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THE CAFFEINE BREATH TEST: A LESS INVASIVE METHOD

TO MEASURE CYTOCHROME P450-1A ACTIVITY IN BIRDS

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Lori Ann Feyk

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THE CAFFEINE BREATH TEST: A LESS INVASIVE METHOD TO MEASURE CYTOCHROME P450-1A ACTIVITY IN BIRDS

By

Lori Ann Feyk

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ABSTRACT

THE CAFFEINE BREATH TEST: A LESS INVASIVE METHOD TO MEASURE CYTOCHROME P450-1A ACTIVITY IN BIRDS

By

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Cytochrome P450-1A activity is often used as a biomarker of exposure of wildlife to planar polychlorinated diaromatic hydrocarbons, and is usually measured *ex vivo* in liver tissue. The purpose of this study was to develop a less invasive breath test to measure P450-1A activity in birds. This would allow an assessment of P450-1A activity without the need to kill birds, and would allow measurement on the same individual over time. Caffeine is specifically metabolized by P450-1A. The caffeine breath test (CBT) was performed by injecting ^{14}C-labelled caffeine and measuring the ^{14}C activity of respired $^{14}CO_2$. Domestic chickens (*Gallus domesticus*) were used to develop the CBT, and several ^{14}C-labelled caffeine isomers were tested. The CBT procedure was also performed in the field with three species of Great Lakes fish-eating birds. The CBT was an effective method for measuring P450-1A activity. Chickens induced with 2,3,7,8-TCDD metabolized the ^{14}C-caffeine up to ten times more rapidly than controls. Tri-labelled caffeine (1,3,7-^{14}C-trimethylxanthine) and 3-methyl-^{14}C-caffeine were effective substrates in the CBT.

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INTRODUCTION

Great Lakes fish-eating birds: A historical perspective

Fish-eating birds on the Laurentian Great Lakes of North America have experienced population size fluctuations in response to a variety of factors (1), including exposure to synthetic halogenated hydrocarbons (HHs) (2). HHs such as DDT were present in the Great Lakes at sufficiently great concentrations during the 1960s and 1970s to cause reproductive impairment in several bird species (3-7). Since then concentrations of HHs in the Great Lakes have declined (8), and as a result populations of some birds such as the Double-crested Cormorant (*Phalacrocorax auritus*) (DCC) and the Herring Gull (*Larus argentatus*) (HG) have increased (9, 10). However, some HHs are still present in the Great Lakes ecosystem, where they continue to exert subtle toxic effects on aquatic top predators such as fish-eating birds (11-14). Polychlorinated diaromatic hydrocarbons (PCDHs) are of particular concern. Of the PCDHs, those that can attain a semi-planar configuration are among the most toxic compounds (15). Some of the currently observed adverse outcomes in populations of Great Lakes colonial fish-eating water birds (GLCFEWB) are attributed to planar PCDHs (*p*PCDHs) (13, 16-18).

***Planar polychlorinated diaromatic hydrocarbons (p*PCDHs)**

A number of molecules found in the environment have similar structural features and can be classified as *p*PCDHs (15). Structural features common to these molecules are two aromatic rings that can assume a planar configuration and substitution by one or more chlorine atoms. Examples of *p*PCDHs include some polychlorinated biphenyl (PCB) congeners, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). *p*PCDHs are persistent in the environment and bioaccumulate in aquatic food chains due to their lipophilicity.

Various *p*PCDH compounds exert their effects on organisms through a common mechanism (19). In the cell, *p*PCDHs bind to the aryl hydrocarbon receptor (*Ah-r*) (20). The *p*PCDH/*Ah-r* complex then binds to a translocating protein which transports the activated complex into the cell nucleus. The complex binds to specific DNA sequences called dioxin responsive enhancers (DREs) (21). This interaction with DNA results in an alteration of gene expression, which mediates the responses of the organism to *p*PCDHs (15, 20). Transcription of the drug-metabolizing cytochrome P450-1A1 and cytochrome P450-1A2 enzymes is enhanced (15). An increase in these enzymes or in their activity is often used as an indication of exposure to *p*PCDHs (22).

*p*PCDH-stimulated alterations in gene expression can lead to a variety of harmful metabolic changes which include alterations in vitamin metabolism and endocrine function (23). For example, exposure to *p*PCDHs can lead to altered

metabolism of retinoids (24), thyroid involution (25), immunosuppression (26) and behavioral changes (27, 28). Other metabolic imbalances which can result from *p*PCDH exposure include porphyria (29) and wasting syndrome (30). *In ovo* exposure to *p*PCDHs can produce teratogenic effects or cause embryo lethality in birds (31, 32).

*p*PCDHs are present in aquatic environments as complex mixtures. These mixtures change as a function of space and time (17), due to processes such as environmental weathering and metabolism by organisms. The relative concentrations of the various *p*PCDH congeners in aquatic organisms are different from one trophic level to another (33). As a result, the complex mixtures to which top predators such as fish-eating birds are exposed are different in composition than the mixtures which were originally released into the aquatic environment (17). Therefore, it is nearly impossible to use the results of studies of dose-response relationships of technical mixtures which are performed under laboratory conditions to predict the effects of those mixtures in wildlife. The potency of a complex *p*PCDH mixture in an environmental compartment is often determined by assessing the overall response of a biological system to the mixture as extracted from the compartment and then expressing the potency in terms of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) (15, 34).

Monitoring techniques

A monitoring technique is needed in order to assess current physiological responses of GLCFEWB to chemicals in their environment (2, 35). Monitoring can be used to determine the status and trends in GLCFEWB populations, especially relative to exposure to *p*PCDHs (36). Following exposure to toxic agents, biochemical alterations often occur prior to population-level effects. A biomonitoring program may be able to detect the physiological influence of *p*PCDHs on birds at the biochemical level before more serious toxic effects which impact bird populations occur. Such a monitoring technique could serve as an early warning indicator, perhaps making preventative management to protect wildlife resources possible.

Cytochrome P450-1A activity as a biomarker

The most commonly used biochemical endpoint to assess exposure of organisms to *p*PCDHs is cytochrome P450-1A activity (15). The induction of P450-1A activity by *p*PCDHs is a well characterized response which is mediated through *Ah-r* (15). As such, P450-1A activity serves as an overall summed measurement of the impact of many *p*PCDH congeners which are present in the environment as a complex mixture but which act through a common mechanism (34). Therefore, measurement of P450-1A complements the direct chemical measurement of *p*PCDHs in tissues, and can be less expensive and easier to interpret. Induction of P450-1A activity is a sensitive biomarker. Enzyme activity can be increased by several orders of magnitude

following *p*PCDH exposure (37-39). The most common method used to measure P450-1A activity requires a sample of liver tissue from the test organism, from which microsomes are isolated and the activity of one or more cytochrome P450-1A-requiring monooxygenase enzymes are assessed (40). One substrate which has been used to determine P450-1A monooxygenase activity is ethoxyresorufin. Ethoxyresorufin-o-deethylase (EROD) catalyzes the reduction of ethoxyresorufin to resorufin.

Rationale for a breath test to measure cytochrome P450-1A activity

Breath test methodology is less invasive than the collection of liver tissue for determination of P450-1A activity. The use of a breath test would obviate the need to kill animals to determine their P450-1A induction status. Also, the existence of less invasive methods could make possible the monitoring of threatened and endangered species in the Great Lakes, such as the Bald Eagle and the Caspian Tern. These are the species managers are most interested in protecting, and yet are currently also the species about which they can gather the least information. A breath test would also be useful because it would allow researchers to make multiple measurements of P450-1A activity over time in the same individual, making it possible to study the kinetics of P450-1A activity in response to varying *p*PCDH exposure regimes. This would be an improvement over present laboratory testing methods because it would reduce inter-individual variation, which can be quite large.

A breath test has already been developed to measure P450-1A activity in mammals. In that system labelled caffeine was used as a substrate (41-44). The caffeine breath test has successfully detected the induction of P450-1A in human smokers (45) and in marmoset monkeys dosed with *p*PCDHs (44). The research on which we report here is an extension of that methodology to use with birds.

The Caffeine Breath Test (CBT)

The basic principle on which the CBT is based is that P450-1A monooxygenase enzymes catalyze the N-demethylation of the caffeine molecule, and after cycling through the one-carbon metabolic pool the cleaved methyl group appears in the breath as labelled CO_2 (45). To perform the CBT, labelled caffeine is administered orally or by injection and the rate of labelled CO_2 exhalation is measured. The amount of label appearing in the breath is proportional to the rate of P450-1A activity. Caffeine molecules must be labelled in the 1, 3 or 7-methyl positions, or in a combination of the methyl positions, with either ^{14}C or ^{13}C.

Differences in caffeine metabolism occur between various mammalian species. Cleavage at the three methyl positions have different orders of relative prominence (46), and the P450-1A1 and P450-1A2 isoforms may have different relative involvements in caffeine N-demethylation in various mammalian species (47). In humans N-3 demethylation of caffeine by P450-

1A2 is the primary metabolic pathway (48). Formation of other demethylated metabolites is mediated, at least in part, by other P450 enzymes (49).

The applicability of the CBT to birds depends on whether caffeine is N-demethylated by an enzyme dependent on a cytochrome P450-1A isoform, or whether the induction of several isoforms by *p*PCDHs may be correlated with the evolution of labelled CO_2. The pathway for metabolism of caffeine in birds is presently poorly characterized. Therefore, one of the goals of the present research was to determine whether caffeine is N-demethylated in a cytochrome P450-1A-dependent manner.

The types of cytochrome P450-1A isoforms which are present in birds are also not well characterized. While a P450-1A isoform has been found in most bird species studied, due to a lack of biochemical probes for avian species it has not been possible to definitively discriminate between the 1A1 and 1A2 isoforms (50). Preliminary studies suggest that most birds have a P450-1A isoform which cross-reacts with rat P450-1A1 (51). In animals which have both the 1A1 and 1A2 isoforms both isoforms are generally induced simultaneously by *p*PCDHs, but the 1A2 isoform is generally induced to a lesser degree (52).

Goal of Research

The goal of the present research was to develop a CBT for use in birds, and to determine whether the test provides a useful measure of cytochrome

P450-1A activity in birds. This question was addressed by testing the following null hypotheses:

H_o: Rates of $^{14}CO_2$ exhalation are not greater in birds which have been induced with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) relative to controls.

and

H_o: The results of the caffeine breath test (i.e., $^{14}CO_2$ activity in exhaled breath) are not positively correlated with the results of the *ex vivo* hepatic EROD assay (activity expressed as pmole resorufin/min/mg protein).

METHODS

Overview

Several experiments were conducted in order to develop and characterize the CBT. The first experiments were part of a pilot study designed to determine whether the breath test could be performed with chickens. Fourteen female White Leghorn chickens (*Gallus domesticus*) (WLCs) were used (Appendix A). The appropriate dose of caffeine and the quantity of radioactive tracer necessary for use in chickens were determined. Investigations centered on whether there were significant differences in the rate of caffeine metabolism between control and TCDD-exposed chickens. In addition, the rate of caffeine metabolism was compared to *ex vivo* hepatic EROD activity of the chickens. Since different animal species exhibit variation in the location of maximum N-demethylation of the caffeine molecule, a tri-labelled caffeine molecule with ^{14}C at the 1, 3 and 7-methyl positions was used in order to maximize the likelihood of observing a cytochrome P450-1A-mediated response (Figure 1). The tri-labelled caffeine molecule was synthesized as a part of this project since it was not commercially available.

The second phase of the project was a field study designed to determine whether the CBT was workable in the field, to investigate the response of wild

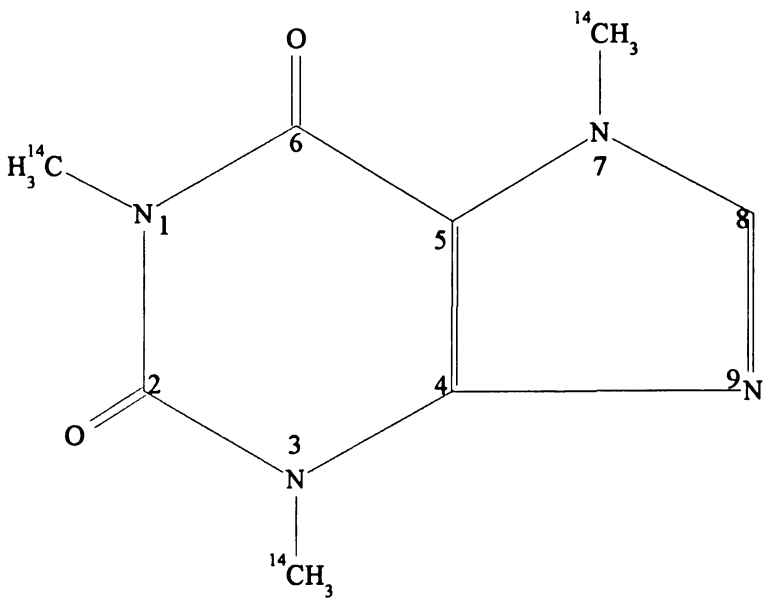


Figure 1. 1,3,7-^{14}C-trimethylxanthine (tri-labelled caffeine).

birds to the technique, and to gather some data on cytochrome P450-1A activity in Great Lakes fish-eating birds. The breath test was performed on nine HG, five Ring-billed Gull (*Larus delawarensis*) (RBG) and three DCC nestlings between 26 to 40 days of age. Rates of caffeine metabolism were compared to *ex vivo* hepatic EROD activity of the same individual birds. Sampling was performed in the North Channel of Lake Huron and on Kidney Island in Saginaw Bay, Lake Huron. Kidney Island is a confined disposal facility (CDF) for dredged sediments from the Saginaw River, which are known to be contaminated with *p*PCDHs (12).

The third phase of the project was performed to determine whether commercially available mono-labelled caffeine could be used as a substrate for the CBT in birds. The mono-labelled caffeine molecules tested were 1-methyl-^{14}C-caffeine (Moravek Biochemicals, Brea CA, Lot 108-215-059, 0.250 mCi in 2.5 ml ethanol) and 3-methyl-^{14}C-caffeine (Moravek Biochemicals, Brea CA, Lot 111-137-054, 0.05 mCi in 1 ml ethanol). Rates of caffeine metabolism were compared to *ex vivo* hepatic EROD activity. Ten female Silver-spangled Hamburg chickens (*Gallus domesticus*) (SSHCs) were used in this laboratory study (Appendix A).

Synthesis of radiolabelled caffeine (1,3,7-^{14}C-trimethylxanthine)

The radiolabelled caffeine synthesis was based on the methods of Kotake *et al.* (45). A reaction mixture was prepared by adding 3.6 μmole xanthine,

0.5 ml dimethyl sulfoxide (DMSO), and a micro-stir bar to a 5 ml pear-shaped flask (Kontes, Vineland NJ) . The mixture was stirred until all xanthine was dissolved. An additional 0.5 ml DMSO was then added to the flask along with 36 μmole powdered potassium carbonate (K_2CO_3). The flask was attached to a vacuum manifold and immersed in a dry ice/isopropanol slurry (Figure 2). An ampule containing 1 mCi of ^{14}C-methyl iodide (specific activity 54.7 mCi/mmole, Amersham Corp., Arlington Heights IL) was immersed in a dry ice/isopropanol slurry and attached to the vacuum manifold after inserting a magnetic hammer. All vacuum manifold ground glass joints were sealed with Dow High Vacuum Silicon Grease. A water aspirator was used to pull a vacuum on the vacuum manifold system. The system was then closed to maintain the vacuum, and the ^{14}C-methyl iodide ampule was removed from the dry ice slurry. The magnetic hammer was dropped to shear the ampule hook, which released the gaseous ^{14}C-methyl iodide into the manifold. The ^{14}C-methyl iodide gas was allowed to transfer to the cooled reaction flask for one hour. Transfer was aided by warming the ampule and manifold with a hand-held hair dryer or heat tape. The reaction flask was then removed from the manifold, sealed with a ground glass stopper, wrapped in aluminum foil to exclude light, and left on a stir-plate at room temperature for 48 hr.

