamination.

The effect of contaminants on microorganisms in the intestinal tract of birds has been proposed as a potential new nondestructive biomarker of exposure to bioaccumulative compounds of concern (BCCs). Due to their lipophilic properties, these compounds, which include polychlorinated biphenyls (PCBs), dioxins, furans, and organochlorine pesticides, accumulate and concentrate in the fatty tissue of organisms feeding at high trophic levels. They have been of particular concern to fish-eating birds and mammals. These compounds generate a variety of detrimental effects, even at small concentrations, including reproductive and developmental abnormalities, and immune and nervous system effects (4).

Alteration of the intestinal flora by environmental contaminants may occur either directly by pollutant effects on the microbes or indirectly by suppressing the immune system of the host organism. Studies concerning direct effects of contaminants on microbes are limited. The primary interest for microorganisms in toxicology is on their ability to breakdown or transform chemical compounds and aid in bioremediation. Studies concerning the effects of BCCs on bacteria have shown them to have both beneficial and detrimental effects, depending on the type of bacteria affected (5, 7, 26, 28).

Immune system suppression may alter normal intestinal flora populations by allowing abnormal and/or more pathogenic strains to colonize the intestinal tract. The colonization and the immune surveillance of the intestinal tract are largely regulated by

immunoglobulins (20). Absence or suppression of immunoglobulins could result in the formation of an abnormal flora population.

In addition to affecting the types of bacteria present in the avian gut, BCCs may also influence the antibiotic susceptibility of those compounds. Several studies have shown that genes encoding for antibiotic resistance may be selected for in the presence of heavy metals, organic solvents, and biocides (1). There have also been studies showing genetic linkage between virulence determinants and antibiotic resistance (18, 27, 29). Therefore, with the colonization of more pathogenic species, there is the potential for a change in antibiotic resistance as well.

The effects of BCCs on avian intestinal flora has been examined in two laboratory experiments using domestic chickens (*Gallus domesticus*). In these experiments, chicken were exposed *in ovo* to several concentrations of environmental contaminants, the first PCB 126, the second an extract taken from double-crested cormorant (*Phalacrocorax auritis*) eggs that contained a mixture of contaminants representative of those found in the Great Lakes region. These experiments demonstrated some effect of bioaccumulative compounds on both the composition of bacteria in the intestinal tract and the antibiotic susceptibility of several bacterial species.

In this study, the effects of bioaccumulative contaminants on the intestinal flora of juvenile bald eagles were examined. Cloacal swabs of nestling eagles were taken during normal banding and sampling activities and the intestinal flora was compared to blood plasma contaminant levels. The objectives were to determine the effects of environmental contaminants on 1. the composition of microorganisms in the intestinal tract of nestling

bald eagles; and 2. the antibiotic susceptibility of those organisms. The potential of this technique to serve as a biomarker of contaminant exposure in avian species is examined.

## Materials and Methods

### Sampling

Sampling of nestling eagles occurred coincidental to annual banding and sampling activities as part of the Michigan Bald Eagle Sentinel Project. Nestling bald eagles aged 4-9 weeks were sampled from mid-May through the end of June, 2002. The sampling region consisted of the lower and upper peninsulas of Michigan and Voyageurs National Park (VNP), Minnesota. Sampling regions in Michigan were distinguished as Inland or Great Lakes (within 8 km of a Great Lakes shoreline). A total of 61 birds were sampled among the three regions (Inland n=27, Great Lakes n=19, VNP n=15).

Eagle nests with young were identified by aerial surveys conducted by the Michigan Department of Natural Resources and the National Park Service. Once at the nest, a climber ascended the nest tree and lowered a nestling to the ground in an eagle bag. On the ground, the eagle was weighed, banded, and morphometric measurements were taken to estimate age and sex of nestlings (6). Using sterile techniques, approximately 10-12cc of blood were drawn via the brachial vein, using heparinized syringes and a 22 gauge needle (8). Blood was immediately transferred to 12 ml heparinized vacutainer tubes and stored on ice packs until transport back to the field lab. A cloacal swab was taken prior to release of the bird. The cloacal region was disinfected with a 70% isopropanol wipe and a sterile, cotton-tipped swab was inserted approximately 2.54 cm into the cloaca. A sufficient amount of fecal material was

removed and the swab was then used to inoculate a Columbia CNA agar plate and a Levine EMB agar plate. Sterile agar plates were kept in a cooler on ice packs before use and were placed in a separate cooler containing no ice (ambient temperature) following inoculation. After all procedures were completed, the nestling was raised back into the nest and the climber descended the tree.

At the end of the day the agar plates were transported back to the field lab where they were streaked for colony isolation and placed in a 37°C incubator. Following 20-24 h of incubation, the plates were removed and examined for colony growth. All colony observations were recorded and if needed, colonies were subcultured to obtain a pure culture. After pure cultures were obtained, each colony type was subcultured onto a Trypticase Soy Agar (TSA) slant. Slants were further incubated for 20-24 h and stored in a refrigerated cooler (~5°C) for the remainder of field season. After transport back to Clemson University, South Carolina the samples were placed in a refrigerator until analysis.

Once at the field lab, blood samples were centrifuged to separate plasma from red and white blood cells. The plasma layer was then decanted into a new heparinized vacutainer tube, sealed, and frozen. Plasma samples were shipped to the U.S. Fish and Wildlife Service East Lansing Field Office for storage until analysis at Clemson.

### Bacterial Analysis

Each bacterial sample was inoculated from the TSA slant onto a Columbia CNA or Levine EMB agar plate and incubated 20-24 h at 35°C. Colonies were then analyzed using MicroScan® Dried Gram Negative Panel 22 and Dried Gram Positive Panel 14 (Dade Behring, Inc., Deerfield, IL). Dried Gram Negative Panel 22 included the

following antibiotics: penicillin, kanamycin, nitrofurantoin, colistin, cephalothin, ampicillin/sulbactam, aztreonam, ceftazidime, trimethoprim/sulfamethoxazole, ciprofloxacin, cefpodoxime, ampicillin, piperacillin, cefazolin, cefoxitin, cefuroxime, ceftriaxone, cefotaxime, gentamicin, tobramycin, amikacin, levofloxacin, meropenem, imipenem. The Dried Gram Positive Panel 14 included the following antibiotics: streptomycin (synergy screen), gentamicin (synergy screen), penicillin, tetracycline, amoxicillin/clavulanate, trimethoprim/sulfamethoxazole, ampicillin, erythromycin, clindamycin, gentamicin, oxacillin, vancomycin, cefazolin, cefotaxime, ciprofloxacin, levofloxacin, norfloxacin, imipenem, cephalothin, nitrofurantoin, trovafloxacin, rifampin. Quality control organisms *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were analyzed concurrently. Identification to species level and antibiotic susceptibility profiles were determined following MicroScan® Procedural Manual guidelines. For several species, additional testing was needed to obtain positive identification. Identification to the species level was obtained for the majority of samples. Minimum inhibitory concentrations (MICs) for comparing antibiotic susceptibility were determined from National Committee for Clinical Laboratory Standards (NCCLS) Interpretive Breakpoints as provided in the MicroScan® manual (22, 23).

#### Plasma Analysis

Plasma samples were analyzed for organochlorine pesticides and PCBs following Clemson Institute of Environmental Toxicology Standard Operating Procedure (SOP) 401-78-01. Briefly, one mL of plasma was denatured with methanol, extracted with methylene chloride, and purified with alumina and silica solid phase extraction. Quality control samples were analyzed concurrently and internal gas chromatography standards

were added to each sample. Samples were analyzed by gas chromatography with electron-capture detection (GC-ECD). The quantification level was 2 ng/g (25).

