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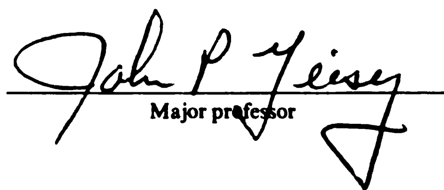

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CHARACTERIZATION OF CYTOCHROME P450-1A (CYP1A) ENZYMES IN THREE AVIAN SPECIES AND THE DEVELOPMENT OF A CAFFEINE BREATH TEST TO MEASURE CYP1A ACTIVITY *IN VIVO*

By

Lori Ann Feyk

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ABSTRACT

CHARACTERIZATION OF CYTOCHROME P450-1A (CYP1A) ENZYMES IN THREE AVIAN SPECIES AND THE DEVELOPMENT OF A CAFFEINE BREATH TEST TO MEASURE CYP1A ACTIVITY *IN VIVO*

By

Lori Ann Feyk

Cytochrome P450-1A (CYP1A) activity is often used as a biomarker of exposure of wildlife to polyhalogenated diaromatic hydrocarbons (PHDHs), and is usually measured *ex vivo* in liver tissue. A caffeine breath test with radiolabelled substrate (^{14}C-CBT) has been developed to measure *in vivo* avian CYP1A activity. Research goals were to characterize avian CYP1A enzymes, develop stable isotope methods (^{13}C-CBT), determine dose-response relationships, and assess the relative utility of the CBT and *ex vivo* ethoxyresorufin-*o*-deethylase (EROD) assay.

^{13}C-CBT methods were developed with twenty chickens (*Gallus domesticus*). Chickens received three intraperitoneal injections of 0, 1, 5, or 50 μg 3,3',4,4',5-pentachlorobiphenyl (PCB 126)/kg body weight, and caffeine N-demethylation (CNDM) was quantified by measurement of $^{13}CO_2/^{12}CO_2$ in expired breath. The ^{13}C-CBT was not as sensitive or specific as the EROD assay. Constitutive CNDM of great inter-individual variability was observed, and the magnitude of induction was greater for EROD activity than for CNDM (approximately 1000- and 2-fold respectively).

Thirty Common Tern (*Sterna hirundu*) hatchlings were fed fish containing

PCB 126 and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) at 0, 100 and 1000 pg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents/g fish for 21 d. The ^{14}C-CBT was performed twice during development in half the birds. Week 1 and 2 CNDM and day 21 EROD activity were elevated in birds that received the greatest PCB dose. There was lesser constitutive and greater induction of EROD activity than CNDM. The ^{14}C-CBT was less invasive than the EROD assay. No alterations of survival, growth or morphological development occurred in CBT subjects.

Catalytic and immunochemical properties of avian hepatic microsomal CYP1A enzymes were characterized following treatment with β-naphthoflavone (BNF) and/or isosafrole. CYP1A of the Herring Gull (*Larus argentatus*) and Double-crested Cormorant (*Phalacrocorax auritus*) but not the chicken was induced by isosafrole. Patterns of alkoxyresorufin-*o*-dealkylase activities varied among avian species. In all three species, BNF induced a protein immunoreactive with monoclonal antibody to CYP1A1 from the marine fish *Stenotomus chrysops* (scup), but not with polyclonal antibodies to mammalian CYP1A2.

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LIST OF ABBREVIATIONS AND ACRONYMS