High pressure liquid chromatography (HPLC) was utilized to purify the radiolabelled caffeine (Appendix B). The synthesis flask volume was increased to 3 ml by adding 2 ml of the HPLC solvent (1:4 methanol:water containing 1% acetic acid), and the contents of the flask were mixed. The reaction

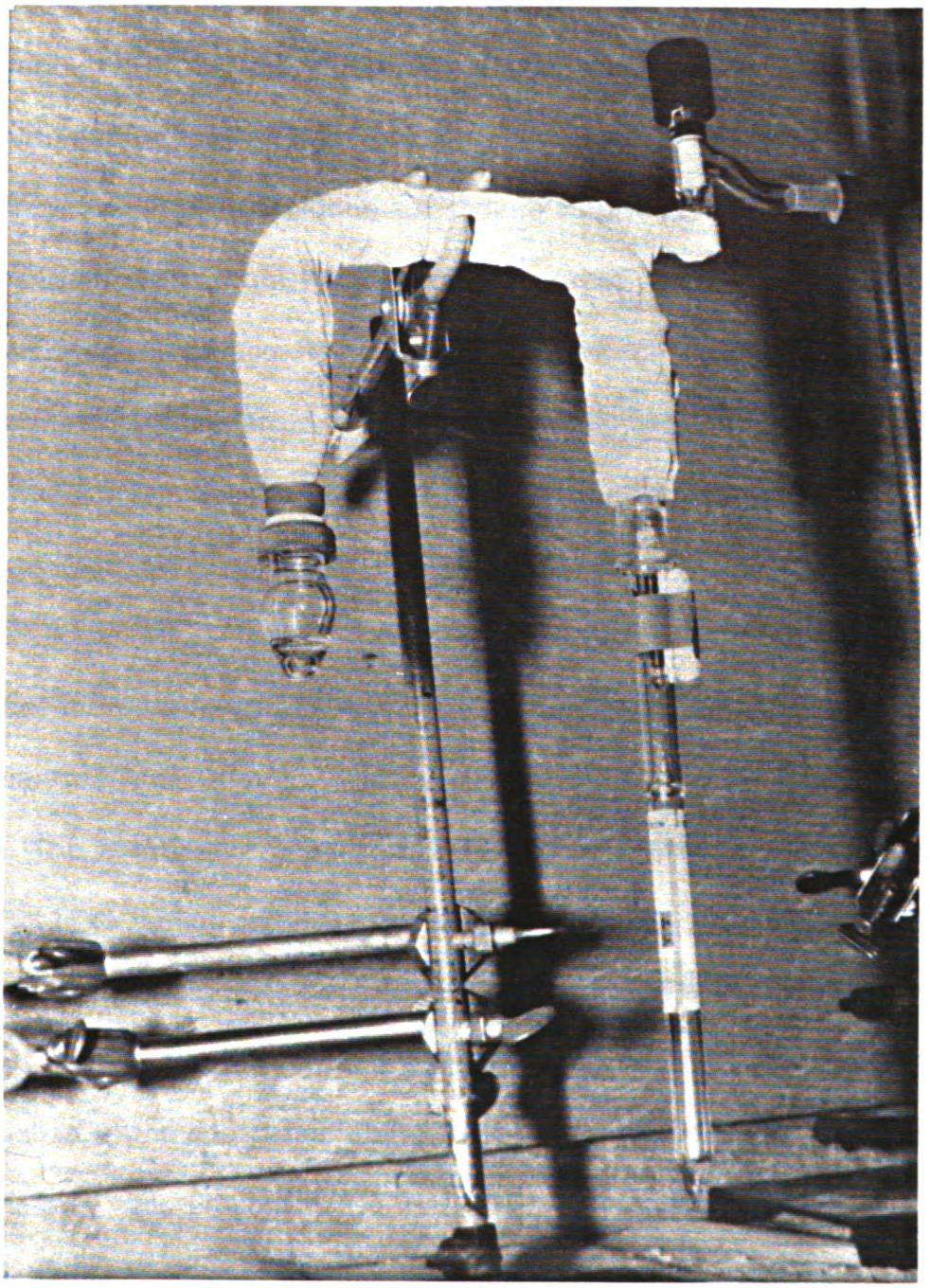


Figure 2. 1,3,7-^{14}C-trimethylxanthine synthesis.

mixture was then injected into the HPLC in 200 µl increments, and the caffeine peaks were collected. The radiolabelled caffeine was extracted from the HPLC solvent into HPLC-grade chloroform. Three 40 ml aliquots of chloroform were used to extract the caffeine from approximately 100 ml of 1:4 methanol:water collected during HPLC elution. A sodium sulfate drying column was used to remove all water from the chloroform extract, and the extract was rotory evaporated to reduce its volume to a few ml. The caffeine was then quantitatively transferred to a 50 ml volumetric flask and stored in chloroform at -20° C. The flask was wrapped in aluminum foil for storage.

Caffeine breath test (CBT)

Apparatus

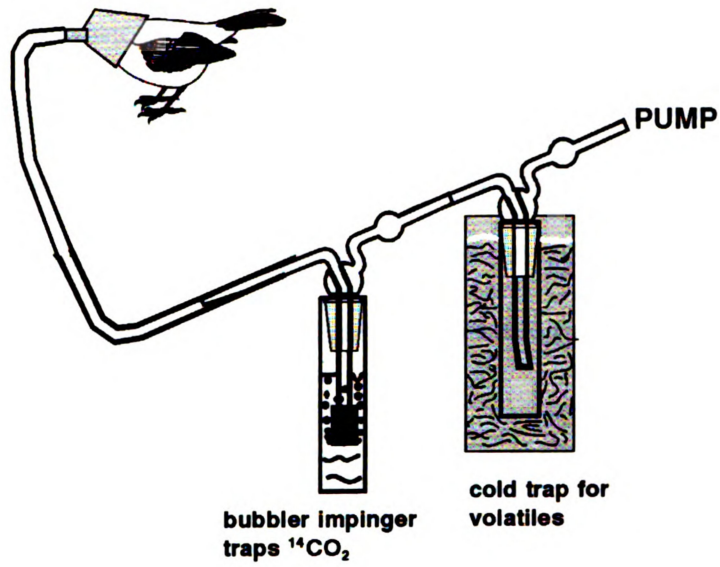
The breath test apparatus consisted of a number of components. A 16"x11"x8" airline carrier cage was used to hold the chicken during laboratory tests. A lightweight leather mask was constructed which had several Velcro® straps for custom fitting, and a plastic cone for the beak to fit into which attached to the apparatus tubing. The mask was not airtight, so a sufficient air supply was present. The mask was connected to flexible latex tubing which was connected to teflon tubing which was anchored to the cage. The teflon tubing was connected to one or two 30 ml bubbler impingers (SKC Inc., Eighty Four PA, 225-36-2), then to a glass cold trap, and finally to a sample pump (SKC Inc., Eighty Four PA, 224-43XR) with an adjustable low-flow

controller (SKC Inc., Eighty Four PA, 224-26-01) (Figure 3). During a breath test the impingers were each filled with 12 ml of 10% ethanolamine in methanol, and the cold trap was immersed in a dry ice/methanol slurry to trap any remaining volatile compounds. The pump pulled the expired breath through the apparatus at a constant rate of less than 500 ml/min.

Injection solutions

The radiolabelled caffeine injection solutions were prepared in the following manner. The ^{14}C-labelled caffeine was placed into an empty, sterile Vacutainer® tube and the carrier solvent was evaporated under a gentle stream of nitrogen. Cold caffeine and sterile saline (0.9%) were then added to the tube. Each dose consisted of 5 μCi of ^{14}C activity with approximately 1 mg caffeine/kg body weight in 1 ml physiological saline.

The absolute caffeine dose used varied among experiments due to the difference in body weights of the test species. However, solutions were always prepared to deliver 5 μCi of ^{14}C activity and approximately 1 mg caffeine/kg body weight in a volume of 1 ml sterile saline. In the experiments using tri-labelled caffeine (1,3,7-^{14}C-trimethylxanthine) and WLCs, injection solutions contained 1.5 mg caffeine (specific activity 0.65 μCi/μmole). In the field study nestlings were expected to have an average weight of 600 g, so the injection solutions contained 0.60 mg caffeine (specific activity 1.61 μCi/μmole). The RBG nestlings weighed 375 - 450 g, so the injection volume was changed to 0.7 ml for that species to avoid a caffeine overdose.



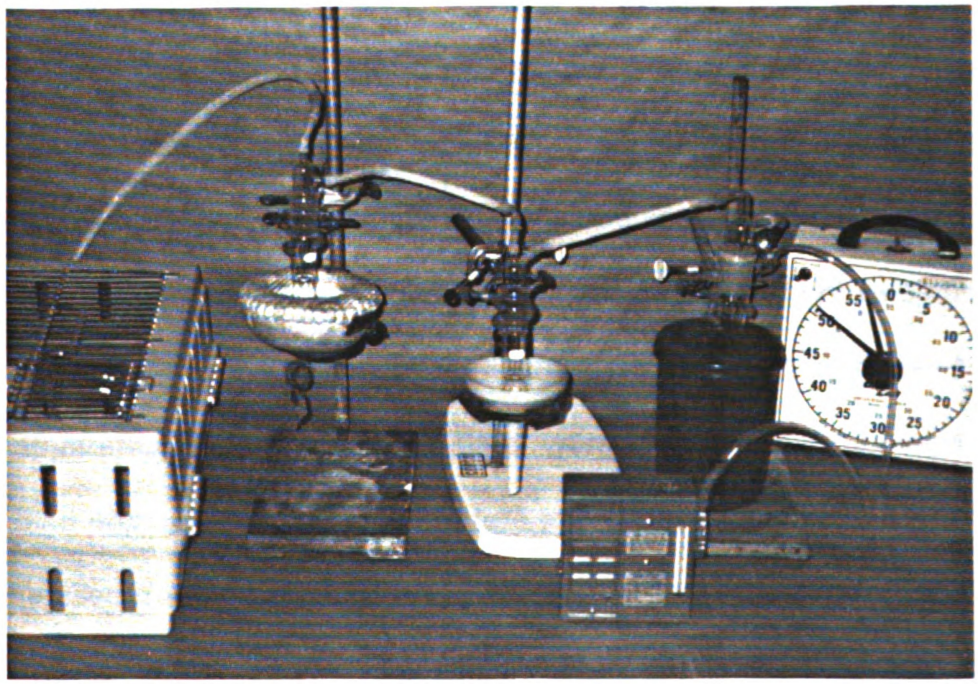


Figure 3. Caffeine breath test apparatus.

Therefore, they were only administered 3.5 μCi of ^{14}C. The DCCs which were tested had an average weight of 1550 g, and they received a 1 ml injection which contained 0.39 mg caffeine/kg body weight with an activity of 5 μCi of ^{14}C. In the laboratory study of mono-labelled caffeine compounds using SSHCs, the doses of tri-labelled caffeine and 1-methyl-^{14}C-caffeine contained 1.5 mg caffeine. The dose was modified for the study of 3-methyl-^{14}C-caffeine to 1.2 mg caffeine (specific activity 0.81 μCi/μmole) because the average weight of the SSHCs was approximately 1.2 kg.

Procedure

Each breath test was performed in the following manner. Prior to beginning a breath test a dry ice/methanol slurry was prepared for the cold trap, 12 ml of trapping solution was placed in both impingers, and all tubing was connected. The bird was weighed, and a ten-minute pre-injection breath sample was obtained. Subsequently 1 ml of a solution which contained 5 μCi of ^{14}C and approximately 1 mg caffeine/kg body weight in physiological saline was injected into the brachial vein of the bird. The bird was then hooked to the apparatus, and the pump and timer were started. The trapping solution in the lead impinger was changed before each interval sample. A 4 ml aliquant of the used trapping solution was placed in a 20 ml scintillation vial along with 16 ml of Safety-Solve® High Flash Point Cocktail (Research Products International Corporation, Mount Prospect IL), and the ^{14}C activity (from respired $^{14}CO_2$) was determined by liquid scintillation. The time periods for

analysis during laboratory experiments were 10, 20, 30, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 min post-injection. In the field, data were only collected at the 10, 20, 30 and 40 min time periods.

Following the breath test, most birds were killed by cervical dislocation or decapitation. The liver and gall bladder were immediately removed after death, and the gall bladder was carefully separated from the liver without puncture. The liver was then weighed, wrapped in aluminum foil, and quick-frozen in liquid nitrogen, where it remained stored until EROD enzyme activity was determined. During field experiments the livers were placed immediately on dry ice and then transferred to liquid nitrogen within 12 hr.

Calculations

The total mass of caffeine metabolized over the first 40 min of the test was used to calculate rates of caffeine metabolism. This was done by summing the activities (Bq) of $^{14}CO_2$ expired during 40 min post-injection, and then converting the total activity to mass caffeine (pmole) using the specific activity of the caffeine. The total mass of caffeine metabolized over the first 40 min of the test was divided by 40 min to represent the average rate of caffeine metabolism over that time period.

Induction experiments

TCDD was administered to some birds to induce the cytochrome P450-IA enzyme prior to performing the breath test. The TCDD was dissolved in corn

oil, and was administered in two interperitoneal injections 72 hr and 48 hr prior to the breath test. The doses of TCDD that were tested during the tri-labelled caffeine experiments were 1, 3, 5.3, and 17.8 μg TCDD/kg body weight. The only TCDD dose administered during the mono-labelled caffeine study was 3 μg TCDD/kg body weight.

Ex vivo *measurement of hepatic cytochrome P450-1A activity*

Preparation of liver microsomes

A microsomal fraction was prepared from the liver tissue collected from each bird following the breath test by the methods of Bellward *et al.* (40). Tissue samples were removed from liquid nitrogen to isolate a 0.75 gram subsample for the procedure. All subsequent steps were performed at 4° C. The subsample was homogenized with Tris buffer (0.05 M Tris, 1.15% KCl, pH 7.5) using a Tri-R Stir-R® (Tri-R Instruments, Inc., Rockville Centre, NY) with a teflon stir stick, and the homogenate was centrifuged at 10,000 x g for 20 min. The precipitate was discarded and the supernatant was centrifuged at 100,000 x g for 60 min. The supernatant was discarded and the microsomal pellet was resuspended in EDTA buffer (10 mM EDTA, 1.15% KCl, pH 7.4) using a Tekmar® Tissumizer (Tekmar Co., Cincinnati OH). This suspension was centrifuged at 100,000 x g for 60 min. The supernatant was discarded and the microsomal pellet was resuspended in a microsomal stabilizing buffer (20% glycerol, 0.1 M KH_2PO_4, 1 mM EDTA, 1 mM dithiothreitol, pH 7.25) using a Tekmar® blender. The suspension was placed

in several eppendorf tubes in 100 µl aliquots and stored in a -80° C freezer until the EROD enzyme activity assay could be performed. The EROD assay was performed within one week of the microsomal preparation.

