EFFECTS OF BINARY MIXTURES OF SIX XENOBIOTICS ON HORMONE CONCENTRATIONS AND MORPHOMETRIC ENDPOINTS OF NORTHERN BOBWHITE QUAIL (*COLINUS VIRGINIANUS*)

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Introduction

Bobwhite quail, (*Colinus virginianus*) a wild avian species, was selected to investigate the effects of six xenobiotics on survival, hormone concentrations, and morphometric endpoints. Doses were chosen to encompass a range of environmental contaminant concentrations and toxic concentrations of several chemicals. The six xenobitoics selected were 2,3,7,8-TCDD (TCDD), ethynyl estradiol, o,p'-DDE, p,p'-DDE, coumestrol, and indole-3-carbinol. TCDD is a dioxin that is considered anti-estrogenic[1,2] and anti-androgenic[2,3]. TCDD is considered approximately 1000 times more toxic than most substances with an LD_{50} equal to 1 ppb[4]. Ethynyl estradiol is a synthetic estrogen that has been used in birth control pills[5]. In this study, ethynyl estradiol is placed within the same potency range as TCDD. Coumestrol is a phytoestrogen[6,7] found in clover species, alfalfa, and soy beans. Indole-3-carbinol is a phyto anti-estrogen[8] found in broccoli, brussel sprouts, and cabbage. The next two compounds are isomeric degradation products of the insecticide DDT, o,p'-DDE and p,p'-DDE. o,p'-DDE is considered weakly estrogenic compound. p,p'-DDE is considered anti-androgenic[9].

Studies on single compounds are not enough. Investigation of chemical interactions is very important, because chemical mixtures are not predictable and may act differently than either component alone. In the environment, chemicals are mixed with phytochemicals, pesticides, pharmaceuticals, foods, and other xenobiotics and natural compounds. Toxicity has been reported for various types of chemical interactions[10-12].

The purpose of this research was to perform a preliminary investigation of the effects of eight binary mixtures of six xenobiotics to investigate mixture relationships. Endpoints investigated were survival, plasma testosterone and plasma estradiol concentrations, hormone ratios (estradiol/testosterone), as well as somatic indices of liver, kidney, brain, spleen, gonads, and measurements of weight gain.

Materials and Methods

Quail eggs were obtained from Quail Valley of North Carolina. Ten eggs were used per dose per treatment. Chemicals included a control (vehicle/corn oil), coumestrol (3.0 mg/kg), indole-3-carbinol (3.0 mg/kg), p,p'-DDE (1.0 mg/kg), o,p'-DDE (1.0 mg/kg), ethynyl estradiol (3.0 μg/kg), and TCDD (0.1μg/kg). Injections were performed in a sterile hood (5 μl/10 g). The injection site was wiped with isopropyl alcohol prior to injection, injected below the air cell, and injection hole sealed with a small drop of melted paraffin (100%). Eggs were incubated on rotating shelves at

37.5°C and 65% relative humidity for 21 days, placed in hatcher set at 37°C and 70% relative humidity, hatched, dried, weighed, measured, banded, and place in a brooder with similarly dosed chicks. Chicks were given water and food *ad libitum*. Local heaters were set for 95°C week 1, 90°C week 2, and 85°C week 3. The room light cycle was 12:12.

Chicks (21 days old) were weighed, anesthetized with carbon dioxide (CO_2) gas, bled, euthanized, and measured. Tissues (liver, kidney, brain, gonads, oviduct, and spleen) were collected and weighed. Heparinized blood was centrifuged at 2500 rpm for 15 minutes. Plasma was transferred to clean Eppendorf tubes and frozen (-20°C) until analysis.

Hormone concentrations were determined by radioimmunoassay using ^{125}I labeled hormones (10,000 cpm). Duplicate samples (80 μl) were prepared by liquid-liquid extraction using ethyl acetate and hexane followed by solid phase extraction using isooctane, methanol, ethyl acetate with Alumina B columns. Estradiol and testosterone fractions were collected separately, reconstituted, and assayed. Samples for recovery determination were prepared with assay samples. Assays were incubated with antibody overnight (4°C). Charcoal was used to separate free from bound hormone. Samples were counted in a scintillation counter for 2 minutes.