### Statistical Analysis

Plasma contaminant concentrations were compared to microbial composition, biochemical profiles and antibiotic susceptibility profiles using logistic regression. In several analyses, there appeared to be outliers present. When this occurred, the x-axis (contaminant concentration) was collapsed into three categories (low, medium, and high) and the data was analyzed by both a chi-square and Fisher's Exact test to determine if the results were indeed significant. If p values for these tests appeared significant then the results of the logistic regression were assumed to be significant and were reported. Biochemical profiles were also compared to antibiotic susceptibility profiles using Fisher's Exact test.

## Results

### Plasma Analysis

Detectable concentrations of PCBs and several organochlorine pesticides including *p,p'*-DDE, chlordane, and dieldrin were observed among the samples, as shown in Table 15. Mean concentrations of total PCBs, *p,p'*-DDE, and dieldrin were significantly greater in samples of the Great Lakes region than in Inland Michigan or VNP ( $p < 0.0001$  for total PCBs,  $p = 0.0057$  for *p,p'*-DDE,  $p < 0.0001$  for dieldrin).

Table 15. Blood plasma concentrations of PCBs and organochlorine pesticides from nestling bald eagle samples, reported in ng/g.

Region	Total PCBs		<i>p,p'</i> -DDE		Dieldrin		Chlordane	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Inland MI	19.55	ND – 83.66	17.52	<2.00 – 89.01	<2.00	ND – 2.20	<2.00	ND – 2.96
Great Lakes	84.09	10.08 – 217.48	32.57	5.6 – 128.72	2.18	ND – 6.56	<2.00	ND – 3.35
VNP	24.77	ND – 155.12	14.25	3.01 – 56.26	<2.00	ND – 2.01	<2.00	ND – 0.96
Total	40.84	ND – 217.48	22.96	<2.00 – 128.72	<2.00	ND – 6.56	<2.00	ND – 3.35

#### Microbial Composition

A total of 28 bacterial species were identified from 13 genera, as shown in Table 16. The majority of species belong to genera of the *Enterobacteriaceae* family, as well as the genera *Staphylococcus* and *Enterococcus*. The predominant species encountered were *Escherichia coli* and *Enterococcus faecalis*. Several colonies were only able to be identified to the genus level.

Table 16. Type and prevalence of bacteria identified in nestling bald eagles.

Bacteria	Number of positive swabs
<i>Acinetobacter lwoffii</i>	3
<i>Aerococcus viridans</i>	1
<i>Cedecea</i> spp.	2
<i>C. davisae</i>	1
<i>C. species 5</i>	1
<i>Edwardsiella tarda</i>	1
<i>Enterobacter cloacae</i>	6
<i>Enterococcus</i> spp.	57
<i>E. casseliflavus</i>	1
<i>E. durans hirae</i>	1
<i>E. faecalis</i>	54
<i>E. faecium</i>	1
<i>Escherichia</i> spp.	61
<i>E. coli</i>	59
<i>E. fergusonii</i>	1
<i>E. hermannii</i>	1
<i>Kluyvera ascorbata</i>	1
<i>Leclercia adecarboxy</i>	1
<i>Micrococcus</i> spp.	6
<i>Proteus</i> spp.	15
<i>P. mirabilis</i>	13
<i>P. vulgaris</i>	2
<i>Serratia odorifera 1</i>	1
<i>Staphylococcus</i> spp.	64
<i>S. aureus</i>	13
<i>S. auricularis</i>	10
<i>S. capitis</i> subspecies <i>capitis</i>	1
<i>S. hyicus</i>	10
<i>S. intermedius</i>	17
<i>S. schleiferi</i>	2
<i>S. sciuri</i>	5
<i>S. simulans</i>	2
<i>S. warneri</i>	3
<i>S. xylosus</i>	1

No significant differences were seen for presence of bacterial species by concentration of any contaminant. However, two genera did show significant differences for presence by concentration of total PCBs. Bacteria belonging to the genera *Cedecea* and *Micrococcus* were significantly more likely to be observed in birds with high plasma PCB concentrations ( $p=0.0341$  for *Cedecea*,  $p=0.0054$  for *Micrococcus*).

No significant differences were found among biochemical profiles of the bacteria when compared to contaminant concentrations. There were also no significant differences by contaminant concentration when compared with age and sex of the birds, as well as the number of bacterial species present per bird. A significant difference was noted when comparing number of bacterial species by age (in weeks) (Figure 22). The number of bacterial species present per bird tends to decrease with increasing age ( $p=0.0185$ ).

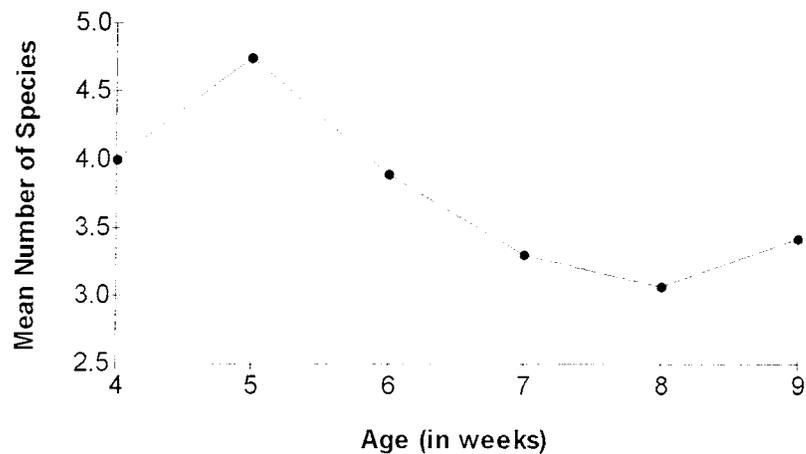


Figure 22. Mean number of bacterial species present per bird by age (in weeks) in nestling bald eagles.

### Antibiotic Susceptibility Profiles

Significant differences were noted in *Escherichia coli* susceptibility to several antibiotics when compared to plasma PCB concentrations. Resistance of *Escherichia coli* isolates to ampicillin and piperacillin decreased as plasma PCB levels increased ( $p=0.0375$  for both). In contrast, resistance of *Escherichia coli* isolates to ampicillin/sulbactam increased as plasma PCBs increased ( $p=0.0253$ ). Similar trends occurred with concentrations of *p,p'*-DDE, dieldrin, and chlordane, although these comparisons were not significant. Overall, little antibiotic resistance was seen among *Escherichia coli* isolates, as well as the other species of bacteria. Of the 59 strains of *Escherichia coli* identified, only six showed resistance to one or more antibiotic. Of these six, five isolates were resistant to six or more of the tested antibiotics. These multi-resistant bacteria were found in birds containing both low and high concentrations of contaminants and in all three geographic regions.

A significant difference was also observed for susceptibility of *Enterococcus faecalis* to tetracycline when compared to plasma PCB concentrations. Resistance to tetracycline tends to decrease with increasing PCB concentration ( $p=0.0269$ ). As with *Escherichia coli*, very little antibiotic resistance was seen among the samples. Of the 54 isolates of *Enterococcus faecalis*, six were resistant to at least one antibiotic, the most common being erythromycin. Again, these resistant bacteria were found in birds containing both low and high concentration of contaminants and in both the Inland and Great Lakes regions of Michigan.

Several significant correlations were noted among antibiotic susceptibility profiles and biochemical results. For *Escherichia coli*, ability to hydrolyze esculin was

significantly correlated with antibiotic susceptibility to colistin ( $p=0.0088$ ), cephalothin ( $p=0.0228$ ), aztreonam ( $p=0.0041$ ), ceftazidime ( $p=0.0058$ ), cefpodoxime ( $p=0.0088$ ), cefoxitin ( $p=0.0088$ ), and amikacin ( $p=0.0035$ ). In addition, nitrofurantoin susceptibility was correlated with urease production ( $p=0.0169$ ) and production of hydrogen sulfide gas ( $p=0.0169$ ). Finally, susceptibility of *Escherichia coli* to kanamycin was correlated with decarboxylation of arginine ( $p=0.0267$ ) (Table 17). Susceptibility of *Proteus mirabilis* to cephalothin ( $p<0.0001$ ), aztreonam ( $p=0.0047$ ), ceftazidime ( $p=0.0350$ ), cefoxitin ( $p=0.0070$ ), gentamicin ( $p=0.0070$ ), tobramycin ( $p=0.0070$ ), and amikacin ( $p=0.0070$ ) was also correlated with esculin hydrolysis. Susceptibility of these bacteria to cephalothin and aztreonam was also correlated to decarboxylation of arginine ( $p=0.0070$  and  $p=0.0210$  respectively) (Table 18). Correlations between biochemical profiles and antibiotic susceptibility were also noted for *Staphylococcus intermedius*. Tetracycline and erythromycin susceptibility were correlated with ribose fermentation ( $p=0.0083$  for both) (Table 19).