Ethoxyresorufin-o-deethylase (EROD) enzyme activity assay

The protein concentration of the microsomal preparation was determined using one of the 100 µl aliquots. A bovine serum albumin (BSA) standard curve was developed using concentrations ranging from 20 to 80 µg/100 µl, and each sample was diluted five-fold in order to fall within that range. Three test tubes were prepared for each sample and standard by adding 100 µl of the sample or standard to 5 ml of a dye solution (1: 8 Bio-Rad Protein Assay:water) in each tube, and a blank was also prepared. After mixing the contents of the tubes and allowing them to sit for 10 min, the optical density at 595 nm was determined.

A 96-well micro-titer plate was prepared for the EROD activity assay. Each well had a 200 µl capacity. Three replicates (wells) were prepared for a blank and each sample and resorufin standard. Six resorufin standard concentrations were analyzed ranging from 2.5 to 60 pmole/200 µl. Standard wells contained resorufin, Tris buffer, and 50 µl of ethoxyresorufin (10 µM). Blank wells contained 50 µl of ethoxyresorufin (10 µM) and 150 µl of Tris buffer. Sample wells contained 10 µg protein, 50 µl of ethoxyresorufin (10 µM), 50 µl of NADPH (0.5 mM) and Tris buffer. Florescence of resorufin was measured in each well every 5 min for one hour by use of a Cytofluor 2300

(Millipore Corp., Carlsbad CA). EROD activity was determined kinetically and reported as pmole resorufin/min/mg protein.

RESULTS

Experiments using 1,3,7-^{14}C-trimethylxanthine

Breath test with unexposed White Leghorn chickens (WLCs)

In order to obtain baseline data on the rate of cytochrome P450-IA-catalyzed N-demethylation of caffeine in uninduced chickens, the CBT was performed on seven WLCs which had not been exposed to TCDD (Appendix C). The rate of caffeine metabolism in these chickens tended to increase steadily during the first three hours following the injection of ^{14}C-labelled caffeine, and then it began to decline (Figure 4). Among-chicken variability in the amount of caffeine metabolized per minute was greatest 120 min after the injection of the caffeine (cv = 47.6%) (Appendix D).

Breath test with TCDD-induced WLCs

The rates of $^{14}CO_2$ exhalation in eight TCDD-exposed WLCs were greater than those of unexposed controls during the CBT (Appendix C). This is indicative of a greater rate of caffeine metabolism in the TCDD-exposed WLCs than in the controls. All doses of TCDD tested resulted in greater rates of caffeine metabolism (Figure 5), but dose-related differences in caffeine

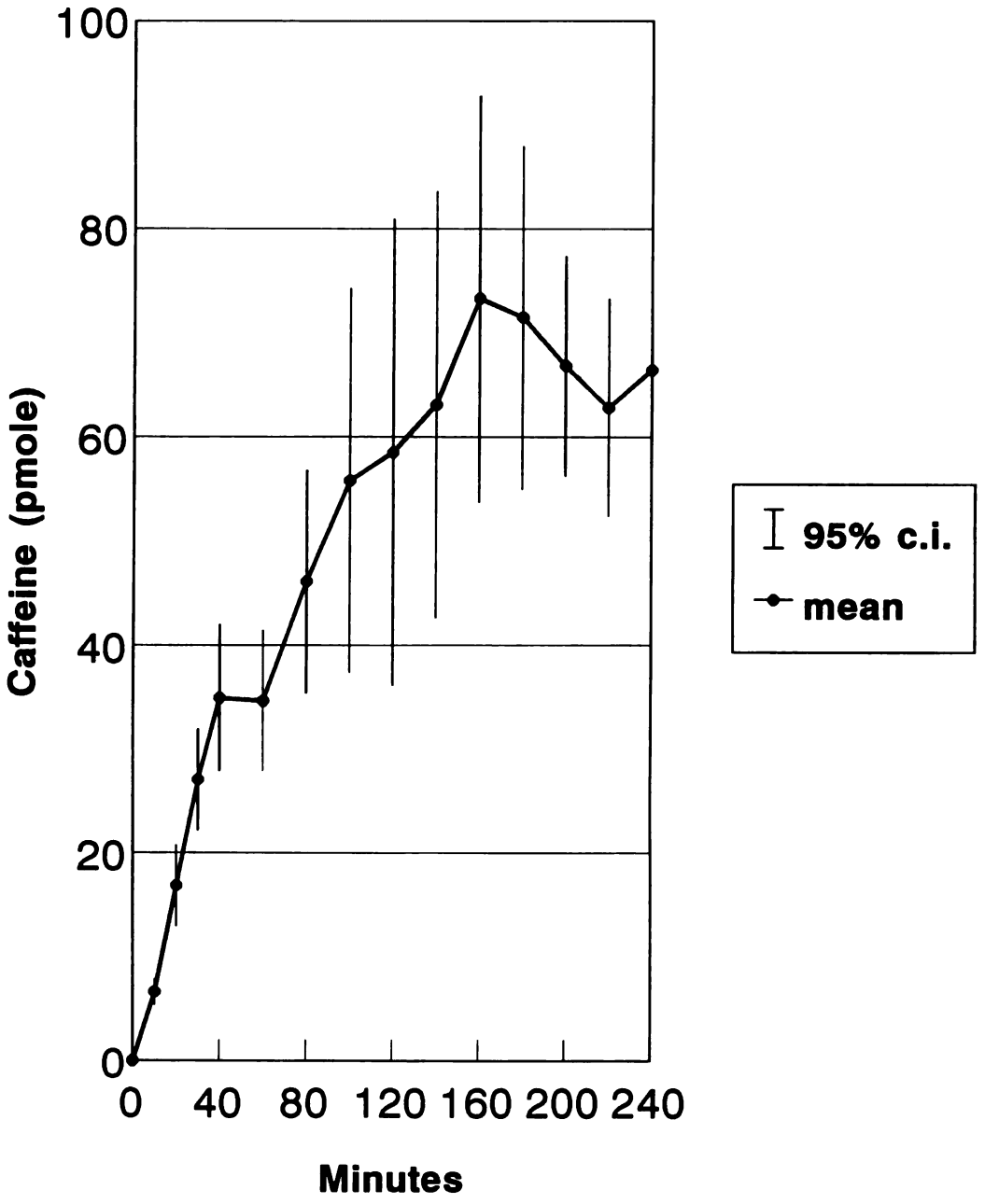


Figure 4. $^{14}CO_2$ exhaled, expressed as pmole caffeine metabolized by control WLCs during the CBT when 1,3,7-^{14}C-trimethylxanthine was used. Points represent means of six uninduced WLCs, while the error bars represent ± 1.96 s.e. (95% c.i.).

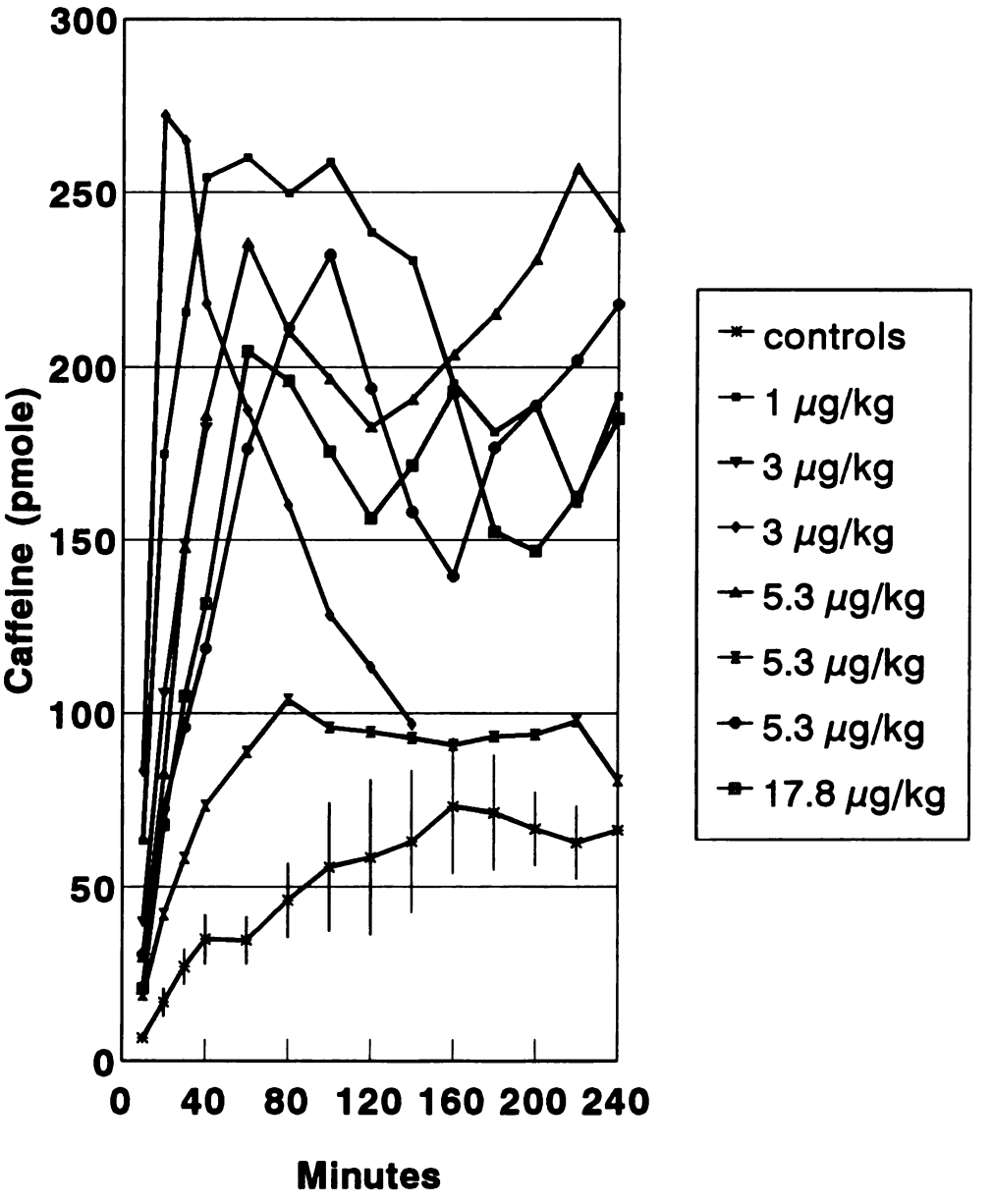


Figure 5. $^{14}CO_2$ exhaled, expressed as pmole caffeine metabolized by WLCs during the CBT when 1,3,7-^{14}C-trimethylxanthine was used. The "controls" line represents the means of six uninduced chickens ± 1.96 s.e. (95% c.i.). All other lines show the response of individual WLCs induced with the indicated dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) 72 hr and 48 hr prior to administering the CBT.

metabolism were not observed among the TCDD-exposed WLCs. With the exception of one individual (chicken #9) which was only moderately induced after receiving a dose of 5.3 μg TCDD/kg body weight, the pmole of caffeine metabolized per minute for all TCDD-exposed WLCs was significantly greater than that of WLCs which were not induced with TCDD. The greatest difference in caffeine metabolism by an induced WLC relative to the controls occurred with chicken #18, which received a 3 μg TCDD/kg body weight dose. Over the first 40 min after injection with tri-labelled ^{14}C-caffeine chicken #18 metabolized 8.32×10^3 pmole caffeine, while the average amount of caffeine metabolized by uninduced WLCs during the same time period was 8.32×10^2 pmole. The pattern of caffeine metabolism over time was different in the TCDD-induced WLCs than in the controls. The rate of caffeine metabolism by the TCDD-induced WLCs increased and decreased as a function of time (Figure 5). The rate in induced WLCs typically increased sharply for a period ranging from 20 to 100 min, and then decreased sharply for approximately one hour, and then increased sharply again. In contrast, during the four-hour breath test period more than one peak was not apparent in the time course of caffeine metabolism by uninduced WLCs.

Multiple breath tests with the same WLC

When three breath tests were performed with chicken #13, consisting of a test prior to exposure to TCDD, a test following induction with 5.3 μg TCDD/kg body weight and a test one week after the initial test following

induction, the rate of caffeine metabolism was greater immediately following induction with TCDD (Figure 6). The cumulative quantity of caffeine metabolized by the chicken during the first 40 min of the control breath test was 763 pmole. When the bird was freshly induced by TCDD the amount of caffeine metabolized was 4463 pmole. One week following that test during maximum induction, the cumulative amount of caffeine metabolized during the first 40 min had subsided to an intermediate value of 4144 pmole. The characteristic pattern of large multiple increases and decreases in the rate of caffeine metabolism typically observed in induced birds was not evident during the CBT performed one week following peak induction.

Breath test with Silver-spangled Hamburg chickens (SSHCs)

Metabolism of tri-labelled ^{14}C-caffeine by SSHCs was compared to that of WLCs. The SSHC which was induced with 3 μg TCDD/kg body weight metabolized the labelled caffeine more rapidly than the control SSHC (Figure 7). This difference in metabolic rate was more apparent after the first hour post-injection (Appendix E). The uninduced SSHC expressed a greater rate of basal caffeine metabolism (3363 pmole in the first 40 min) than did the uninduced WLCs (855 pmole in the first 40 min).

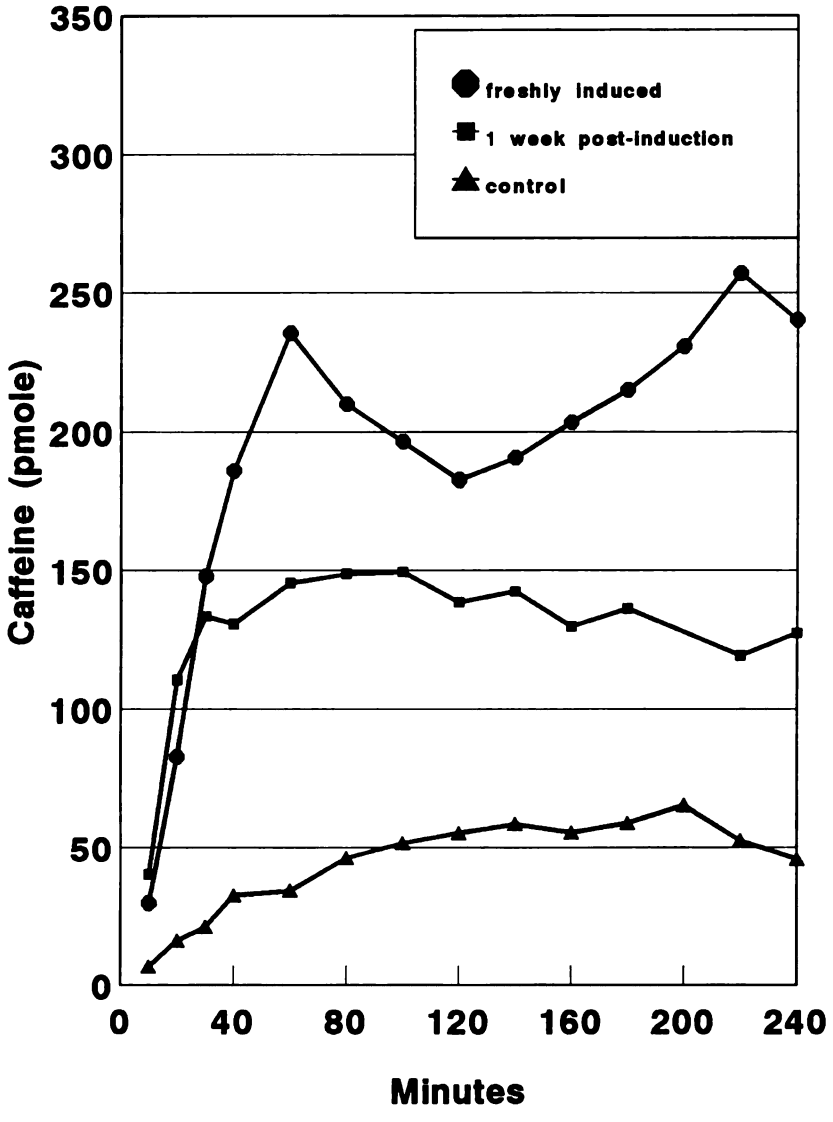


Figure 6. $^{14}CO_2$ exhaled, expressed as pmole caffeine metabolized by WLC #13 during three CBTs when 1,3,7-^{14}C-trimethylxanthine was used. The "control" line represents the response of the WLC prior to induction. The "freshly induced" line represents the response of the WLC after i.p. injection with 5.3 μg TCDD/kg body weight 72 hr and 48 hr prior to administering the CBT. The "one week later" line represents the response of the WLC in a CBT performed one week after the CBT following TCDD induction, when no further exposure to TCDD occurred.