3-MC	3-methylcholanthrene
ABP	4-aminobiphenyl
AHH	aromatic hydrocarbon hydroxylase
AhR	aromatic hydrocarbon receptor
alk-ROD	alkoxyresorufin-*O*-dealkylase
APE	atom percent excess
BNF	β-naphthoflavone
BR	benzyloxyresorufin
BROD	benzyloxyresorufin-*O*-debenzylase
BSA	bovine serum albumin
BW	body weight (kilograms)
CBT	caffeine breath test
^{13}C-CBT	caffeine breath test with stable isotopically-labelled substrate
^{14}C-CBT	caffeine breath test with radiolabelled substrate
CNDM	caffeine N-demethylation
CSPD	substituted 1,2-dioxetane-phosophate
CUMPCD	cumulative percent of caffeine dose
CV	coefficient of variation
CYP	cytochrome P450
CYP1A	cytochrome P450-1A
DCC	Double-crested Cormorant (*Phalacrocorax auritus*)
DDT	1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
DTT	dithiothrietol
ECD	electron capture detection
EDTA	(ethylenedinitrilo)tetraacetic acid
ER	ethoxyresorufin
EROD	ethoxyresorufin-*O*-deethylase
GC	gas chromatograph
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HG	Herring Gull (*Larus argentatus*)
HH	halogenated hydrocarbon
i.p.	intraperitoneal
i.v.	intravenous

IgG	immunoglobulin G
IRMS	isotope ratio mass spectroscopy
MAb	monoclonal antibody
MFO	mixed-function oxygenase
MR	methoxyresorufin
MROD	methoxyresorufin-*O*-demethylase
MS	mass spectroscopy
MV	minute volume (liters/minute)
MW	molecular weight
NADPH	β-nicotinamide adenine dinucleotide phosphate
OC	organochlorine
PADM	percent administered dose metabolized
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCB 30	2,4,6-trichlorobiphenyl
PCB 77	3,3',4,4'-tetrachlorobiphenyl
PCB 126	3,3',4,4',5-pentachlorobiphenyl
PCB 153	2,2',4,4',5,5'-hexachlorobiphenyl
PCD	percent caffeine dose metabolized per minute
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
PDB	calcium carbonate standard (limestone fossil)
PEG	polyclonal antibody against mouse CYP1A1
PHDH	polyhalogenated diaromatic hydrocarbon
PR	pentoxyresorufin
PROD	pentoxyresorufin-*O*-depentylase
RITOX	Research Institute of Toxicology
RYE	polyclonal antibody against mouse CYP1A2
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalency factor
TEQ	2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent
Tris	Tris(hydroxymethyl)aminomethane
WHOI	Woods Hole Oceanographic Institution

INTRODUCTION

Problem Statement

Fish-eating birds on the Great Lakes have experienced reproductive impairment and reduced fitness as a result of exposure to environmental contaminants in their diet. Polyhalogenated diaromatic hydrocarbons (PHDHs) such as polychlorinated dioxins, furans and biphenyls (PCBs) continue to exert subtle toxic effects on birds in contaminated areas, such as immunosuppression and an increased incidence of congenital deformities (Giesy et al., 1994).

Biomonitoring techniques are used to assess the health status and trends of Great Lakes fish-eating bird populations, especially relative to exposure to these problematic PHDH compounds (Gilbertson, 1988; Fox et al., 1991). Biomonitoring programs often focus on biochemical endpoints of PHDH exposure, because biochemical alterations often occur prior to population-level effects and may serve as early warning indicators. The most commonly used biochemical endpoint to assess exposure of organisms to PHDHs is the induction of cytochrome P450-1A (CYP1A) activity, which is a sensitive and well characterized response (Brunström and Andersson, 1988; Safe, 1990; Bosveld et al., 1992). CYP1A activity is usually measured in liver tissue from

the test organism, from which microsomes are isolated and the rate of catalysis of one or more CYP1A substrates such as ethoxyresorufin-*O*-deethylase (EROD) are assessed (Bellward et al., 1990). While in some cases liver biopsies can be performed, test organisms are usually killed to obtain liver samples for this analysis.

A non-lethal method of measurement of CYP1A activity such as a caffeine breath test (CBT) would be a useful tool for avian toxicologists and wildlife managers. The use of a breath test would obviate the need to kill birds to determine their CYP1A induction status. Also, the existence of less invasive methods could make possible the monitoring of CYP1A activity in threatened or endangered avian species from PHDH-contaminated areas of the Great Lakes. A breath test would also be useful because it would allow researchers to make multiple measurements of CYP1A activity over time in the same individual. This would enable the study of CYP1A induction kinetics in response to varying PHDH exposure regimes, endogenous factors or environmental conditions.