Table 17. Correlations between biochemical reactions and antibiotic susceptibility of *Escherichia coli* isolates from nestling bald eagles.

Antibiotic	Esculin Hydrolysis		Urease Production		H <sub>2</sub> S Production		Arginine Decarboxylation	
	+	-	+	-	+	-	+	-
Colistin	R	S						
Cephalothin	V	V						
Aztreonam	R	S						
Ceftazidime	I	S						
Cefopodoxime	R	S						
Cefoxitin	R	S						
Amikacin	R	S						
Nitrofurantoin			R	S	R	S		
Kanamycin							R	V

\*S =  $\geq 75\%$  of samples are susceptible, I =  $\geq 75\%$  of samples are intermediate, R =  $\geq 75\%$  of samples are resistant, V = resistance phenotype is variable (i.e. no correlation).

Table 18. Correlations between biochemical reactions and antibiotic susceptibility of *Proteus mirabilis* isolates from nestling bald eagles.

Antibiotic	Esculin Hydrolysis		Arginine Decarboxylation	
	+	-	+	-
Cephalothin	I	S	R	S
Aztreonam	R	S	V	V
Ceftazidime	V	S		
Cefoxitin	R	S		
Gentamicin	R	S		
Tobramycin	R	S		
Amikacin	R	S		

\*S =  $\geq 75\%$  of samples are susceptible, I =  $\geq 75\%$  of samples are intermediate, R =  $\geq 75\%$  of samples are resistant, V = resistance phenotype is variable (i.e. no correlation).

Table 19. Correlations between biochemical reactions and antibiotic susceptibility of *Staphylococcus intermedius* isolates from nestling bald eagles.

Antibiotic	Ribose Fermentation	
	+	-
Erythromycin	S	R
Tetracycline	S	V

\*S =  $\geq 75\%$  of samples are susceptible. R =  $\geq 75\%$  of samples are resistant, V = resistance phenotype is variable (i.e. no correlation).

## Discussion

### Plasma Analysis

Contaminant concentrations observed in the samples are similar to the concentrations observed in previous years in these regions (8, 25). Great Lakes samples had higher plasma concentrations levels for all compounds. This region has had consistently higher concentrations than inland sites in previous years.

### Microbial Composition

No previous studies have been conducted on the normal intestinal flora of bald eagles. Therefore, this study provides baseline data for the intestinal flora of this species. The majority of species observed were enteric bacteria, along with several *Staphylococcus* and *Enterococcus* species. Two bacterial species, *Escherichia coli* and *Enterococcus faecalis*, were observed in almost all of the samples. This is not surprising, as both species are ubiquitous and are associated with animal feces and carcasses (10).

The only significant differences found in microbial composition as compared to contaminant concentrations were the differences in presence of the genera *Cedecea* and

*Micrococcus*. Both of these genera were significantly more likely to occur in birds with high plasma PCB concentrations. The *Micrococcus* isolates were identified in the Great Lakes and VNP samples and the *Cedecea* isolates were identified in samples from a pair of neighboring Great Lakes islands. It is possible that the differences seen among samples could be due to regional variation in bacterial populations. Since only a few isolates were observed among the samples for both of these genera it is difficult to state with certainty that the contaminant levels are the factor influencing their presence. To the author's knowledge there is no literature on the effects of environmental contaminants on these genera so it is difficult to speculate on what, if any, effect the contaminants may be having on these bacteria. There is also no literature to suggest that these bacteria would be more prone to colonize in immunosuppressed organisms, although *Cedecea* species have been associated with infection in humans (21).

No significant differences were found between microbial diversity and contaminant concentration, so it appears that contaminant levels do not affect the number of species present in the eagles' intestinal flora. However, the age of the birds was significantly correlated with species diversity, in that the total number of bacterial species observed per bird was greater in the younger birds. Numerous studies have shown that in the domestic chicken, intestinal flora varies with age (3, 11, 19) as the bacteria compete and interact to form the normal flora. It is reasonable to assume that eagle flora may develop in the same manner. It is important to note that this study only examined the aerobic flora and that the anaerobic flora may not follow the same patterns.

Compared to the results obtained from the laboratory dosing experiments, several similarities and also some key differences were observed. In both laboratory

experiments, as well as the field experiment, *Escherichia coli* was the predominant bacterial species observed. *Enterococcus faecalis* was also a predominant species in chickens as well as eagles. These results are not surprising, as these species are known to be common inhabitants of normal chicken flora and as mentioned earlier, are ubiquitous species. The presence of *Staphylococcus* species was noted in both bird species as well, the eagles as a whole had a greater variety of *Staphylococcus* species than the chickens.

There was, in general, greater species diversity in the eagle flora, with a total of 28 different species observed as compared to 18 in the double-crested cormorant extract laboratory experiment. These results are also unsurprising, as the nestling eagles would be exposed to a greater variety of bacteria in their diet and their environment than would young chickens. The diet of nestling eagles is composed of animal carcasses and carrion which would harbor a variety of bacterial species.

There were two species common in the lab samples, *Klebsiella pneumoniae* and *Enterococcus gallinarum*, that were notably absent from the eagle flora. Both of these species are associated with intestinal flora. *Enterococcus* species as a whole were more common in chicken flora than in eagles. Brittingham et al. (10) observed four species of *Enterococcus* in wild songbirds but these bacteria did not make up a large percentage of total isolates. This study did not look for presence of *Klebsiella* and to the author's knowledge there is no other literature reporting this bacteria in wild species. These two genera may be more prevalent in humans and domestic animals than in wild species.

While several significant differences were found among samples for presence of certain species by dose in the chicken experiments, no such results were seen for the eagle samples, with the exception of the two genera. The two species that showed the

most significant differences by presence in the lab experiments, *K. pneumoniae* and *Enterococcus gallinarum*, were absent from the eagle flora. Comparisons between the flora of the two bird species is difficult since many of the bacteria observed were only seen in one species and most bacterial species were only isolated from a few birds.

#### Antibiotic Susceptibility Profiles

Overall, the number of antibiotic resistant bacteria observed in the samples was relatively low. These results might be expected, as the eagles are not directly exposed to the battery of antibiotics used in domestic animals and humans. However, several studies have shown the presence of antibiotic resistant bacteria in wild animals, including fish and rodents (13, 30), and the discovery of these bacteria is becoming an increasing concern.

The overall resistance of bacteria does not seem to be correlated with plasma contaminant levels or regions, as resistant strains were found in all three regions and from birds with a range of contaminant concentrations. Perhaps some other factor may be involved in the selection of the resistance phenotypes seen. The sites where these samples were collected may have regional contamination of pharmaceuticals from agricultural facilities, hospitals, or some other facility. A study by Goni-Urriza et al. (14) has shown urban effluent to cause increased antibiotic resistance in riverine bacterial populations. Another explanation may be the existence of metal contamination in these areas. Several studies have shown correlations between the presence of metals such as mercury and higher incidence of antibiotic resistant bacteria (1, 30). The correlations seen in these and other studies have been linked to the presence of metal resistance and antibiotic resistance genes on common bacterial plasmids (17, 24).