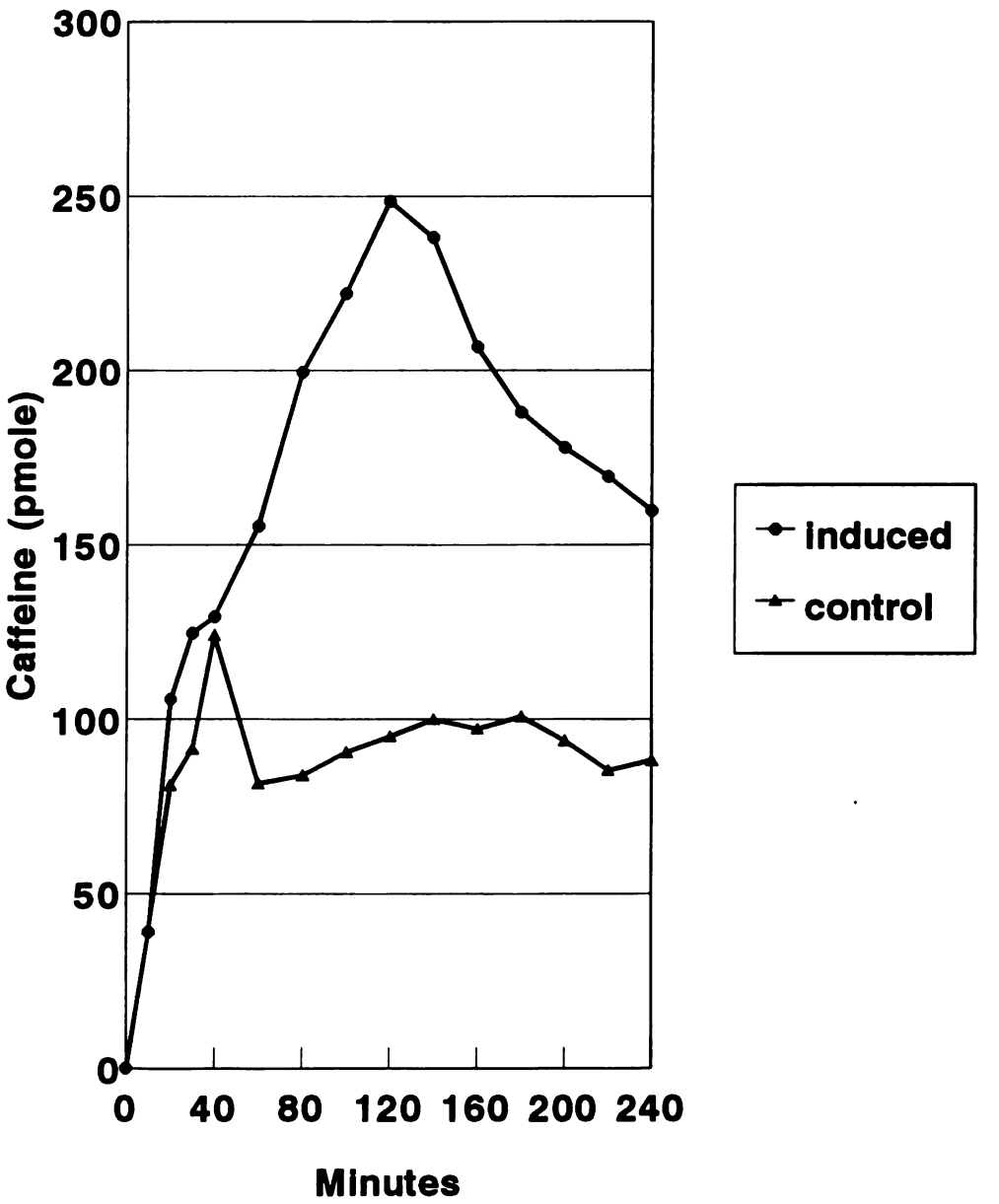


Figure 7. $^{14}CO_2$ exhaled, expressed as pmole caffeine metabolized by two individual SSHCs during the CBT when 1,3,7-^{14}C-trimethylxanthine was used. The "control" line represents the response of an uninduced SSHC. The "induced" line represents the response of a SSHC after i.p. injection with 3 μg TCDD/kg body weight 72 hr and 48 hr prior to administering the CBT.

Field experiment with Great Lakes fish-eating birds

The CBT was performed successfully on nine HG, five RBG and three DCC nestlings at several Great Lakes field locations (Appendix F). Individuals of all three species tolerated the breath test procedure, and in general were calmer than chickens which were studied under laboratory conditions. Caffeine was metabolized to $^{14}CO_2$ at a relatively small but measurable rate by all of the birds. The rate of $^{14}CO_2$ exhalation varied among species (Figure 8). RBGs exhibited the greatest variability of $^{14}CO_2$ exhalation. Three of the RBGs exhibited little $^{14}CO_2$ exhalation, corresponding to a degree of caffeine metabolism within the range observed for the two other species (9 to 200 pmole caffeine over 40 min). Two RBGs exhaled $^{14}CO_2$ at a greater rate during the CBT than any of the other wild birds tested, corresponding to the metabolism of 572 and 1762 pmole caffeine over 40 min. One of these birds was from the North Channel of Lake Huron, while the other was from Kidney Island (confined disposal facility) in Saginaw Bay. All of the HGs tested exhibited relatively little P450-1A activity as determined by either the CBT (9 to 196 pmole caffeine over 40 min) or the *ex vivo* hepatic EROD assay (<1.6 to 15 pmole resorufin/min/mg protein). DCCs exhibited the widest range of *ex vivo* hepatic EROD activity (2.0 to 33.7 pmole resorufin/min/mg protein). However, the rate of $^{14}CO_2$ exhalation by DCCs during the CBT was small relative to the other species, and corresponded to the metabolism of approximately 10 pmole caffeine over 40 min.

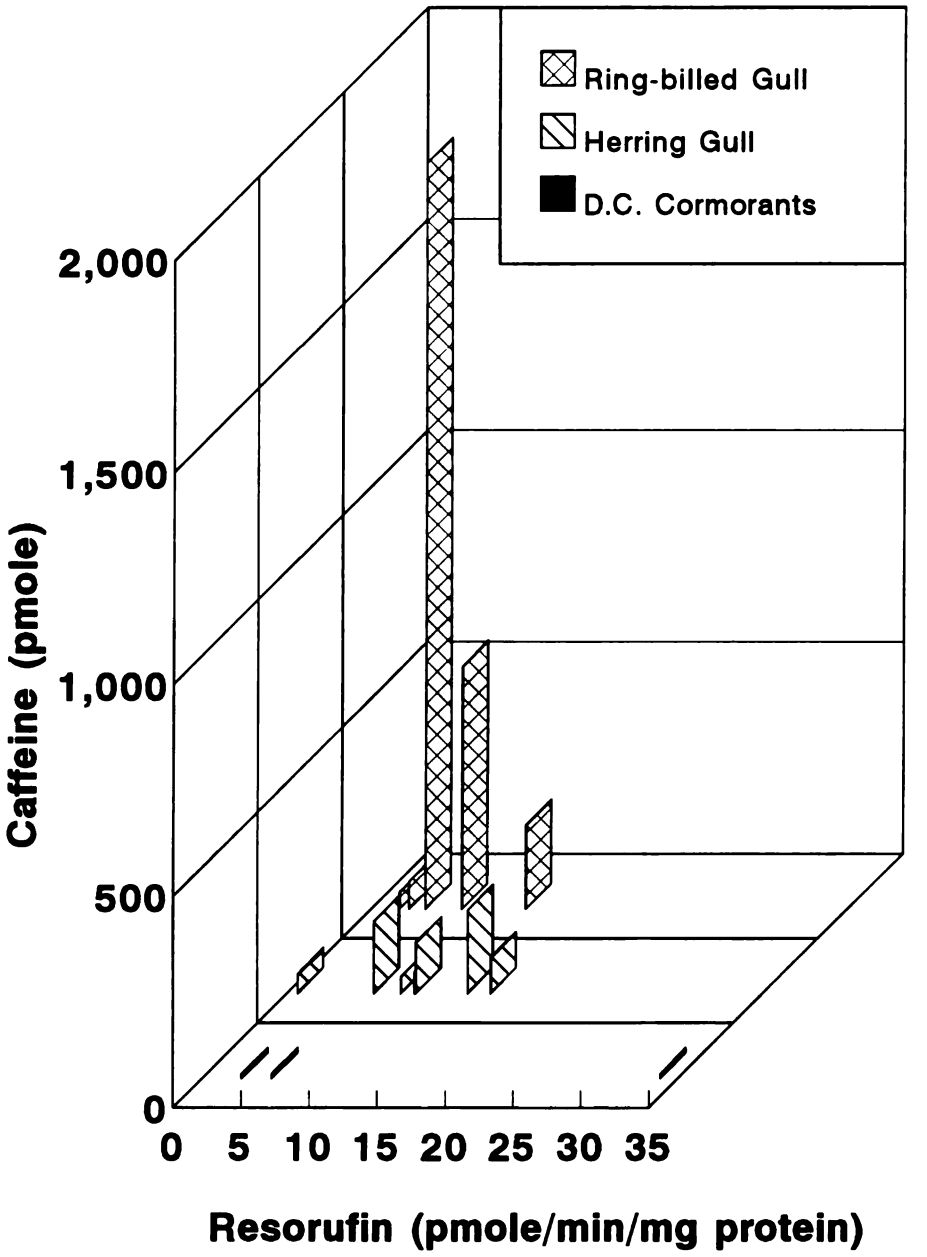


Figure 8. Cumulative $^{14}CO_2$ exhaled during the first 40 min of the CBT when 1,3,7-^{14}C-trimethylxanthine was used, expressed as pmole caffeine metabolized by individual HGs, RBGs and DCCs in comparison to *ex vivo* hepatic tissue activity of ethoxyresorufin-*o*-deethylase expressed as pmole resorufin/min/mg microsomal protein.

Metabolism of mono-labelled caffeine compounds

1-methyl-^{14}C-caffeine

The CBT was performed on four SSHCs using 1-methyl-^{14}C-caffeine. Three of the SSHCs were induced with 3 μg TCDD/kg body weight 72 hr and 48 hr prior to the CBT, while the fourth SSHC was an uninduced control. While there appears to be some difference in the 1-N-demethylation of caffeine between control and induced SSHCs, the results using this substrate are difficult to interpret (Figure 9). The initial rate of 1-N-demethylation of caffeine as measured by $^{14}CO_2$ exhalation was greater in the induced birds relative to the control. However, the control bird continued to increase in the rate of $^{14}CO_2$ exhalation after the rate of $^{14}CO_2$ exhalation in induced birds began to drop, and by the 120 min time point the amount of $^{14}CO_2$ exhaled was higher in the control bird than in any of the induced birds. The level of peak $^{14}CO_2$ exhalation during the 4 hr test was greater in the control than in two of the three induced birds.

3-methyl-^{14}C-caffeine

The CBT was performed on four SSHCs using 3-methyl-^{14}C-caffeine. Three of the SSHCs were induced with 3 μg TCDD/kg body weight 72 hr and 48 hr prior to the breath test, while the fourth SSHC was an uninduced control. The three TCDD-induced SSHCs had greater rates of 3-N-demethylation of caffeine as measured by $^{14}CO_2$ exhalation than the control SSHC did (Figure 10).

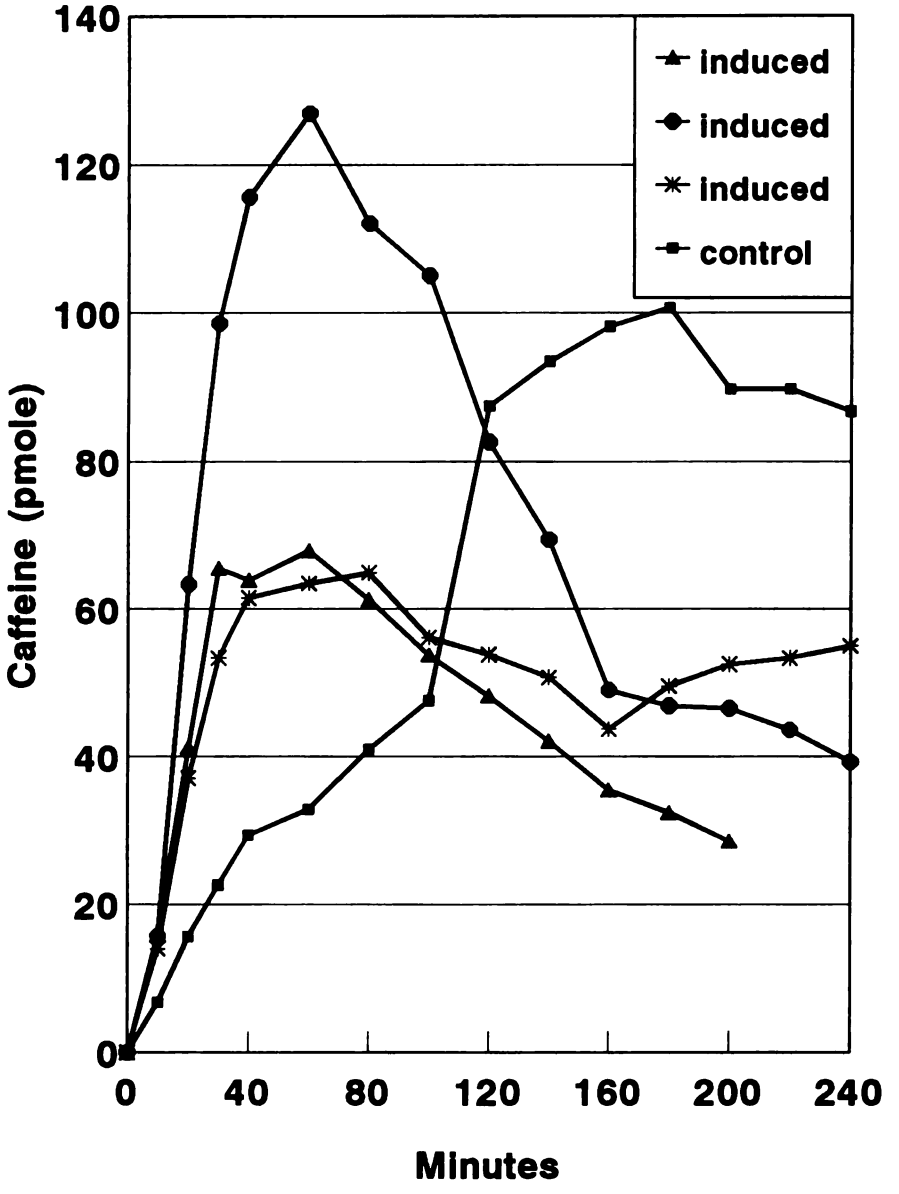


Figure 9. $^{14}CO_2$ exhaled, expressed as pmole caffeine metabolized by four individual SSHCs during the CBT when 1-methyl-^{14}C-caffeine was used. The "control" line represents the response of an uninduced SSHC. The "induced" lines represent the response of individual SSHCs after i.p. injection with 3 μg TCDD/kg body weight 72 hr and 48 hr prior to administering the CBT.