Curve fit and plasma hormone sample concentration determination were performed by IMMUNOFIT® (Beckman) using a four parameter logistic or linear fit when applicable. Normality and varianece tests used were Shapiro-Wilks and Levene's tests, respectively. Significant differences in hormone concentration and ratios, tissue somatic indices, and measurements among doses or chemicals were determined by two-way General Linear Model (GLM) ($p<0.05$). Data failing normality and variance tests were log transformed. If they still failed normality and variance tests, then data were ranked prior to performing a GLM. Least significant difference was used for *post-hoc* analysis. Survival data for treatment groups of mixtures, mixture components, and controls were analyzed using Fisher's Exact.

Results and Discussion

Survival

Quail in this study experienced an unexplainably high incidence of mortality that appeared to occur in the last four days of incubation. The vehicle, corn oil, is considered less harmful to developing avian embryos than ethanol[13].

Significant survival differences existed among mixtures, components, and controls of coumestrol + TCDD, indole-3-carbinol + TCDD, and ethynyl estradiol + TCDD (Tables 1-3). This is largely a result of control and TCDD survival difference of 60%. The TCDD survival was very high compared to survival of the controls and the mean survival percent of all of the treatments (40.6 +/- 19.5 SD). This tendency of TCDD to improve survival at the 0.1 μg/kg concentration has been observed in our dose-response data (unpublished) of TCDD dosed quail eggs and in our dose-response data (unpublished) of TCDD dosed alligator eggs at the same concentrations.

Table 1. Percent survival of eggs dosed with vehicle (V), coumestrol (C), TCDD (T), or coumestrol + TCDD (CT) ($p<0.05$).

Treatment	V	C	T	CT
Group Size	20	10	10	10
# Survived	6	7	9	2
% Survival	30	70	90	20

Table 2. Percent survival of eggs dosed with vehicle (V), indole-3-carbinol (I), TCDD (T), or indole-3-carbinol + TCDD (IT) ($p<0.05$).

Treatment	V	I	T	IT
Group Size	20	10	10	10
# Survived	6	4	9	4
% Survival	30	40	90	40

Table 3. Percent survival of eggs dosed with vehicle (V), ethynyl estradiol (E), TCDD (T), or ethynyl estradiol + TCDD (ET) ($p<0.05$).

Treatment	V	E	T	ET
Group Size	20	10	10	10
# Survived	6	6	9	3
% Survival	30	60	90	30

Somatic Indices and Hormone Concentrations

Plasma estradiol concentrations were significantly different between the coumestrol + ethynyl estradiol mixture and each of its components. None of the groups were significantly different from the control values. Coumestrol (3 mg/kg) produced greater plasma estradiol concentrations than ethynyl estradiol (3 μg/kg) at the parallel concentration (not significant). The mixture of these two chemicals at these concentrations appears to antagonize the increase of estrogen concentrations in the plasma that result from the estrogens. This may result from competitive inhibition at the estrogen receptor where both of these chemicals act[5]. Or, excess estrogens binding the estrogen receptor, may have fed back to the pituitary-hypothalamus axis and decreased the synthesis of estradiol, thus lowering the plasma estradiol concentrations

Ethynyl estradiol significantly increased liver somatic index of quail compared to coumestrol + ethynyl estradiol dosed quail eggs. A previously reported effect of ethynyl estradiol is synthesis of hepatic proteins: coagulation factors, sex hormone binding globulin, and angiotensinogen[14]. This increase in protein production may be responsible for the increased liver somatic index.

p,p'-DDE + ethynyl estradiol mixture was significantly higher for kidney somatic index than the vehicle control. Based on the kidney somatic index of the p,p'-DDE dosed group, ethynyl estradiol and p,p'-DDE appear to have additive or synergistic effects when administered at these concentrations. Ferreira et al.[15] report p,p'-DDE (50 nmol DDE/mg protein) inhibits succinate dehydrogenase (complex II), which decreases respiration rate and decreases mitochondrial transmembrane potential. Perhaps observed effects in the kidney are related to this inhibition of

mitochondrial respiration. Ethynyl estradiol may have inhibited this effect when present with p,p'-DDE.

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