The generally low frequency of antibiotic resistance among the eagle samples is encouraging. These results may suggest that the nestlings' environments are not heavily contaminated by antibiotics and also that the bioaccumulative contaminants are not playing a role in the selection of antibiotic resistance in the birds' microflora.

Several significant correlations were observed between plasma PCB levels and antibiotic susceptibility of *Escherichia coli* and *Enterococcus faecalis*. Resistance of *Escherichia coli* to ampicillin and piperacillin tended to decrease with increasing plasma PCBs, while resistance to ampicillin/sulbactam tended to increase. These results are the product of only a few samples, however, and while significantly different by both a logistic regression analysis and Fisher's Exact test, may not be entirely indicative of the true population. The resistance of *Enterococcus faecalis* isolates to tetracycline also tended to decrease with increasing PCB concentrations, but again only a few samples were responsible for this difference.

Several significant differences in the chicken samples are similar to what was observed in the bald eagle flora. In the double-crested cormorant extract experiment, the presence of ampicillin and piperacillin resistant strains of *Escherichia coli* were significantly less in the groups that were exposed to the extract, and strains resistant to ampicillin/sulbactam were more frequently observed in the dosed groups. These results are similar to what was observed in the *Escherichia coli* isolates from the eagle flora. The commonality of these results between experiments may point to a predictable effect of the contaminants on the antibiotic susceptibility of this species. However, because the correlations in eagle data were based only on a few samples and the laboratory data does not agree between the two chicken studies, further study would be needed to authenticate

these results. Antibiotic susceptibility profiles as a whole differed significantly between nestling eagles and chickens, in that the frequency of resistant strains was much lower in the eagles than in the chickens.

As in the chicken experiments, several biochemical results were significantly correlated to the antibiotic susceptibility profiles in certain bacteria. The correlations seen in the field samples did not include any of the same correlations seen the laboratory cultures. Different correlations were also observed between the two laboratory experiments. These results suggest that the correlations seen are not universal and therefore biochemical profiles could not be used as an indicator of antibiotic susceptibility. As discussed in the laboratory experiments, the correlations seen could be due to the presence of the two phenotypes on the same bacterial plasmid. Plasmids have been discovered that confer antibiotic susceptibility along with other traits, such as ability to ferment sucrose (2, 16). Since the number of occurring bacterial plasmids is vast and there are many plasmids that have not been characterized, these results are not surprising.

### General Conclusions

Overall, the results of this study suggest that bioaccumulative contaminants such as PCBs and organochlorine pesticides do not have a significant impact on the microbial composition or antibiotic susceptibility of bald eagle intestinal flora. The few significant correlations that were observed could indicate a potential for these contaminants to have some effect on the microbial flora, but without further study, no conclusive statements can be made. Alterations in microbial flora do not hold much promise as an indicator of contaminant exposure, at least with the methods used in this study. There may be some changes occurring with the bacteria at a more molecular level that could potentially be

predictive. Without further study, the application of this technique as a biomarker of contaminant exposure would not be valuable.

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## CONCLUSIONS

The objectives of this project were to examine the effects of bioaccumulative contaminants on the intestinal flora of avian species and to determine the potential for those effects to act as biomarkers of exposure to bioaccumulative contaminants. Results obtained from two laboratory experiments using domestic chickens and a field experiment with bald eagles were used to evaluate these objectives.

Significant differences were observed among dose groups in both laboratory experiments for microbial composition, biochemical reactions, and antibiotic susceptibility. However, the correlations observed differed between the two experiments, even when concerning the same bacterial species. Significant correlations were also observed between biochemical reactions and antibiotic susceptibility profiles in both experiments, although the same correlations were not observed in both experiments. Differences seen in both experiments were not clearly dose-dependent. Literature concerning toxic effects of bioaccumulative contaminants on bacteria and antibiotic susceptibility is scarce. The mechanism governing the alterations observed could not be determined.

A few significant correlations were observed for microbial composition and antibiotic susceptibility as compared to plasma contaminant levels in nestling bald eagles. However, the results seen were not clear evidence that bioaccumulative contaminants exert significant effects on the eagle intestinal flora. The most beneficial information obtained from the field study was baseline data on the intestinal flora of bald eagles, on which no published data exists. In addition, it was observed that the bacterial flora of

nestling eagles contained very few antibiotic resistant bacterial isolates. As the incidence of multiple antibiotic resistant strains is becoming an increasing concern, even in wildlife species, this information is valuable.

The potential of intestinal flora alteration to serve as a potential biomarker of exposure to bioaccumulative contaminants was examined by comparing laboratory and field studies. While the compounds tested did seem to exert an effect on the intestinal flora of domestic chickens in a laboratory setting, these results did not correlate well with what was observed in the field samples. Although the techniques used in this study were relatively easy, inexpensive, and noninvasive, the use of the techniques as a biomarker of exposure is not feasible at this time. Several of the criteria for evaluating field biomarkers outlined in the introduction, including understanding the natural variability associated with the biomarker, whether or not the biomarker responds in a dose-dependent manner, and whether or not the physiological changes can be interpreted, are not met with the technique used in this study. The discovery of significant differences in this experiment may indicate that the contaminants are exerting an effect on avian intestinal flora. If further studies can ascertain the mechanism behind these alterations, there may be some potential for this technique to act as a biomarker in the future.

## **Appendix II**

### **Effects of the DCC Extract on Growth Parameters in the Chicken Embryo**

**Effect of Double-Crested Cormorant Egg Extract on Growth Parameters of the  
Chick Embryo**

Gwyn Bochringer and Keith Grasman

Department of Biological Sciences  
Wright State University  
Dayton, OH

## **Abstract**

Studies have shown that Great Lakes organisms are being adversely affected by contaminants in the environment. However, establishing a cause and effect relationship between a chemical and an adverse effect is difficult to do because organisms are exposed to many contaminants in the wild. The extraction of possible causative agents from the tissue or eggs of adversely affected wildlife and subsequent re-introduction of this extract into laboratory animals can help prove the cause and effect relationship. In this study, double-crested cormorant egg extract was injected into chicken eggs (day 0 incubation) at doses of 0.0625 egg equivalent (EE), 0.125 EE, 0.1875 EE, and 0.250 EE. A PCB dose of 0.84 ng/g was used as a positive control. Embryos were removed prior to hatch, and organ masses and femur lengths were measured as an indication of growth. Although not all parameters were significantly different than the vehicle control at each dose, a general dose-related decrease in mass or length was seen, except for heart mass which increased. Only the bursa and heart indices were significant, indicating that changes in these masses were not due solely to changes in the body mass. The liver, thymus, and bursa indices showed a dose-related decrease, suggesting that the organ masses were decreasing at a faster rate than the body mass. Further studies need to be done to elucidate the cause and effect relationship between chemicals seen in egg extracts and adverse effects seen in the wild.

## **List of Abbreviations**

1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, DDT; 1,2,3,7,8-pentachlorodibenzodioxin, PeCDD; 2,3,4,7,8-Pentachlorodibenzofuran, PeCDF; 2,3,7,8-tetrachlorodibenzodioxin, TCDD; egg equivalent, EE; Great Lakes Embryo Mortality, Edema, and Deformities Syndrome, GLEMEDS; polychlorinated biphenyl, PCB

## **Introduction**

Congenital deformities and declines in Great Lakes wildlife populations including birds, turtles, and ranch minks have been documented since the 1960's and 1970's. Deformities include crossed bills, edema, spinal deformities, dwarfed appendages, growth retardation, and wasting syndrome (reviewed in Ludwig et al, 1996; and Powell et al, 1997).

Eggshell thinning was also seen in Bald Eagles and other birds, which resulted in significant declines in populations. In piscivorous birds, deformities were occurring at such an alarming rate that the term Great Lakes Embryo Mortality, Edema, and Deformities Syndrome (GLEMEDS) was developed to describe the problem. It was soon hypothesized that GLEMEDS and other adverse effects were somehow correlated with the concentration of contaminants that were present in the Great Lakes basin. Although population declines have diminished over the past decade as clean up of the lakes has taken place, deformities and reproductive problems still exist (reviewed in Ludwig et al., 1996).