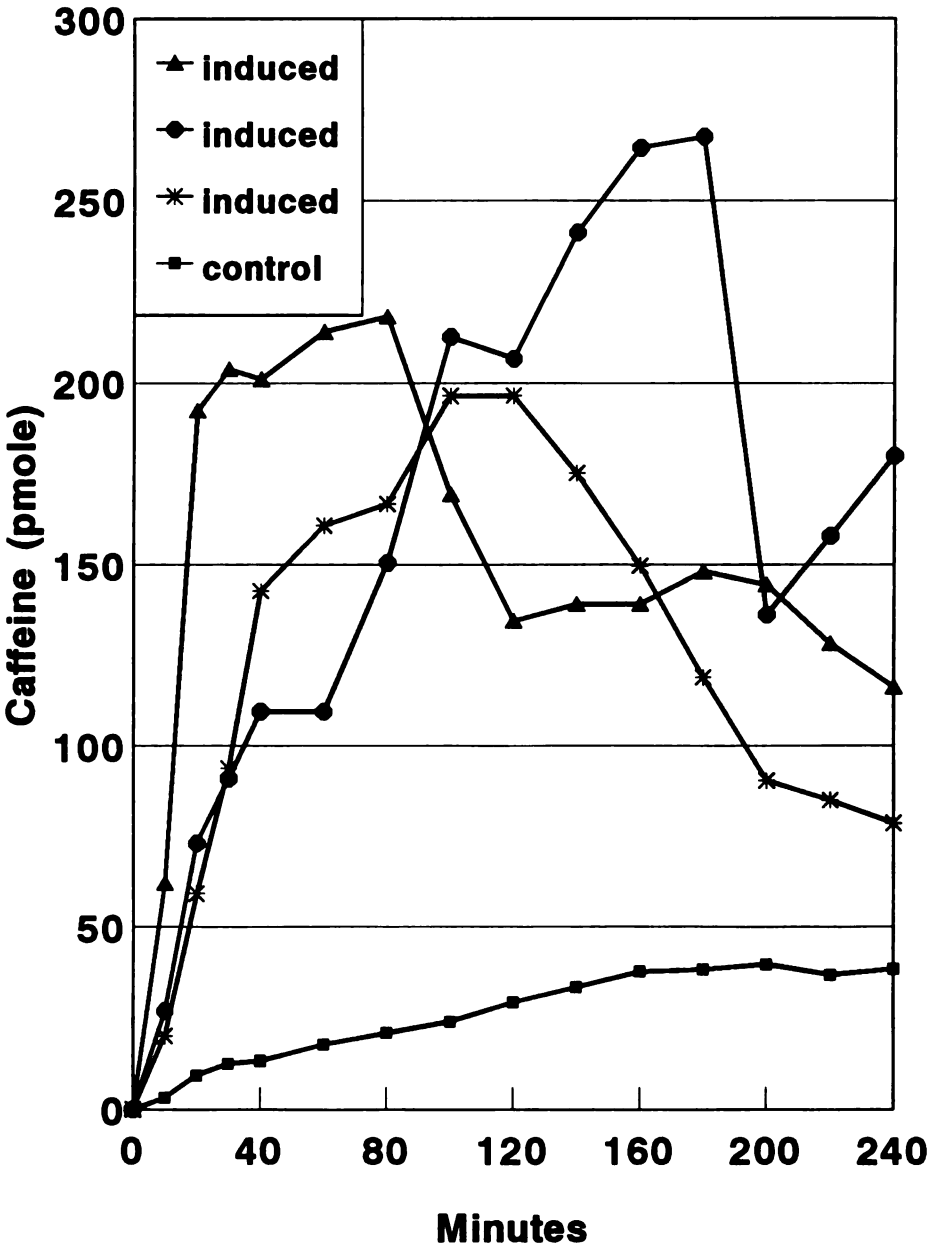


Figure 10. $^{14}CO_2$ exhaled, expressed as pmole caffeine metabolized by four individual SSHCs during the CBT when 3-methyl-^{14}C-caffeine was used. The "control" line represents the response of an uninduced SSHC. The "induced" lines represent the response of individual SSHCs after i.p. injection with 3 μg TCDD/kg body weight 72 hr and 48 hr prior to administering the CBT.

Caffeine metabolism vs.* ex vivo *hepatic P450-1A activity

1,3,7-^{14}C-trimethylxanthine in WLCs

$^{14}CO_2$ expiration in the CBT was compared to *ex vivo* hepatic P450-1A (EROD) activity in five WLCs. The liver of a sixth WLC (#18) was in such poor condition following induction with TCDD that a suitable microsomal preparation could not be made. A linear relationship between $^{14}CO_2$ expiration and *ex vivo* hepatic EROD activity was not apparent (Figure 11). The *ex vivo* hepatic EROD activity and $^{14}CO_2$ expiration were both less in the WLC that did not receive TCDD than in the TCDD-induced WLCs (Appendix G).

Great Lakes fish-eating birds

When the CBT and the *ex vivo* hepatic EROD assay were performed in Great Lakes fish-eating birds, a linear relationship was not apparent between the results of the two assays for any of the three species tested (Figure 8). All nine HGs exhibited relatively little P450-1A activity in both assays (Appendix H). The DCCs exhibited little caffeine metabolism (Appendix H). In two DCCs the EROD activity was also relatively small, however one DCC exhibited relatively great EROD activity (Appendix H). EROD activities of RBGs were relatively small (Appendix H). Three of the RBGs metabolized caffeine at a small rate which was comparable to the other wild birds tested, while two RBGs expressed greater rates of caffeine metabolism (Appendix H).

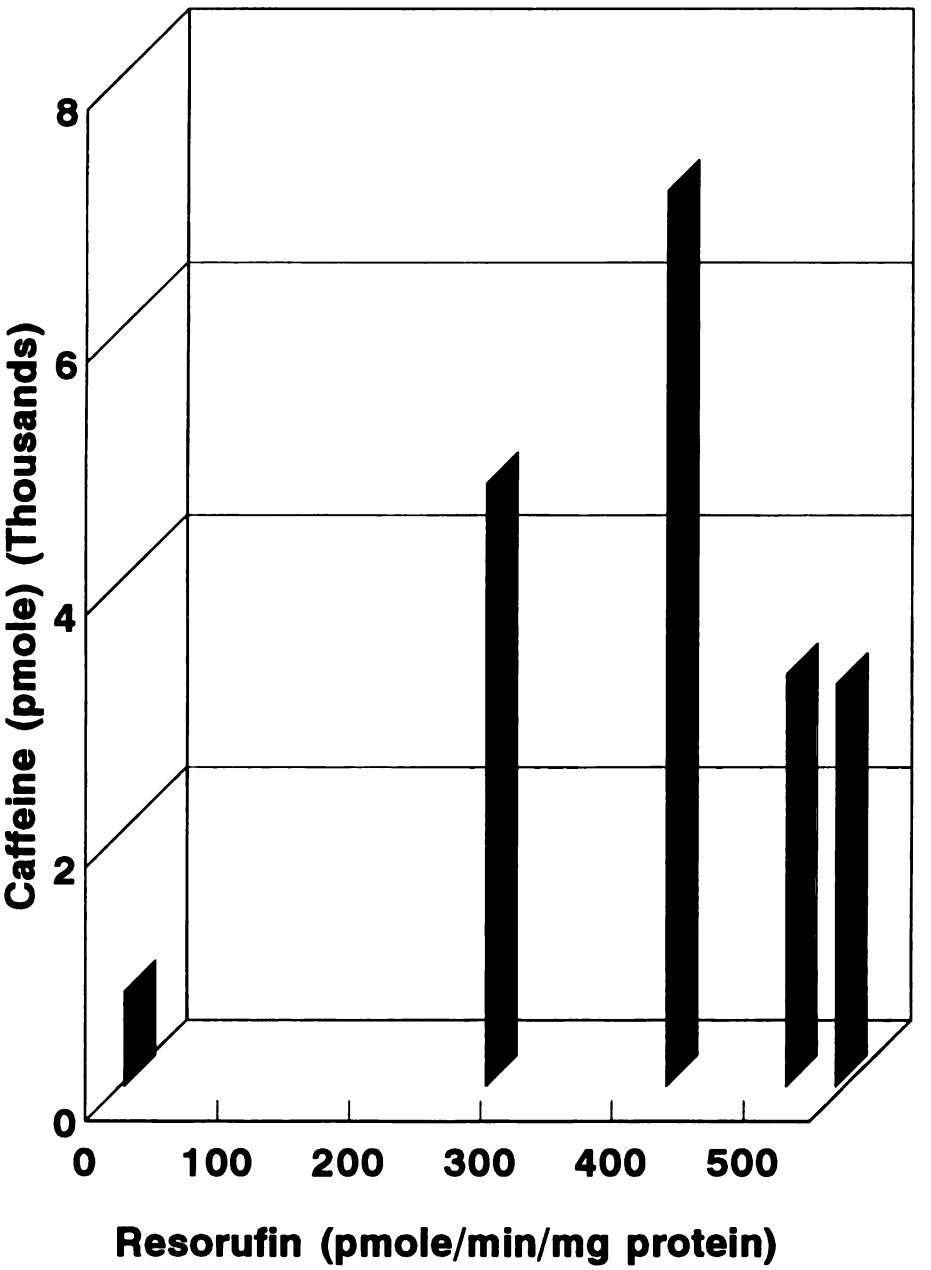


Figure 11. Cumulative $^{14}CO_2$ exhaled during the first 40 min of the CBT when 1,3,7-^{14}C-trimethylxanthine was used, expressed as pmole caffeine metabolized by five individual WLCs in comparison to *ex vivo* hepatic tissue activity of ethoxyresorufin-*o*-deethylase expressed as pmole resorufin/min/mg microsomal protein.

Mono-labelled caffeine in SSHCs

Ex vivo hepatic EROD activity was compared with the metabolism of 3-methyl-^{14}C-caffeine, because this mono-labelled caffeine was most effective at discriminating differences in metabolism between control and TCDD-induced SSHCs. As with the tri-labelled caffeine, a linear relationship between the results of the two assays was not apparent (Figure 12). However, both assays were able to discriminate between the uninduced SSHC and the TCDD-treated SSHCs (Appendix I).

Mortality during the CBT

During the initial experiment using WLCs and 1,3,7-^{14}C-trimethylxanthine some WLCs died during the breath test, apparently due to stress-related heart attacks. One out of seven of the control WLCs died, while three out of eight TCDD-induced WLCs died during the test. Three out of the four deaths occurred towards the end of the study period, when the WLCs had attained an age of approximately eighteen months.

During the experiment using mono-labelled caffeine and SSHCs, two out of eleven SSHCs died during the breath test. One of the SSHCs which died had been induced with 3 μg TCDD/kg body weight 72 hr and 48 hr before the test, and died towards the end of the four hour post-injection breath collection period. The other SSHC was an uninduced control which died several minutes into the pre-test before any caffeine was injected.

In contrast, no birds died during the breath test procedure in the field. All three species of field birds tested seemed calmer, stronger and more tolerant of the breath test procedure than the domestic chickens.

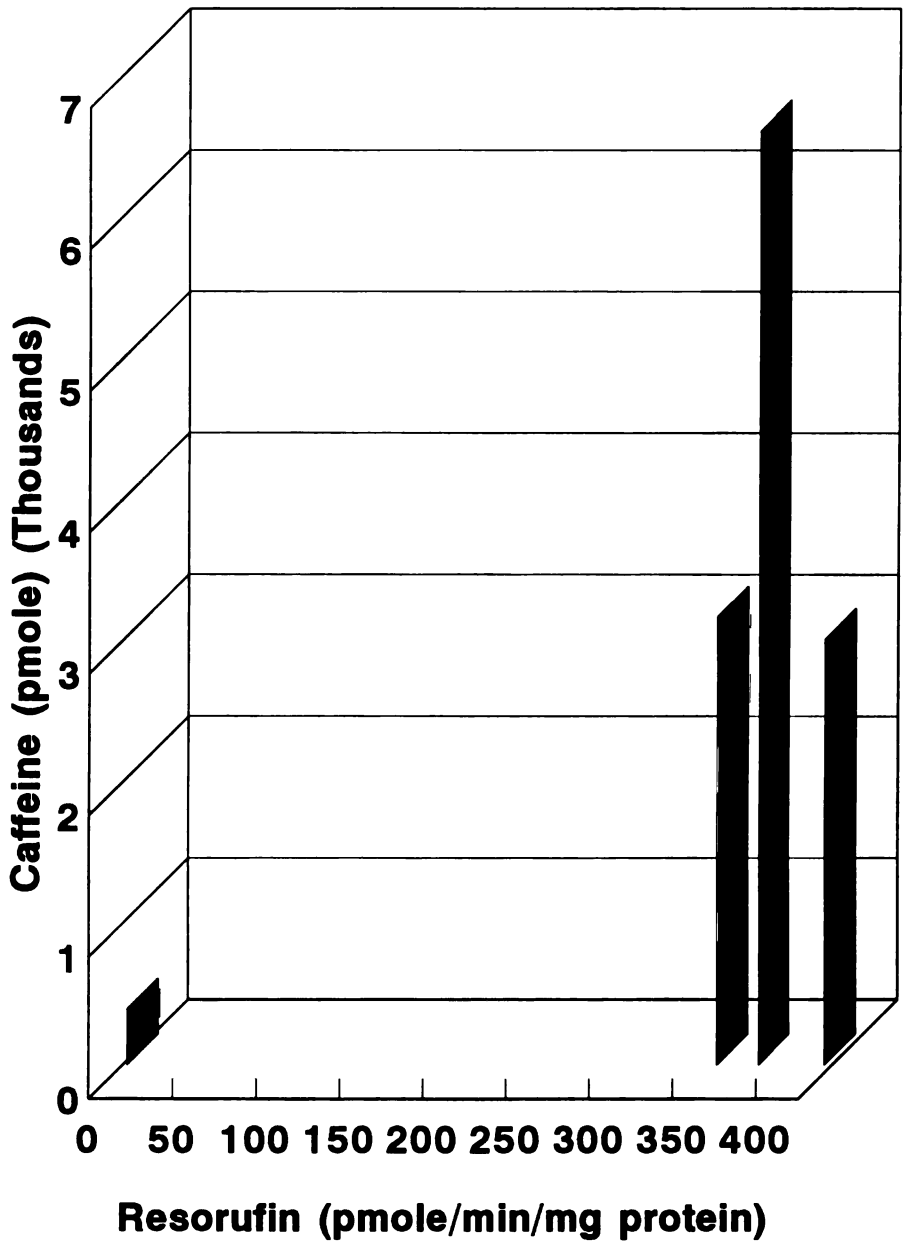


Figure 12. Cumulative $^{14}CO_2$ exhaled during the first 40 min of the CBT when 3-methyl-^{14}C-caffeine was used, expressed as pmole caffeine metabolized by four individual SSHCs in comparison to *ex vivo* hepatic tissue activity of ethoxyresorufin-*o*-deethylase expressed as pmole resorufin/min/mg microsomal protein.