Contaminants in the Great Lakes basin include such chemicals as polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzodioxin (TCDD), and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT). These chemicals are lipophilic in nature, and persistent in the environment. Exposure to humans and wildlife can occur through ingestion, inhalation, or dermal exposures. Once an organism has been exposed to a lipophilic compound, the chemical will accumulate in the adipose tissue and biomagnify up through the food chain. Birds can pass on contaminants to their offspring through deposition in the yolk or through deposition from their feathers onto incubating eggs. Humans and mammals can pass on the contaminants to their offspring through breast milk.

Many of the effects seen in Great Lakes wildlife are consistent with those seen in laboratory animals exposed to chemicals found in the Great Lakes basin. Exposure to TCDD and PCBs in fish, chicken, and rats have caused edema, hemorrhages, compromised immune systems, altered hormone homeostasis, and embryo mortality (Walker and Catron, 2000). Rats exposed to PCB 126 had embryos with significantly shorter femur and humeri, impaired bone strength, and altered retinoid homeostasis (Lind et al., 2000). Chicken embryos exposed to PCBs experienced growth retardation, decreased body masses, and decreased femur lengths (Gould et al., 1996; Gould et al., 1997). A dose-related increase in heart mass and altered cardiac output were also seen in chicken embryos exposed to TCDD and PCB 126 (Walker and Catron, 2000).

Piscivorous birds of the Great Lakes basin have been significantly affected by contaminants, and congenital deformities have been seen most often in the double-crested cormorant (*Phalacrocorax auritus*). Because the cormorant is a sensitive species, it is often targeted as a species to monitor in the region (Ryckman et al. 1998). The cormorant migrates between the Atlantic coast and Gulf of Mexico to nesting sites in the Great Lakes, and contaminants found in cormorant eggs correlate with contaminants accumulated from the Great Lakes basin (Ludwig et al. 1996). However, it is difficult to determine a cause and effect relationship between a chemical and an adverse effect because wildlife is exposed to numerous chemicals in the environment which could be contributing to the problem. While chemicals such as PCBs and TCDD may act alone, most likely they are acting with other chemicals to cause a synergistic or antagonistic effect (Meadows et al., 1996).

The extraction of possible causative agents from the tissue or eggs of adversely affected wildlife and subsequent re-introduction of this extract into laboratory animals can help prove the cause and effect relationship. If the same effects are seen in laboratory animals, this link can be established. Cormorant eggs collected from Spider Island near Lake Michigan, an area known to be contaminated with chemicals such as TCDD and PCBs, were pooled together and processed to isolate the possible causative agents. Chemical analysis on the extract determined that it was comprised of PCB 126 (73%), 1,2,3,7,8-pentachlorodibenzodioxin (PeCDD) (7%), PCB 77 (5%), TCDD (4%), PCB 105 (4%), and 2,3,4,7,8-Pentachlorodibenzofuran (PeCDF) (3%). The extract was injected into chicken eggs to determine mortality rates and abnormalities in the chicks at

3 weeks after hatching. Doses were based on an egg equivalent (EE), which is the amount of extract present in one average cormorant egg. From the study, significant differences were seen in the highest dose of 1 EE, with a higher mortality rate, smaller body mass, smaller brain and bursa masses, and a higher relative brain mass than the controls. No significant differences were seen in abnormalities in any of the dose groups (Powell et al., 1997).

In this study, the egg extract collected from the Spider Island study was injected into domestic chicken eggs (*Gallus domesticus*), and growth parameters were measured to determine potential effects from the extract. The chicken was used due to its sensitivity to the chemicals found in the extract (Powell et al., 1997).

## **Materials and Methods**

### *Egg Extract Injection Doses*

Doses were based on a cormorant extract study conducted at Clemson University, and adjusted to account for high mortality rates seen in the Clemson study. Doses included a vehicle control, which received 175  $\mu$ l sunflower oil (n = 31), 0.0625 EE which received 0.001 EE/g/egg (n = 26), 0.125 EE which received 0.002 EE/g/egg (n = 29), 0.1875 EE which received 0.003 EE/g/egg (n = 37), and 0.250 EE which received 0.004 EE/g/egg (n = 37). A PCB 126 dose of 0.84 ng/g/egg (n = 41) was used as a positive control because this amount corresponds to the amount of PCB 126 found in 0.250 EE. The 0.0625 EE and 0.250 EE doses were received from Clemson University. For the 0.125 EE and 0.1875 EE doses, egg extract received from Clemson University was used by Wright

State University to prepare the doses. Egg extract used by Clemson and Wright State were obtained from the Spider Island study (Powell et al., 1997).

#### *Egg Preparation, Injections, Incubation, and Candling*

White Leghorn chicken (*Gallus domesticus*) eggs were candled to check for damage and to locate the air cell. The eggs were labeled, weighed, and randomly assigned to treatment groups. A sterile dissecting probe was used to make a small hole in the shell over the air cell, and a 26 gauge needle was used to inject the dose into the egg. The hole was then sealed with melted paraffin wax, and placed in an incubator at the humidity range of 48-59%, dry bulb temperature of 37.5°C, and wet bulb temperature of 28-30°C. Incubator temperature and humidity were monitored daily and recorded. The eggs were candled on incubation days 4, 11, and 18 to check for embryo viability and damage. Dead embryos were removed from the shell and examined for stage of death. On day 18 of incubation, the eggs were placed on their sides, the dry bulb temperature was decreased to 37.0°C, and the wet bulb temperature was increased to 31-32°C.

#### *Embryo Removal, Dissection, and Organ Measurement*

On day 20 of incubation, the eggs were removed from the incubator for dissection. For each dissection, egg mass was recorded, and the embryo was removed from the egg and decapitated. Body and yolk mass was recorded, and the yolk sac was detached from the body and weighed. The body mass was recorded, and the abdominal cavity was opened. The gallbladder was carefully removed, and the liver lobes were removed and weighed separately. The thymus lobes were removed and weighed separately, as were the thyroid

glands. The bursa of Fabricius was then removed and weighed, followed by the brain and heart. Where organs could not be removed, a note was made in the record.

Following organ removal, the left and right femur were measured twice with a sliding caliper to the nearest 0.05 mm, and the measurements were averaged. The embryo was then placed in a specimen cup with buffered formalin for preservation.

### *Statistical Analysis*

All statistical results were made using SAS Institute Inc. JMP IN version 4.04 statistical software. Statistical analyses were conducted on total organ masses measured as absolute weight as well as on the relative weight of the organ as a percentage of the body, expressed as the index measurement. The index measurement was calculated as (organ mass/body mass) x 100. Percent mortality was calculated by subtracting the number of excluded or infertile eggs from the sample number, dividing the number of dead embryos by this number, and multiplying by 100. Variances between all means and the vehicle control were made using a one-way ANOVA test where equal variances and normality could be shown, with the Tukey-Kramer HSD Test used to show significant differences between means. Variances were tested using the Bartlett Test, and normality was tested using the Shapiro-Wilks Test. Where equal variances and normality were questioned, the non-parametric Kruskal-Wallis one-way ChiSquare approximation was used. To test a linear relationship, a linear regression using Analysis of Covariance was conducted, using body mass as the covariant. To show linear trends, means  $\pm$  standard error were graphed for each parameter, with a linear regression trendline added. For all statistics, the level of significance used was 0.05.