DISCUSSION

Experiments using 1,3,7-^{14}C-trimethylxanthine

Comparison of CBT in control and TCDD-exposed WLCs

Since caffeine metabolism was greater in WLCs exposed to TCDD than in controls, caffeine metabolism may be useful as a biomarker of *p*PCDH exposure in birds. The rate of metabolism of caffeine in TCDD-exposed WLCs was characterized by increases and decreases, while this pattern was not observed in control WLCs. Since the maximum and minimum rates of caffeine metabolism were reached after different post-injection times in the WLCs, it was not possible to choose one time point for analysis of relative caffeine metabolism. Instead, the total mass of caffeine metabolized over the first 40 min of the test was used in calculations.

Multiple breath tests with the same WLC

A major advantage of the CBT over *ex vivo* assessments of hepatic EROD activity is that measurements of cytochrome P450-1A activity can be made on the same individual as a function of time. This is important because of among-individual variation. To test the ability of the CBT to assess the dynamics of

cytochrome P450-1A activity as a function of time, the CBT was repeated three times on the same WLC. Several TCDD exposure regimes were assessed. CBTs included a control test (pre-exposure), a test 72 hr following TCDD injection (as described elsewhere), and a test one week later without additional exposure to TCDD. The expected pattern of caffeine metabolism was observed in this experiment. Little metabolism of caffeine was observed prior to exposure of the chicken to TCDD. Following TCDD exposure the rate of caffeine metabolism was greater, and was characterized by the increases and decreases which are typically observed in induced birds. One week following maximum induction, the rate of caffeine metabolism in the chicken was intermediate between the control and greatly induced rates of metabolism. Thus, the CBT can successfully detect changes in P450-1A activity over time in the same individual.

Breath test with SSHCs

SSHCs exhibited both similarities and differences in caffeine metabolism relative to WLCs. SSHCs, like the WLCs, also metabolized tri-labelled caffeine more rapidly following treatment with TCDD. However, the SSHCs appeared to exhibit a greater basal rate of caffeine metabolism than the WLCs. This may be due to inherent species differences. Alternatively, SSHCs may have had moderately induced enzyme activity due to their lifelong exposure to coccidiostat in their food. While I am unaware of literature on the effect of

antibiotics on cytochrome P450 activity in chickens, it is possible that coccidiostat could induce the enzyme system since a variety of drugs are known to do so (50). It was necessary to supply the SSHCs with coccidiostat because prior to this experiment they had been housed on the floor, which enhanced their susceptibility to coccidiosis. In contrast, the WLCs had always been housed in individual cages so they were never treated with coccidiostat.

Field experiment with Great Lakes fish-eating birds

The CBT procedure can be performed successfully in the field. All of the breath test components are small and portable, and fit into a standard cooler for transport. The pump is battery-operated. However, the test could be modified for improved field use by eliminating the fragile glass components, such as the bubbler impingers and cold trap, and by eliminating the need for dry ice. Perhaps a solid material could be used to trap the $^{14}CO_2$. These refinements of the breath test apparatus could be a focus of future research on the breath test.

Weather conditions can present several challenges to breath test success. The weather can change quickly on the Great Lakes, and 20 min are needed to set up or take down the CBT. The breath test should never be performed in the rain, as it would probably stress test birds too much. If the weather is hot and sunny, test birds must be shaded to avoid overheating. This is especially important because the mask on the bird's head diminishes the bird's

ability to thermoregulate. In addition, it was discovered that the sunlight caused a temporary photoactivation of the scintillation cocktail. This resulted in a chemiluminescence which interfered with the measurement of ^{14}C activity. After the samples were left in the dark for several weeks the chemiluminescence subsided and the ^{14}C activity in the samples could be quantified. It is preferable to add the scintillation cocktail to the sample vials after returning to the laboratory in order to avoid photoactivation and minimize activities in the field.

Metabolism of mono-labelled caffeine compounds

It would be desirable to replace tri-labelled caffeine with a commercially available mono-labelled caffeine compound in the CBT so that the inconvenient, expensive, time-consuming and difficult synthesis of tri-labelled caffeine by researchers could be avoided. The requirement to synthesize the tri-labelled caffeine is a deterrent for other researchers who would otherwise be interested in performing the CBT. Also, if each researcher were to synthesize their own tri-labelled caffeine, differences in product purity could produce inconsistent results which could lessen the reproducibility of experiments.

The two mono-labelled caffeine compounds which were tested were not equally effective substrates in the CBT. The 1-methyl-^{14}C-caffeine was not a good substrate for the CBT, because differences in caffeine 1-N-demethylation

in control and TCDD-treated birds were small and difficult to interpret. In contrast, 3-methyl-^{14}C-caffeine appeared to be an excellent substrate for the caffeine breath test with SSHCs. This is an indication that caffeine 3-N-demethylation is mediated by cytochrome P450-1A in this species.

The substitution of a mono-labelled compound for the tri-labelled caffeine must be made with caution. It is likely that there are differences in the location of maximum cytochrome P450-1A-mediated N-demethylation of the caffeine molecule among species of birds. This is known to be the case with different species of rodents (46). Therefore, unless the N-demethylation of caffeine has been characterized for a species the most appropriate substrate for the CBT is the tri-labelled form, because a cytochrome P450-1A-mediated N-demethylation in any of the three methyl positions would be detected.

Caffeine metabolism vs.* ex vivo *hepatic P450-1A activity

TCDD-exposed chickens and uninduced chickens could be distinguished by the use of either the *ex vivo* hepatic EROD assay or the CBT. However, distinguishing relative P450-1A activity within birds of the same treatment group was more problematic. The relationship between the rates of ethoxyresorufin and caffeine metabolism was not linear, and the relative rankings of P450-1A activities as determined by the two assays did not coincide within birds of the same treatment group. This may have been

because the power of the CBT to discriminate differences in P450-1A activities was poor. In the CBT, a difference of 1686 pmole caffeine metabolized over 40 min was required before two samples were significantly different (α = 0.10). This value was calculated from the tri-labelled caffeine laboratory study using the following equation:

$$z\left(\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}\right) = \overline{y_1} - \overline{y_2}$$

The small sample size (7 each for induced and control WLCs) contributed to this poor discriminating power, but doubling the sample size in the CBT experiment would not have resulted in major improvements in power. If the standard deviations in the larger experiment were similar to the present data, metabolism of caffeine over 40 min would need to be at least 1150 pmole different in order for the samples to be significantly different (α = 0.10). Since the average mass of caffeine metabolized by TCDD-induced WLCs over 40 min was 3867 pmole greater than that of uninduced WLCs, the CBT had sufficient power to distinguish between the two groups. However, the CBT could not discriminate among enzyme activities of uninduced WLCs, among which the caffeine metabolism of individual WLCs varied a maximum of 565 pmole over 40 min.

The method discrimination power (MDP) for the EROD assay was determined in the different manner. Two avian microsomal samples were

repetitively analyzed, and a pooled standard deviation of the EROD activities was used to calculate the critical value using a t-statistic. For samples with an activity range of 4.1 to 8.4 pmole resorufin/min/mg protein, the EROD activities must be at least 2.0 pmole/min/mg protein different from each other before they can be designated as "different" (α = 0.05). *Ex vivo* hepatic EROD activity of an individual can be expected to have an assay-to-assay coefficient of variation of 25%. In the tri-labelled caffeine experiment with WLCs, EROD activities of TCDD-induced WLCs were roughly two orders of magnitude greater than the EROD activity of an uninduced WLC.

It is difficult to compare the relative discriminating power of the CBT and the EROD assay because they were not calculated in the same manner. The CBT MDP was calculated using the means and standard deviations of enzyme activities of individuals from two treatment groups, and therefore encompassed among-individual variation. In contrast, the EROD assay MDP was calculated using the means and standard deviations from repetitive analysis of individual samples, and therefore described variations in measurement only.

A statistically significant linear relationship was not apparent between $^{14}CO_2$ exhalation during the CBT and *ex vivo* hepatic EROD activity. This poor correlation may be due to insufficient statistical power as a result of small sample sizes and relatively great within- and among-individual variability. Alternatively, there may be biological reasons why the enzyme activities as measured by these two assays were not more closely associated.

In this experiment the TCDD-exposed chickens may have experienced hepatotoxicity, which could have interfered with their ability to metabolize caffeine during the CBT. Degeneration of liver tissue, as indicated by an infirm texture, was often observed in the TCDD-treated chickens. TCDD can cause hepatocellular necrosis, hepatic lipidosis, vacuolization in the cytoplasm of hepatocytes, and hepatocellular swelling (53, 54). HGs from a *p*-PCDH-contaminated area had hepatocytes which were swollen to the point of compressing sinusoids (54). The compression of hepatic sinusoids could impair hepatic blood flow. Caffeine metabolism in TCDD-treated birds during the CBT could be impaired by decreased delivery of caffeine to hepatocytes by the blood. TCDD-exposed birds may have induced P450-1A activity which is evident during the *ex vivo* EROD assay, but which cannot be as readily detected during the *in vivo* CBT due to reduced blood flow. This hypothesis could be tested during future research on the CBT. Histopathological examination of liver tissue should also be conducted during future work with the CBT.

It is also possible that the CBT may not be directly related to *ex vivo* hepatic EROD activity. It is unknown which cytochrome P450-1A isozymes metabolize caffeine or ethoxyresorufin in birds. Furthermore, the position of N-demethylation of caffeine varies among isozymes and species. P450-1A1 metabolizes ethoxyresorufin in most mammals, while caffeine is metabolized by P450-1A2 in humans and some rodents (47, 48). Since both isozymes are

normally induced following TCDD exposure in species which possess them, metabolism of either caffeine or ethoxyresorufin would be an appropriate substrate to use as a functional measure of TCDD exposure. Differential induction of the two isozymes could explain the lack of a strong correlation between the two assays.

There is no strong evidence for the existence of two cytochrome P450-1A isozymes in most bird species (50). In a study of cross reactivity with microsomes from six fish-eating bird species and polyclonal antibodies against purified rat isozymes for P450-1A1 and P450-1A2, strong reactivity was seen in all six species of birds with antibodies against rat P450-1A1. However, only one species in the study, the cormorant *Phalacrocorax carbo*, reacted with the antibodies to P450-1A2. This reactivity with P450-1A2 appeared to involve the same protein which reacted with P450-1A1, which indicated that the cormorant microsomes had a protein with shared epitopes to P450-1A1 and P450-1A2 (51).

A poor correlation between the results of the CBT and *ex vivo* hepatic EROD activity should not lead to the conclusion that the breath test is not an acceptable alternative to the EROD assay. Either assay is capable of discriminating between unexposed and induced birds. When indicators of P450-1A induction are used to assess exposures of individuals to *p*PCDH, differences of orders of magnitude are more meaningful than are small differences, both from a measurement error perspective and a biological

relevance perspective. Both assays are capable of detecting an order of magnitude difference in enzyme activity with confidence.

Literature references for hepatic EROD activity in Great Lakes birds are sparse, but some comparisons can be made with the results of this field study. Hepatic *ex vivo* EROD activities observed in the field during this experiment were an order of magnitude less than those observed in a 1982 study of newly hatched HGs from the Great Lakes (55), and an order of magnitude less than activities observed during the early 1980s in nestling and adult HGs in Newfoundland (22, 56). Literature references could not be found to relate absolute hepatic EROD activities to possible adverse effects. Comparisons of absolute EROD activities measured under different conditions in different laboratories must be made with caution. An interlaboratory comparison of the EROD assay showed that while all of the laboratories in the study could successfully differentiate between high-activity and low-activity samples, the absolute EROD activity detected in the samples varied widely among laboratories (57).

The different bird species tested in the field seemed to exhibit a different relative response in the two assays. RBGs had relatively great caffeine metabolism in the CBT but had relatively small hepatic EROD activity, while DCCs exhibited the opposite trend. This may be due to differences in the existence of the 1A1 and 1A2 isoforms of cytochrome P450 in the different species. Alternatively, it is possible that the dose of caffeine used was too

small for the DCCs. The tested DCCs weighed more than individuals of either gull species tested, averaging 1550 g. Since the injection solution contained only 0.6 mg caffeine, the DCC only received a 0.39 mg/kg dose instead of the desired 1 mg/kg dose.

Site-specific differences in cytochrome P450-1A activity in birds were not apparent in the field, although the studied areas were chosen to encompass both suspected "clean" and "dirty" areas with respect to *p*PCDH contamination (13). The RBG which exhibited the greatest rate of $^{14}CO_2$ exhalation during the CBT was from the confined disposal facility in Saginaw Bay, an area known to have greater concentrations of *p*PCDHs (12, 58). It would be tempting to speculate that this RBG exhibited a greater rate of caffeine metabolism because its cytochrome P450-1A had been induced following *p*PCDH exposure, except that the *ex vivo* hepatic EROD activity of that individual was small and did not support that hypothesis. In fact, the other RBG with a greater rate of $^{14}CO_2$ exhalation also exhibited relatively little *ex vivo* hepatic EROD activity. The bird with the greatest *ex vivo* hepatic EROD activity was a DCC from the North Channel, which was expected to be a relatively "clean" area with respect to *p*PCDH contamination. Unfortunately, DCC were not available to study at the confined disposal facility for comparison.

The P450-1A activities of all of the wild birds in this study were small. The greatest EROD activity of 33.7 pmole/min/mg protein was observed in a DCC. This EROD activity can be contrasted with the levels of EROD activity

observed in TCDD-treated chickens, which ranged from 214 to 543 pmole resorufin/min/mg protein. The greatest rate of caffeine metabolism observed in a wild bird was 1762 pmole over 40 min. This rate of caffeine metabolism can be contrasted with those of TCDD-induced chickens, which usually metabolized over 3000 pmole caffeine in 40 min. Therefore, in the field both assays indicated that the wild birds were relatively uninduced.

Invasiveness of the breath test

The breath test was designed to be a less invasive *in vivo* replacement for the standard *ex vivo* hepatic EROD assay. Test chickens occasionally died during the breath test, however, and this demonstrated that the breath test procedure is not entirely non-invasive. The deaths were apparently caused by stress-related heart attacks. Since a few birds died before caffeine was injected, it is likely that the stress was associated with handling rather than any toxicity of the caffeine injection solution. Most of the deaths occurred near the end of the study period, and the advancing age of the chickens may have made them more sensitive. Also, most of the chickens which died during the breath test had been exposed to TCDD, which may have weakened them.

The breath test seemed to be tolerated well by the wild bird species tested in the field. None of the seventeen gull or cormorant nestlings which were tested died during the breath test procedure. Most of the wild birds which were tested were remarkably calm during the procedure, and when they

were freed following the breath test they calmly started to walk away within seconds of release as if nothing had happened. However, in future research the post-release mortality of wild birds should be monitored following the CBT.

It is likely that different species of birds experience different degrees of handling stress, and may be more or less sensitive to the handling stress associated with the breath test. Therefore, further refinements and investigations of the breath test will be necessary before it can be used in threatened or endangered bird species.

CONCLUSIONS

1) Among individual variation in the rate of N-demethylation of tri-labelled ^{14}C-caffeine in control WLCs was greatest 120 min after injection. The coefficient of variation was 47.6%.

2) WLCs which had been pre-exposed to TCDD in order to induce P450-1A enzymes metabolized the tri-labelled ^{14}C-caffeine more rapidly than unexposed WLCs. The amount of ^{14}C in the breath was as much as an order of magnitude greater in TCDD-exposed WLCs than in controls.

3) Cumulative ^{14}C activity (Bq) measured at the beginning of the CBT was a better indicator of induction status than a single measure of ^{14}C activity (Bq) at one time several hours after the birds were injected with ^{14}C-labelled caffeine.

4) The CBT was successfully performed several times in one individual WLC in order to observe a time course of P450-1A induction. This demonstrated a key advantage of the breath test: the ability to reduce inter-individual variation when studying induction dynamics.

5) The CBT was performed on HG, RBG and DCC nestlings at several Great Lakes field locations.

6) Commercially available 1-methyl-^{14}C-caffeine was not a good substrate for the CBT using SSHCs. The CBT using this caffeine molecule was not effective at distinguishing between control and TCDD-exposed SSHCs.

7) Commercially available 3-methyl-^{14}C-caffeine was an excellent substrate for the CBT using SSHCs. The CBT using this caffeine molecule was very effective at distinguishing between control and TCDD-exposed SSHCs.

8) Both the CBT and the *ex vivo* hepatic EROD assay were effective at distinguishing between TCDD-exposed and unexposed chickens under laboratory conditions. However, there was not a strong linear relationship between the results of the CBT and the *ex vivo* hepatic EROD assay, either in the laboratory or in the field.

9) The CBT is a less invasive method to measure P450-1A activity than the *ex vivo* hepatic EROD assay, but further refinements may be necessary to reduce handling stress during the CBT.

APPENDICES

APPENDIX A

Table 1a. Background and maintenance information for WLCs

Strain: Dekalb XL

Sex: Female

Hatch Date: June 25, 1991

Purchase Date: October 29, 1991

Supplier: Herbrucks Poultry Ranch, Ionia MI

Food: Purina Layena Poultry Feed ad lib.

Water: Tap water

Light cycle: 16 hr light/8 hr dark

Coccidiostat: None administered during lifetime

Housing: Individually caged

Table 1b. Background and maintenance information for SSHCs

Sex: Female

Hatch Date: March 1992

Acquisition Date: September 22, 1993

Supplier: Poultry Science Research & Teaching Center, Michigan State University

Food: Purina Accu-Line Breeder Feed ad lib.

Water: Tap water

Light cycle: 9 hr light/15 hr dark

Coccidiostat: Continuously administered via food throughout lifetime

Housing: Individually caged

APPENDIX B

Table 2. Conditions used for the HPLC purification of 1,3,7-^{14}C-trimethylxanthine

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Solvent: 1:4 methanol:water containing 1% acetic acid

C18 reverse-phase column, 25 cm x 1/4" O.D.

Flow rate: 2 ml/min

Pressure: 4100 p.s.i.

Guard Column in place

uv detector: 272 nm

Detector sensitivity: %T

Detector lamp: deuterium

Linear strip chart recorder

Chart speed: 2 cm/min

APPENDIX C

Table 3. ^{14}C activity (Bq) exhaled per minute by WLCs during the CBT when 1,3,7-^{14}C-trimethylxanthine was used[1]

Time Interval (minutes)	Bird #8 Control	Bird #7[2] Control	Bird #5 Control	Bird #13 Control	Bird #9[3] Control	Bird #10 Control
10	0.15	0.18	0.23	0.16	0.16	0.15
20	0.37	0.41	0.64	0.39	0.24	0.46
30	0.64	0.90	0.80	0.51	0.45	0.64
40	0.77	1.27	1.00	0.78	0.55	0.77
60	1.09		1.05	0.82	0.59	0.66
80	1.46		1.43	1.11	0.59	0.98
100	2.22		1.78	1.24	0.72	1.07
120	2.51		1.91	1.33	0.83	0.99
140	2.45		2.09	1.40	1.06	1.16
160	2.62		2.38	1.33	1.52	1.28
180	2.45		2.23	1.41	1.53	1.24
200	1.53		2.20	1.57	1.53	1.24
220	1.53		2.05	1.26	1.58	1.15
240	1.53		2.40	1.10	1.83	1.20
300	1.25					
360	1.09					
420	2.12					

[1]All Bq shown have been corrected for background activity

[2]Bq shown for WLC #7 have been standardized to an injected ^{14}C activity of 5 μCi. Original values were multiplied by 1.111 since only 4.5 μCi was injected into the WLC. WLC #7 died 50 min post-injection.

[3]Bq shown for WLC #9 have been standardized to an injected ^{14}C activity of 5 μCi. Original values were multiplied by 1.075 since only 4.65 μCi was injected into the WLC.

Table 3 (con't).

Time Interval (minutes)	Bird #13 5.3 μg/kg[1]	Bird #13 1 week post-induction	Bird #9 5.3 μg/kg	Bird #10 5.3 μg/kg	Bird #12 17.8 μg/kg
-10[2]	0.00	0.08	0.01	0.00	0.01
10	0.72	0.96	0.45	0.73	0.50
20	1.99	2.65	1.01	1.75	1.64
30	3.55	3.21	1.41	2.31	2.52
40	4.47	3.14	1.77	2.85	3.16
60	5.66	3.50	2.14	4.24	4.92
80	5.05	3.57	2.50	5.08	4.72
100	4.73	3.59	2.31	5.58	4.22
120	4.39	3.33	2.28	4.66	3.76
140	4.58	3.42	2.24	3.80	4.13
160	4.89	3.12	2.19	3.35	4.64
180	5.17	3.27	2.25	4.25	3.67
200	5.55		2.26	4.54	3.53
220	6.18	2.86	2.35	4.86	3.91
240	5.78	3.06	1.94	5.24	4.45

[1]The listed dose refers to the quantity of TCDD per kg body weight which was injected i.p. into the WLC 72 hr and 48 hr prior to administering the breath test.

[2]The -10 min category refers to the pre-test value.

Table 3 (con't).

Time Interval (minutes)	Bird #18[1] 3 μg/kg	Bird #19 Control	Bird #21 1 μg/kg	Bird #20[2] 3 μg/kg
-10	0.01	0.00	0.01	0.00
10	2.01	0.09	1.52	0.96
20	6.55	0.33	4.21	2.54
30	6.37	0.61	5.19	3.58
40	5.25	0.76	6.12	4.39
60	4.51	0.80	6.25	
80	3.85	1.09	6.01	
100	3.09	1.03	6.22	
120	2.72	0.88	5.74	
140	2.33	0.93	5.54	
160		1.44	4.70	
180		1.45	4.36	
200		1.58	4.55	
220		1.49	3.86	
240		1.52	4.61	

[1]WLC #18 died between the 140 and 160 min time intervals.

[2]WLC #20 died 48 min post-injection. The Bq exhaled per min at time-point 48 min was 4.90.

APPENDIX D

Table 4. Mean, standard deviation and coefficient of variation for pmole caffeine metabolized per minute by control WLCs during the CBT using 1,3,7-^{14}C-trimethylxanthine

Time Interval (Minutes)	Mean	Standard Deviation	Coefficient of Variation (%)	n
10	6.61	1.62	24.5	7
20	16.86	5.18	30.7	7
30	27.06	6.53	24.1	7
40	34.96	9.46	27.0	7
60	34.67	8.40	24.2	6
80	46.15	13.31	28.8	6
100	55.85	23.01	41.2	6
120	58.55	27.90	47.6	6
140	63.13	25.57	40.5	6
160	73.29	24.32	33.2	6
180	71.47	20.56	28.8	6
200	66.83	13.12	19.6	6
220	62.84	12.96	20.6	6
240	66.43	19.68	29.6	6

APPENDIX E

Table 5. ^{14}C activity (Bq) exhaled per minute by SSHCs during the CBT[1]

Time Interval (minutes)	Bird #1 Control Tri-label[2]	Bird #4 Induced[3] Tri-label	Bird #3 Control 1-methyl[4]	Bird #2[5] Induced 1-methyl	Bird #5 Induced 1-methyl
10	0.95	0.94	0.16	0.38	0.38
20	1.95	2.54	0.38	0.99	1.52
30	2.20	3.00	0.54	1.58	2.37
40	2.98	3.11	0.71	1.54	2.78
60	1.96	3.73	0.79	1.63	3.05
80	2.02	4.80	0.98	1.47	2.69
100	2.18	5.34	1.14	1.29	2.52
120	2.28	5.98	2.10	1.16	1.99
140	2.40	5.73	2.25	1.01	1.67
160	2.34	4.97	2.36	0.85	1.18
180	2.42	4.52	2.42	0.78	1.13
200	2.26	4.28	2.16	0.69	1.12
220	2.06	4.08	2.16		1.05
240	2.12	3.84	2.08		0.94

[1]All Bq shown have been corrected for background activity.

[2]"Tri-label" refers to the caffeine substrate used in the test (1,3,7-^{14}C-trimethylxanthine).

[3]"Induced" SSHCs received i.p. injections of 3 μg TCDD/kg body weight 72 hr and 48 hr prior to the breath test.

[4]"1-methyl" refers to the caffeine substrate used in the test (1-methyl-^{14}C-caffeine).

[5]SSHC #2 died 192 min post-injection. The value shown for the 200 min time interval is the Bq collected from time 180 min to 192 min post-injection.

Table 5 (con't).

Time Interval (minutes)	Bird #6 Induced 1-methyl	Bird #11 Control 3-methyl[1]	Bird #8 Induced 3-methyl	Bird #9 Induced 3-methyl	Bird #10 Induced 3-methyl
10	0.34	0.10	0.60	0.81	1.87
20	0.89	0.28	1.78	2.20	5.79
30	1.28	0.38	2.82	2.74	6.14
40	1.48	0.40	4.30	3.29	6.05
60	1.53	0.54	4.84	3.29	6.44
80	1.56	0.63	5.02	4.53	6.57
100	1.35	0.73	5.92	6.40	5.10
120	1.29	0.88	5.92	6.22	4.05
140	1.22	1.01	5.27	7.26	4.19
160	1.05	1.14	4.51	7.97	4.19
180	1.19	1.15	3.58	8.06	4.46
200	1.26	1.20	2.72	4.10	4.34
220	1.28	1.11	2.56	4.76	3.86
240	1.32	1.16	2.36	5.42	3.50

[1]"3-methyl" refers to the caffeine substrate used in the test (3-methyl-^{14}C-caffeine).

APPENDIX F

Table 6. ^{14}C activity (Bq) exhaled per minute by HGs, RBGs and DCCs during the CBT when 1,3,7-^{14}C-trimethylxanthine was used[1]

Bird I.D.	Location	10 min	20 min	30 min	40 min
HG #1[2]	A.I.N.C.[3]	0.09	0.72	0.91	1.06
RB #2[4]	E.I.N.C.[5]	0.19	0.80	1.29	1.58
RB #3	E.I.N.C.	1.54	6.84	11.76	14.15
HG #4	E.I.N.C.	0.17	0.06	0.15	0.18
DCC #5[6]	E.I.N.C.	-0.02	0.12	0.22	0.30
DCC #6	E.I.N.C.	-0.02	0.06	0.21	0.31
RB #7	E.I.N.C.	0.12	0.42	0.63	1.22
HG #8	E.I.N.C.	-0.07	0.50	0.74	1.25
DCC #9	E.I.N.C.	-0.15	-0.02	0.34	0.29
HG #10	C.D.F.[7]	0.23	0.85	1.35	-0.11
HG #11	C.D.F.	0.92	2.23	3.16	3.93
RB #12	C.D.F.	7.97	22.17	30.86	44.61
RB #13	C.D.F.	0.88	2.48	3.59	4.93
HG #14	C.D.F.	1.01	3.01	3.71	4.03
HG #15	C.D.F.	0.09	0.83	2.12	2.00
HG #16	C.D.F.	0.46	1.41	2.48	2.86
HG #17	C.D.F.	0.18	0.48	0.88	1.49

[1]All bq shown have been corrected for background activity
[2]HG = Herring Gull
[3]A.I.N.C. = Anchor Island, North Channel of Lake Huron
[4]RB = Ring-billed Gull
[5]E.I.N.C. = Elm Island, North Channel of Lake Huron
[6]DCC = Double-crested Cormorant
[7]C.D.F. = Confined Disposal Facility, Saginaw Bay of Lake Huron

APPENDIX G

Table 7. Pmole caffeine metabolized during the first 40 min of the CBT when 1,3,7-^{14}C-trimethylxanthine was used and pmole resorufin/min/mg protein from the *ex vivo* hepatic EROD assay for five WLCs

Chicken #	pmole resorufin/min/mg protein	pmole caffeine (first 40 min)
10	543.0	3178.2
12	505.7	3253.3
19	3.0	744.0
20	277.9	4769.2
21	414.9	7083.0

APPENDIX H

Table 8. Pmole caffeine metabolized by individual HGs, RBGs and DCCs during a 40 min CBT when 1,3,7-^{14}C-trimethylxanthine was used, and pmole resorufin/min/mg protein from the *ex vivo* hepatic EROD assay

Bird I.D.	pmole resorufin/min/mg protein	pmole caffeine
HG #1[1]	<1.6	46.31
RB #2[2]	2.8	64.41
RB #3	6.7	571.9
HG #4	<1.6	9.34
DCC #5[3]	5.1	10.68
DCC #6	33.7	9.62
RB #7	2.1	39.89
HG #8	<1.6	41.42
DCC #9	2.9	10.68
HG #10	8.4	38.72
HG #11	6.4	170.8
RB #12	4.0	1762
RB #13	11.4	198.1
HG #14	13.3	196.3
HG #15	15.0	84.08
HG #16	9.5	120.1
HG #17	9.4	50.40

[1]HG = Herring Gull
[2]RB = Ring-billed Gull
[3]DCC = Double-crested Cormorant

APPENDIX I

Table 9. Pmole caffeine metabolized by SSHCs during the first 40 min of the CBT and pmole resorufin/min/mg protein from the *ex vivo* hepatic EROD assay

Bird I.D.	Caffeine substrate	pmole caffeine metabolized	pmole resorufin/min/mg protein
1	tri-labelled[1]	3363	3.15
4	tri-labelled	3987	n.d.[2]
2	1-methyl-^{14}C-caffeine	1865	253
3	1-methyl-^{14}C-caffeine	743.6	n.d.
5	1-methyl-^{14}C-caffeine	2933	214
6	1-methyl-^{14}C-caffeine	1660	387
8	3-methyl-^{14}C-caffeine	3158	356
9	3-methyl-^{14}C-caffeine	3002	420
10	3-methyl-^{14}C-caffeine	6593	381
11	3-methyl-^{14}C-caffeine	386.3	2.56

[1]Tri-labelled caffeine is 1,3,7-^{14}C-trimethylxanthine.