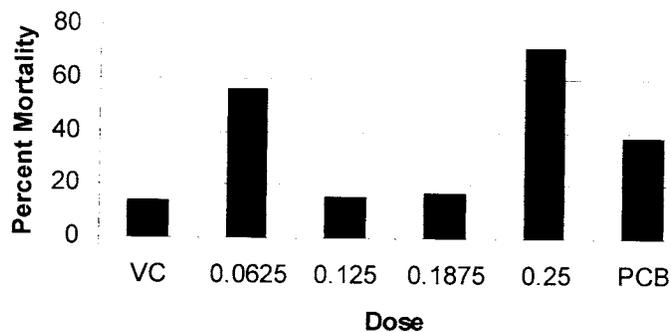
## Results

### *Mortality*

The egg-equivalent doses of 0.0625 and 0.250, as well as the PCB dose produced greater embryo mortality (56%, 71%, and 38%, respectively) than the control (14%) (Table 1 and graph 1). The 0.125 EE and 0.1875 EE doses did not produce mortality rates significantly different than the vehicle control (16% and 17%, respectively). The vehicle control mortality rate is comparable to previous egg-equivalent extraction injections in chicken embryos (Powell et al., 1997).

Dose	Sample Number	Dead	Infertile	Excluded	% Mortality Rate
VC	31	4	1	2	14.29
0.0625	26	14	0	1	56.00
0.125	29	4	3	1	16.00
0.1875	37	6	1	1	17.14
0.250	37	25	1	1	71.43
PCB	41	15	1	1	38.46

**Table 1: Mortality Rates**



**Graph 1: Mortality Rates**

### *Statistical Significant Differences Between Doses*

Parameter means  $\pm$  standard error are shown in Table 2. A one-way ANOVA was conducted to show significant differences between the variance around the individual dose means and the variance around the grand mean. Due to questionable normality and equal variances, the non-parametric Kruskal-Wallis Test was used to determine p values for each parameter. Because making multiple comparisons between means increases the chance of committing a Type I error (declaring significant different when one does not exist), the more conservative Tukey-Kramer HSD Test was used to determine which means were significantly different from the vehicle control. Significant differences were seen between the vehicle control and 0.0625 EE, 0.250 EE, and the PCB dose in the left femur ( $p = 0.0021$ ), right femur ( $p = 0.0018$ ), and body mass ( $p = 0.0065$ ). A significant difference was seen between the vehicle control and 0.0625 EE in the liver mass ( $p = 0.0206$ ). The thymus had significant differences between the vehicle control and the PCB dose ( $p = 0.0013$ ), and the bursa had significant differences between the vehicle control and 0.1875 EE and the PCB dose ( $p = 0.0012$ ). Because the PCB dose was used as a positive control in relationship to the 0.250 EE dose, the PCB dose and 0.250 dose for each parameter were compared for significant differences. There were no significant differences shown between the PCB dose and the 0.250 EE dose for any of the parameters.

A one-way ANOVA using the Tukey-Kramer HSD Test was used to determine significant differences between the vehicle control and each dose for each Somatic Index, and the non-parametric Kruskal-Wallis Test was used to determine the p-value. The

Parameter	VC (N = 24)	0.0625 EE (N = 11)	0.125 EE (N = 21)	0.1875 EE (N = 29)	0.250 EE (N = 10)	PCB (N = 24)
Mean Left Femur ± SE (mm)	31.14 ± 0.30	28.35 ± 0.74 <sup>a</sup>	30.73 ± 0.43	29.39 ± 0.45	27.63 ± 1.37 <sup>a</sup>	28.86 ± 0.65 <sup>a</sup>
Mean Right Femur ± SE (mm)	31.40 ± 0.29	28.44 ± 0.75 <sup>a</sup>	30.88 ± 0.42	29.98 ± 0.46	27.75 ± 1.33 <sup>a</sup>	28.81 ± 0.67 <sup>a</sup>
Mean Egg Mass ± SE (g)	56.80 ± 0.79	57.12 ± 1.26	57.69 ± 0.89	56.07 ± 0.77	56.17 ± 1.32	56.45 ± 0.80
Mean Body + Yolk Mass ± SE (g)	39.91 ± 1.02	35.57 ± 1.79	39.73 ± 0.85	38.88 ± 0.84	34.17 ± 3.11	38.01 ± 1.11
Mean Body Mass ± SE (g)	26.71 ± 0.66	22.55 ± 1.26 <sup>a</sup>	25.94 ± 0.63	24.45 ± 0.71	21.72 ± 1.88 <sup>a</sup>	22.91 ± 0.87 <sup>a</sup>
Mean Yolk Mass ± SE (g)	12.69 ± 0.55	12.66 ± 1.11	13.12 ± 0.62	13.52 ± 0.64	11.92 ± 1.46	14.56 ± 0.62
Mean Liver Mass ± SE (g)	0.548 ± 0.018	0.422 ± 0.034 <sup>a</sup>	0.524 ± 0.029	0.495 ± 0.025	0.421 ± 0.047	0.464 ± 0.026
Mean Thyroid Mass ± SE (g)	0.0062 ± 0.00060	0.0057 ± 0.00117	0.0078 ± 0.00086	0.0070 ± 0.00062	0.0053 ± 0.00147	0.0062 ± 0.00069
Mean Thymus Mass ± SE (g)	0.277 ± 0.0136	0.207 ± 0.0218	0.265 ± 0.0207	0.230 ± 0.0107	0.202 ± 0.0253	0.195 ± 0.0148 <sup>a</sup>
Mean Bursa Mass ± SE (g)	0.037 ± 0.0015	0.029 ± 0.0029	0.033 ± 0.0020	0.028 ± 0.0020 <sup>a</sup>	0.028 ± 0.0032	0.027 ± 0.0022 <sup>a</sup>
Mean Brain Mass ± SE (g)	0.685 ± 0.025	0.613 ± 0.028	0.634 ± 0.027	0.623 ± 0.017	0.586 ± 0.042	0.648 ± 0.022
Mean Heart Mass ± SE (g)	0.197 ± 0.006	0.198 ± 0.014	0.195 ± 0.013	0.220 ± 0.011	0.238 ± 0.017	0.239 ± 0.011
Mean Left Femur Index ± SE	118.61 ± 2.27	128.52 ± 5.05	119.85 ± 2.26	122.03 ± 2.63	131.94 ± 6.23	126.06 ± 2.81
Mean Right Femur Index ± SE	119.64 ± 2.41	129.08 ± 5.57	120.44 ± 2.24	124.44 ± 2.65	132.69 ± 6.38	125.79 ± 2.80
Mean Liver Index ± SE	2.06 ± 0.061	1.86 ± 0.106	2.03 ± 0.099	2.03 ± 0.086	1.90 ± 0.076	2.04 ± 0.111
Mean Thyroid Index ± SE	0.023 ± 0.0022	0.024 ± 0.0046	0.030 ± 0.0031	0.029 ± 0.0025	0.024 ± 0.0062	0.027 ± 0.0032
Mean Thymus Index ± SE	1.04 ± 0.049	0.89 ± 0.068	1.01 ± 0.063	0.94 ± 0.035	0.93 ± 0.075	0.84 ± 0.041
Mean Bursa Index ± SE	0.138 ± 0.0058	0.128 ± 0.0087	0.126 ± 0.0067	0.111 ± 0.0068 <sup>a</sup>	0.120 ± 0.0092	0.113 ± 0.0068
Mean Brain Index ± SE	2.60 ± 0.107	2.68 ± 0.200	2.47 ± 0.100	2.62 ± 0.087	2.83 ± 0.234	2.88 ± 0.125
Mean Heart Index ± SE	0.74 ± 0.020	0.91 ± 0.076	0.75 ± 0.051	0.92 ± 0.054 <sup>a</sup>	1.13 ± 0.062 <sup>a</sup>	1.06 ± 0.052 <sup>a</sup>