[2]n.d. = not determined

LIST OF REFERENCES

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1. Harris, H.J. 1988. Persistent toxic substances and birds and mammals in the Great Lakes. In M.S. Evans, eds., *Toxic contaminants and ecosystem health: a Great Lakes focus*. Volume 21 in *Advances in environmental science and technology*. Wiley, New York, pp. 557-569.

2. Gilbertson, M. 1988. Epidemics in birds and mammals caused by chemicals in the Great Lakes. In M.S. Evans, eds., *Toxic contaminants and ecosystem health: a Great Lakes focus*. Volume 21 in *Advances in environmental science and technology*. Wiley, New York, pp. 133-152.

3. Keith, J.A. 1966. Reproduction in a population of Herring Gulls (*Larus argentatus*) contaminated by DDT. In N.W. Moore, eds., *Pesticides in the environment and their effects on wildlife*. The Journal of Applied Ecology. Blackwell Scientific Publications, Oxford, pp. 57-70.

4. Gilbertson, M. 1974. Pollutants in breeding Herring Gulls in the lower Great Lakes. *Canadian Field-Naturalist* 88:273-280.

5. Ludwig, J.P. and C.S. Tomoff. 1966. Reproductive success and insecticide residues in Lake Michigan Herring Gulls. *Jack-Pine Warbler* 44:77-85.

6. Gilbertson, M. 1975. A Great Lakes tragedy. *Nature Canada* 4:22-25.

7. Gilbertson, M. and G.A. Fox. 1977. Pollutant-associated embryonic mortality of Great Lakes herring gulls. *Environ. Pollut.* 12:211-216.

8. Baumann, P.C. and D.M. Whittle. 1988. The status of selected organics in the Laurentian Great Lakes: an overview of DDT, PCBs, dioxins, furans, and aromatic hydrocarbons. *Aquat. Toxicol.* 11:241-257.

9. Ludwig, J.P. 1984. Decline, resurgence and population dynamics of Michigan and Great Lakes Double-crested Cormorants. *Jack-Pine Warbler* 62:91-102.

10. Price, I.M. and D.V. Weseloh. 1986. Increased numbers and productivity of Double-crested Cormorants, *Phalacrocorax auritus*, on Lake Ontario. *Canadian Field-Naturalist* 100:474-482.

11. Kubiak, T.J., H.J. Harris, L.M. Smith, T.R. Schwartz, D.L. Stalling, J.A. Trick, L. Sileo, D.E. Docherty and T.C. Erdman. 1989. Microcontaminants and reproductive impairment of the Forster's tern on Green Bay, Lake Michigan - 1983. *Arch. Environm. Contam. Toxicol.* 18:706-727.

12. Ludwig, J.P., H.J. Auman, H. Kurita, M.E. Ludwig, L.M. Campbell, J.P. Giesy, D.E. Tillitt, P. Jones, N. Yamashita, S. Tanabe and R. Tatsukawa. 1993. Caspian Tern reproduction in the Saginaw Bay ecosystem following a 100-year flood event. *J. Great Lakes Res.* 19:96-108.

13. Tillitt, D.E., G.T. Ankley, J.P. Giesy, J.P. Ludwig, H. Kurita-Matsuba, D.V. Weseloh, P.S. Ross, C.A. Bishop, L. Sileo, K.L. Stromborg, J. Larson and T.J. Kubiak. 1992. Polychlorinated biphenyl residues and egg mortality in Double-crested Cormorants from the Great Lakes. *Environ. Toxicol. Chem.* 11:1281-1288.

14. Yamashita, N., S. Tanabe, J.P. Ludwig, H. Kurita, M.E. Ludwig and R. Tatsukawa. 1993. Embryonic abnormalities and organochlorine contamination in Double-crested Cormorants (*Phalacrocorax auritus*) and Caspian Terns (*Hydroprogne caspia*) from the upper Great Lakes in 1988. *Environ. Pollut.* 79:163-173.

15. Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* 21:51-88.

16. Tillitt, D.E., G.T. Ankley and J.P. Giesy. 1989. Planar chlorinated hydrocarbons (PCHs) in colonial fish-eating waterbird eggs from the Great Lakes. *Marine Environ. Res.* 28:505-508.

17. Giesy, J.P., J.P. Ludwig and D.E. Tillitt. 1994. Deformities in birds of the Great Lakes region: Assigning causality. *Environ. Sci. Technol.* 28:128-135.

18. Ludwig, J.P., J.P. Giesy, C.L. Summer, W. Bowerman, R. Aulerich, S. Bursian, H.J. Auman, P.D. Jones, L.L. Williams, D.E. Tillitt and M. Gilbertson. 1993. A comparison of water quality criteria for the Great Lakes based on human and wildlife health. *J. Great Lakes Res.* 19:789-807.

19. Poland, A. and J.C. Knutson. 1982. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Ann. Rev. Pharmacol. Toxicol.* 22:517-554.

20. Landers, J.P. and N.J. Bunce. 1991. The *Ah* receptor and the mechanism of dioxin toxicity. *Biochem. J.* 276:273-287.

21. Denison, M.S., P.A. Bank and E.F. Yao. 1992. DNA sequence-specific binding of transformed Ah receptor to a dioxin responsive enhancer: Looks aren't everything. *Chemosphere* 25:33-36.

22. Peakall, D.B., R.J. Norstrom, A.D. Rahimtula and R.D. Butler. 1986. Characterization of mixed-function oxidase systems of the nestling herring gull and its implications for bioeffects monitoring. *Environ. Toxicol. Chem.* 5:379-385.

23. Colborn, T. and C. Clement. 1992. *Chemically-induced alterations in sexual and functional development: The wildlife/human connection*. Vol. XXI. eds. Princeton Scientific Publishing, Princeton, NJ.

24. Spear, P.A., D.H. Bourbonnais, D.B. Peakall and T.W. Moon. 1989. Dove reproduction and retinoid (vitamin A) dynamics in adult females and their eggs following exposure to 3,3',4,4'-tetrachlorobiphenyl. *Can. J. Zool.* 67:908-913.

25. Jefferies, D.J. and J.L.F. Parslow. 1976. Thyroid changes in PCB-dosed guillemots and their indication of one of the mechanisms of action of these materials. *Environ. Pollut.* 10:293-311.

26. Andersson, L., E. Nikolaidis, B. Brunström, Å. Bergman and L. Dencker. 1991. Effects of polychlorinated biphenyls with *Ah* receptor affinity on lymphoid development in the thymus and the bursa of Fabricus of chick embryos *in Ovo* and in mouse thymus anlagen *in Vitro*. *Toxicol. Appl. Pharmacol.* 107:183-188.

27. Fox, G.A., A.P. Gilman, D.B. Peakall and F.W. Anderka. 1978. Behavioral abnormalities of nesting Lake Ontario Herring Gulls. *J. Wildl. Manage.* 42(3):477-483.

28. Fry, D.M., C.K. Toone, S.M. Speich and R.J. Peard. 1987. Sex ratio skew and breeding patterns of gulls: Demographic and toxicological considerations. *Studies in Avian Biology* 10:26-43.

29. Fox, G.A., S.W. Kennedy, R.J. Norstrom and D.C. Wigfield. 1988. Porphyria in herring gulls: a biochemical response to chemical contamination of Great Lakes food chains. *Environ. Toxicol. Chem.* 7:831-839.

30. Weber, L.W.D., M. Lebofsky, B.U. Stahl, J.R. Gorski, G. Muzi and K. Rozman. 1991. Reduced activities of key enzymes of gluconeogenesis as possible cause of acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats. *Toxicology* 66:133-144.

31. Tumasonis, C.F., B. Bush and F.D. Baker. 1973. PCB levels in egg yolks associated with embryonic mortality and deformity of hatched chicks. *Arch. Environm. Contam. Toxicol.* 1:312-324.

32. Brunström, B. 1989. Toxicity of coplanar polychlorinated biphenyls in avian embryos. *Chemosphere* 19:765-768.

33. Jones, P.D., G.T. Ankley, D.A. Best, R. Crawford, N. DeGalan, J.P. Giesy, T.J. Kubiak, J.P. Ludwig, J.L. Newsted, D.E. Tillitt and D.A. Verbrugge. 1993. Biomagnification of bioassay derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents. *Chemosphere* 26:1203-1212.

34. Tillitt, D.E., G.T. Ankley, D.A. Verbrugge, J.P. Giesy, J.P. Ludwig and T.J. Kubiak. 1991. H4IIE rat hepatoma cell bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents in colonial fish-eating waterbird eggs from the Great Lakes. *Arch. Environm. Contam. Toxicol.* 21:91-101.

35. Walker, C.H. 1990. Persistent pollutants in fish-eating sea birds - bioaccumulation, metabolism and effects. *Aquat. Toxicol.* 17:293-324.

36. Fox, G.A., M. Gilbertson, A.P. Gilman and T.J. Kubiak. 1991. A rationale for the use of colonial fish-eating birds to monitor the presence of developmental toxicants in Great Lakes fish. *J. Great Lakes Res.* 17:151-152.

37. Poland, A. and E. Glover. 1973. Chlorinated dibenzo-*p*-dioxins: potent inducers of δ-aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase. II. A study of the structure-activity relationship. *Mol. Pharmacol.* 9:736-747.

38. Brunström, B. and L. Andersson. 1988. Toxicity and 7-ethoxyresorufin O-deethylase-inducing potency of coplanar polychlorinated biphenyls (PCBs) in chick embryos. *Arch. Toxicol.* 62:263-266.

39. Bosveld, B.A.T.C., M. van den Berg and R.M.C. Theelen. 1992. Assessment of the EROD inducing potency of eleven 2,3,7,8-substituted PCDD/Fs and three coplanar PCBs in the chick embryo. *Chemosphere* 25:911-916.

40. Bellward, G.D., R.J. Norstrom, P.E. Whitehead, J.E. Elliott, S.M. Bandiera, C. Dworschak, T. Chang, S. Forbes, B. Cadario, L.E. Hart and K.M. Cheng. 1990. Comparison of polychlorinated dibenzodioxin levels with hepatic mixed-function oxidase induction in Great Blue Herons. *Toxicol. Environ. Health* 30:33-52.

41. Lambert, G.H., D.A. Schoeller, A.N. Kotake, C. Flores and D. Hay. 1986. The effect of age, gender, and sexual maturation on the caffeine breath test. Dev. Pharmacol. Ther. 9:375-388.

42. Wietholtz, H., M. Voegelin, M.J. Arnaud, J. Bircher and R. Preisig. 1981. Assessment of the cytochrome P-448 dependent liver enzyme system by a caffeine breath test. *Eur. J. Clin. Pharmacol.* 21:53-59.

43. Aldridge, A., W.D. Parsons and A.H. Neims. 1977. Stimulation of caffeine metabolism in the rat by 3-methylcholanthrene. *Life Sci.* 21:967-974.

44. Krüger, N., B. Neubert, H. Helge and D. Neubert. 1990. Induction of caffeine-demethylations by 2,3,7,8-TCDD in marmoset monkeys measured with a $^{14}CO_2$ breath-test. *Chemosphere* 20:1173-1176.

45. Kotake, A.N., D.A. Schoeller, G.H. Lambert, A.L. Baker, D.D. Schaffer and H. Josephs. 1982. The caffeine CO_2 breath test: Dose response and route of *N*-demethylation in smokers and nonsmokers. *Clin. Pharmacol. Ther.* 32:261-269.

46. Arnaud, M.J. 1985. Comparative metabolic disposition of [1-Me^{14}C]caffeine in rats, mice, and chinese hamsters. *Drug Metab. Disp.* 13:471-478.

47. Berthou, F., B. Guillois, C. Riche, Y. Dreano, E. Jacqz-Aigrain and P.H. Beaune. 1992. Interspecies variations in caffeine metabolism related to cytochrome P4501A enzymes. *Xenobiotica* 22:671-680.

48. Butler, M.A., M. Iwasake, F.P. Guengerich and F.F. Kadlubar. 1989. Human cytochrome P-450_{PA}(P-450IA2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci. USA* 86:7696-7700.

49. Berthou, F., Jean-P. Flinois, D. Ratanasavanh, P. Beaune, C. Riche and A. Guillouzo. 1991. Evidence for the involvement of several cytochromes P-450 in the first steps of caffeine metabolism by human liver microsomes. *Drug Metab. Disp.* 19:561-567.

50. Ronis, M.J.J. and C.H. Walker. 1989. The microsomal monooxygenases of birds. In E. Hodgson, J.R. Bend and R.M. Philpot, eds., *Reviews in biochemical toxicology*, 10. Elsevier, pp. 301-384.

51. Ronis, M.J.J., T. Hansson, J. Borlakoglu and C.H. Walker. 1989. Cytochromes P-450 of sea birds: cross-reactivity studies with purified rat cytochromes. *Xenobiotica* 19:1167-1173.

52. De Vito, M.J., W.E. Maier, J.J. Diliberto and L.S. Birnbaum. 1993. Comparative ability of various PCBs, PCDFs, and TCDD to induce cytochrome P450 1A1 and 1A2 activity following 4 weeks of treatment. *Fundam. Appl. Toxicol.* 20:125-130.

53. van der Weiden, M.E.J. 1993. Cytochrome P450-1A induction in carp as a biological indicator for the aquatic contamination of chlorinated polyaromatics. Ph.D. thesis. Utrecht University.

54. Gilbertson, M. 1983. Etiology of chick edema disease in herring gulls in the lower Great Lakes. *Chemosphere* 12:357-370.

55. Boersma, D.C., J.A. Ellenton and A. Yagminas. 1986. Investigation of the hepatic mixed-function oxidase system in Herring Gull embryos in relation to environmental contaminants. *Environ. Toxicol. Chem.* 5:309-318.

56. Peakall, D.B., D.A. Jeffrey and D. Boersma. 1987. Mixed-function oxidase activity in seabirds and its relationship to oil pollution. *Comp. Biochem. Physiol.* 88C:151-154.

57. Munkittrick, K.R., M.R. van den Heuvel, D.A. Metner, W.L. Lockhart and J.J. Stegeman. 1993. Interlaboratory comparison and optimization of hepatic microsomal ethoxyresorufin *o*-deethylase activity in white sucker (*Catostomus commersoni*) exposed to bleached kraft pulp mill effluent. *Environ. Toxicol. Chem.* 12:1273-1282.

58. Mora, M.A., H.J. Auman, J.P. Ludwig, J.P. Giesy, D.A. Verbrugge and M.E. Ludwig. 1993. Polychlorinated biphenyls and chlorinated insecticides in plasma of Caspian Terns: Relationships with age, productivity, and colony site tenacity in the Great Lakes. *Arch. Environm. Contam. Toxicol.* 24:320-331.