<sup>a</sup> significantly different from the vehicle control

**Table 2: Parameter and index means ± standard error**

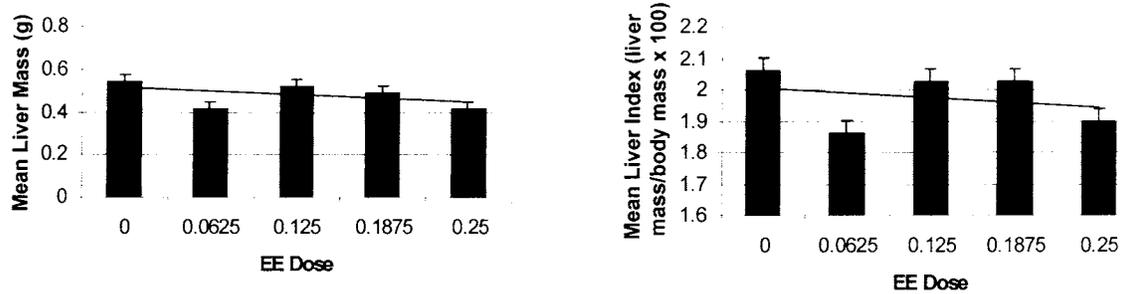
bursa index was significantly different between the vehicle control and 0.1875 EE dose ( $p = 0.0373$ ), and the heart index was significantly different between the vehicle control and 0.1875 EE, 0.250 EE, and PCB ( $p = 0.001$ ). An Analysis of Covariance was ran using body mass as the covariant, and only heart mass was significantly different ( $p = 0.0117$ ).

An ANOVA test was also ran to determine significant differences between dates, in order to assure differences between dose means were not influenced by changes between injection dates. The thyroid mass was significantly different between injection days 3/20/03 and 3/21/03, and 3/27/03 and 3/21/03 ( $p = 0.0126$ ). The thyroid index was significantly different between injection days 3/20/03 and 3/29/03, 3/20/03 and 3/21/03, and 3/19/03 and 3/21/03 ( $p = 0.0038$ ). Brain mass was significantly different between days 3/19/03 and 3/25/03 ( $p = 0.0357$ ).

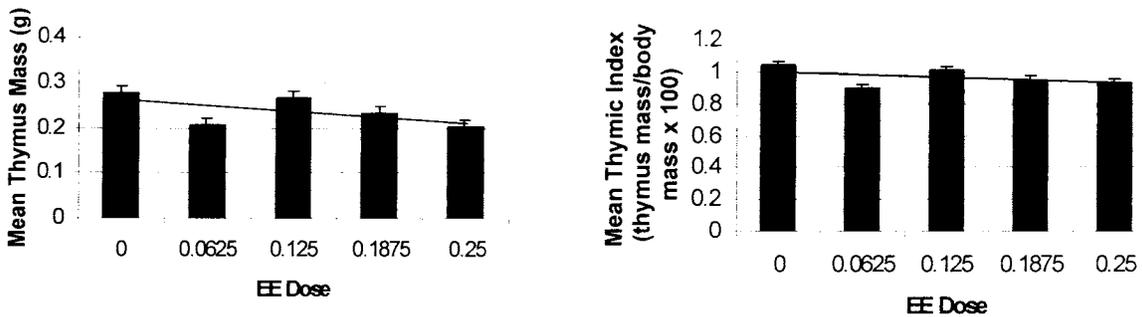
### *Linear Trends*

In order to determine the linear relationship between the vehicle control and the doses, the PCB data was removed and an Analysis of Covariance using body mass as the covariant was conducted to show linear trends. Body mass was significant ( $p = 0.001$ ), as were the bursa mass ( $p = 0.0181$ ), and heart mass ( $p = 0.0037$ ). The linear trend for each parameter was shown by graphing the parameter mean  $\pm$  the standard error by dose, and adding a trend line. As can be seen in Table 2, each parameter excluding the heart mass shows a general dose-response relationship with the length or mass decreasing as the dose increases. The heart mass experiences an increase in mass as the dose increases. The dose-response relationship for the left femur, right femur, thyroid, and brain shows

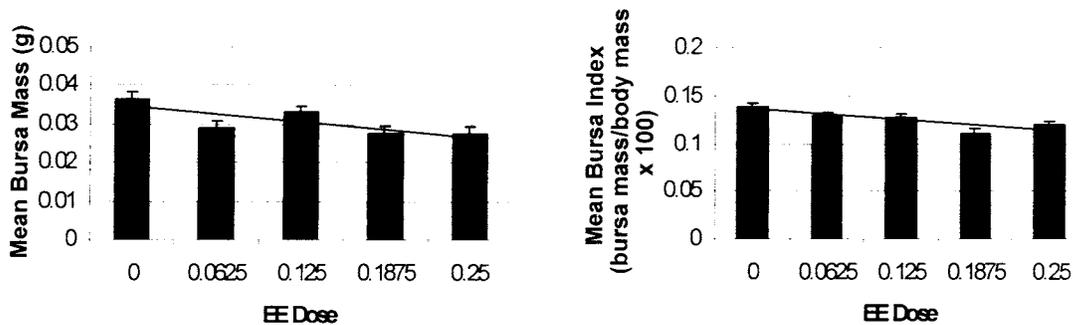
the index increasing while the dose increases. The liver (Graph 2), thymus (Graph 3), and bursa (Graph 3) show a decrease in the index as the dose increases, and the heart (Graph 4) shows an increase in the index as the dose increases.



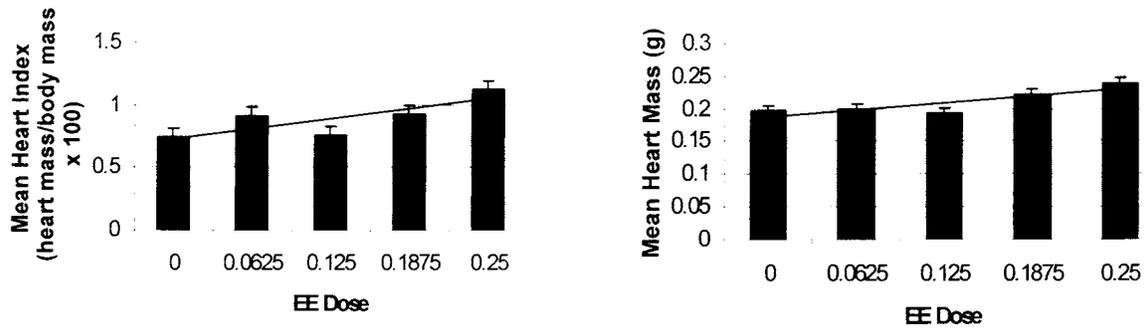
**Graph 2: Mean liver mass and index  $\pm$  standard error**



**Graph 3: Mean thymic mass and index  $\pm$  standard error**



**Graph 4: Mean bursa mass and index  $\pm$  standard error**



**Graph 5: Mean heart mass and index ± standard error**

## Discussion

### *Mortality*

The mortality rates for the 0.0625 EE, 0.250 EE, and PCB doses, but not the 0.125 EE and 0.1875 EE doses, were higher than the vehicle control. Because the 0.0625 EE dose produced higher mortality than the control, it should be expected that the 0.125 EE and 0.1875 EE doses would produce an increasing higher mortality rate than the control and the 0.0625 EE dose. This lack of increasing mortality may be due to the 0.125 EE and 0.1875 EE doses being made at a different time and different location, with potential differences in the way the doses were made. However, in a previous cormorant egg extraction study, doses of 0.001, 0.01, and 0.1 EE did not produce mortality significantly different than the vehicle control (Powell et al., 1997).

### *Organ Weights and Measurements*

Significant differences between days were seen for the bursa and brain masses. These were the only organs affected, and the days which differed varied. Because these organs

did not experience significant differences between doses, the differences between days did not appear to be an influencing factor in the overall findings of the study.

Statistically significant differences seen between parameters and doses varied, as did the significant differences between the indices and doses. A significant effect was seen on body weight in the 0.0625 EE, 0.250 EE, and PCB doses. Low doses of PCBs injected into chicken eggs have been seen to cause significantly lower body masses, and this study confirms this finding (Gould et al., 1997; and Gould et al., 1999). Because the egg extract is comprised of 73% PCB 126, similar findings were expected from the egg extract doses. The lack of significant differences between the vehicle control and the 0.125 EE and 0.1875 EE doses may be due to the manner in which the doses were prepared. However, in a previous egg extract study the body weights in one, two, and three week old chicks have been shown to be significantly lower in chicks receiving 1.0 EE, but no significant differences were seen in body mass at hatching in any of the doses (Powell et al., 1997).

Because body mass is shown to decrease with increasing dose, it is expected that organ masses and lengths should also decrease at a similar rate. Body mass and femur lengths were significantly different from the vehicle control at the 0.0625 EE, 0.250 EE, and PCB doses. Chick and rat femur lengths have been shown to decrease significantly when exposed to PCBs and TCDD (Gould et al., 1999; Kubiak et al., 1989; and Lind et al., 2000). The results of this study confirm a decrease in length with an increase in dose. However, this decrease in length may be due solely to the decrease in body mass, so body

mass must be considered prior to concluding that the decrease is due to the dose.

Micromelia, or dwarfed appendages, has been seen in Great Lakes birds (Ludwig et al., 1996). However, the femur indices in this study indicate that the decrease in length is due to the decrease in body mass, so micromelia was not seen.

Chemicals such as PCBs and TCDD have been shown to cause atrophy of the bursa and thymus in laboratory tests, and atrophy of these organs has also been seen in cormorants and other piscivorous birds in the Great Lakes basin. The thymus and bursa are the sites of maturation of immune system lymphocytes, therefore atrophy of these organs may cause a decrease in immune function leaving the organism open to infectious diseases (Grasman, 2002). In the present study, the PCB dosed thymus and bursa masses were significantly lower than the vehicle control. However, only the 0.1875 EE dose produced bursas with significantly lower masses than the vehicle control. In a previous egg extract study, only the 1.0 EE produced bursa masses that were significantly lower than the control (Powell et al., 1997). The bursa index was significant, suggesting that the decrease in mass was not due solely to the decrease in body mass. Both the thymus and bursa indices showed a decreasing linear trend with an increase in dose. This decreasing trend indicates that the organ mass is decreasing at a faster rate than the body mass, even though significant differences were not seen between the vehicle control and all the doses.

TCDD and PCB 126 have been shown to cause a dose-related increase in heart wet weight in chick embryos (Walker et al., 1997; and Walker and Catron, 2000). This is

consistent with the findings in this study. Using the Analysis of Covariance, the heart mass was the only parameter that was significant when using body mass as the covariant, indicating that heart mass is the only parameter not dependent on body mass. However, no significant differences were seen in heart mass between the vehicle control and any of the doses. Edema, one of the most common deformities seen in Great Lakes birds, may be related to heart failure and subsequent pooling of interstitial fluids in peripheral tissues. Chick cardiac development is similar to mammalian cardiac development, and the chick embryo is highly susceptible to TCDD-induced cardiac deformities (Ivnitski et al., 2001). Previous studies in chick embryos have shown an increase in heart wet and dry weight after exposure to TCDD and PCBs, which shows that the increase in mass is due to hypertrophy of the cardiac muscle and not solely due to pericardial edema. An increase in mortality, edema, and hemorrhages combined with decreases in body mass and increases in heart masses suggests that the embryos may develop cardiac failure and subsequent edema and death (Walker et al., 1997; and Walker and Catron, 2000). Findings from this study were not compared for correlation with deformities such as edemas and hemorrhages, and future studies are needed to determine if such deformities are related solely to cardiac enlargement.

Growth of an embryo is dependent upon different factors, and many parameters must be examined to determine the cause of growth retardation. Normal growth is influenced by thyroxine, which is produced by the thyroid gland. Chick embryos exposed to PCBs and TCDD have been shown to experience decreases in plasma thyroxine, and disruptions to the thyroid function is commonly seen in Great Lakes birds (Fox, 1993; and Janz and

Bellward, 1996). TCDD has also been shown to cause an increase in thyroid mass in rats (Janz and Bellward, 1996). Lack of significant differences seen in this study may be due to dose, or inability to remove all of the organ.

Enlargement of the liver has been seen in Great Lakes birds, which is in contrast to the findings of this study (Fox, 1993; and Kubiak, 1989). The liver is responsible for production of mixed function oxidase enzymes, which assist in making endogenous and exogenous agents more polar and easier to eliminate from the body. Many of these enzymes are biomarkers of exposure, because an increase in their activity has been directly correlated to exposure to TCDD and related chemicals. Alterations in liver mass may be an endpoint of exposure, and may affect an organism's ability to detoxify harmful chemicals. In this study, a general decrease in organ mass was observed as dose increased. However, only the lowest EE dose was significantly different from the vehicle control. The liver index showed a decreasing linear trend with an increase in dose. This decreasing trend indicates that the organ mass is decreasing at a faster rate than the body mass, even though significant differences were not seen between the vehicle control and all the doses. However, a previous egg extract study did not show significant differences in the liver mass at any of the doses, including the highest dose of 1.0 EE (Powell et al., 1997).

## **Conclusions**

TCDD and related chemicals can cause growth retardation in chick embryos, and growth retardation is also seen in piscivorous birds of the Great Lakes basin. Therefore, growth

parameters such as organ masses and skeletal measurements are important endpoints to measure when conducting studies on chemical exposures. Smaller size can diminish the ability of an animal to survive, to defend themselves, to forage for food, and to mate. Smaller embryos may also have a more difficult time hatching (Gould et al., 1997). Growth retardation may also have an effect on organ size, and contribute to a decrease in hormones or other needed organ functions. This can be seen in the decrease in thyroid hormones and immune system lymphocytes upon exposure to TCDD and related chemicals.

Proving a cause and effect relationship between TCDD or related chemicals and adverse effects such as growth retardation is difficult due to the number of chemicals organisms in the wild are exposed to. Using egg extracts from cormorant populations that are known to be adversely affected can help prove a cause and effect relationship. In this study, several growth parameter endpoints were significantly affected by the egg extraction and PCB doses, and this confirms findings of previous studies. However, there were also findings that conflicted with previous studies, and these need to be studied further. Future studies using egg extracts should also examine correlation with biomarkers of exposure such as enzyme induction, hormone levels, and retinoid levels. These parameters have been shown to be adversely affected in wildlife exposed to contaminants, and shown to correlate with contaminant exposure in the lab. If egg extract studies show a correlation between these endpoints and the doses, a cause and effect relationship should be easier to establish.

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## **Tentative List of Publication Titles**

Associations between thyroid hormones, vitamin A, and vitamin E and environmental contaminant levels in plasma of nestling bald eagles.

Investigation of the ACTH stress response test as a potential biomarker of contaminant exposure in nestling bald eagles.

Bald eagle intestinal flora: species composition, antibiotic susceptibility profiles, and correlations with environmental contaminants.

Effects of PCB 126 and an extract derived from double-crested cormorant (*Phalacrocorax auritis*) eggs on chickens (*Gallus domesticus*) dosed *in ovo*: mortality, deformities, and growth.

Effects of PCB 126 and an extract derived from double-crested cormorant (*Phalacrocorax auritis*) eggs on the plasma thyroid hormones and vitamin levels of chickens dosed *in ovo*.

Effects of *in ovo* exposure to PCB 126 and an extract derived from double-crested cormorant eggs (*Phalacrocorax auritis*) on immune function in juvenile chickens (*Gallus domesticus*).

Cytochrome P450 and Phase II enzyme induction in chickens (*Gallus domesticus*) dosed *in ovo* with PCB 126 and an extract derived from double-crested cormorant (*Phalacrocorax auritis*) eggs.

Effects of PCB 126 and an extract derived from double-crested cormorant (*Phalacrocorax auritis*) eggs on the intestinal flora of chickens (*Gallus domesticus*) dosed *in ovo*.