Avian cytochrome P450 enzymes are not as well characterized as are their mammalian and piscine counterparts. Knowledge of avian P450 enzymes, including their activities, inducibilities and relatedness to mammalian and piscine forms, would provide insight into many aspects of avian toxicology. For example, differences in avian species sensitivities to PHDHs might be due to differences in metabolic capabilities. Also, the substrates that are most effective at detection of PHDH-related induction in enzyme activity assays

might vary among avian species. Before the CBT can be accepted as an alternative to the more invasive EROD assay, it must be determined whether caffeine is metabolized by a PHDH-inducible enzyme in birds.

Background Information

Great Lakes fish-eating birds: A historical perspective

Fish-eating birds on the Laurentian Great Lakes of North America have experienced fluctuations in the size of populations in response to a variety of factors. These factors include water level fluctuations, habitat changes, human disturbance, predation, a changing fishery prey base (Harris, 1988), and exposure to synthetic halogenated hydrocarbons (HHs) (Gilbertson, 1988). 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 (Keith, 1966; Ludwig and Tomoff, 1966; Gilbertson, 1974, 1975; Gilbertson and Fox, 1977). Since then concentrations of HHs in the Great Lakes have decreased (Baumann and Whittle, 1988), 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 (Ludwig, 1984; Price and Weseloh, 1986). 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 (Kubiak et al., 1989; Tillitt et al., 1992; Ludwig et al., 1993a; Yamashita et al., 1993). Some of the currently observed adverse outcomes observed in

populations of Great Lakes colonial fish-eating water birds are attributed to PHDHs (Tillitt et al., 1989, 1992; Ludwig et al., 1993b; Giesy et al., 1994).

Polyhalogenated diaromatic hydrocarbons (PHDHs)

A number of molecules found in the environment have similar structural features and can be classified as PHDHs (Safe, 1990). Structural features common to these molecules are two aromatic rings and substitution by one or more halogen atoms. Those PHDHs that can assume a planar configuration are the most biologically potent, and include some PCB congeners, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). PHDHs are persistent in the environment and bioaccumulate in aquatic food chains due to their lipophilic nature and the fact that they are poorly metabolized (Walker, 1990).

Various PHDH compounds exert their effects on organisms through a common mechanism (Poland and Knutson, 1982). In the cell cytosol, PHDHs bind to the aromatic hydrocarbon receptor (AhR) (Landers and Bunce, 1991). The PHDH/AhR complex then dissociates from heat shock protein 90 and binds to the AhR nuclear translocator. The complex binds to specific DNA sequences called dioxin responsive enhancers (Denison et al., 1992). This interaction with DNA results in an alteration of gene expression, which mediates the responses of the organism to PHDHs (Safe, 1990; Landers and Bunce, 1991). Transcription of the drug-metabolizing CYP1A1 and CYP1A2 enzymes is enhanced (Safe, 1990). An increase in these enzymes or in their

activity is often used as an indication of exposure to PHDHs (Peakall et al., 1986).

PHDH-stimulated alterations in gene expression can lead to a variety of harmful metabolic changes which include alterations in vitamin metabolism and endocrine function (Colborn and Clement, 1992). For example, exposure to PHDHs can lead to altered metabolism of retinoids (Spear et al., 1989), thyroid involution (Jefferies and Parslow, 1976), immunosuppression (Andersson et al., 1991) and behavioral changes (Fox et al., 1978; Fry et al., 1987). Other metabolic imbalances which can result from PHDH exposure include porphyria (Fox et al., 1988) and wasting syndrome (Weber et al., 1991). *In ovo* exposure to PHDHs can produce teratogenic effects or cause embryo lethality in birds (Tumasonis et al., 1973; Brunström, 1989).

PHDHs are present in aquatic environments as complex mixtures. These mixtures change as a function of space and time (Giesy et al., 1994), due to processes such as environmental weathering and metabolism by organisms. The relative concentrations of the various PHDH congeners in aquatic organisms are different from one trophic level to another (Jones et al., 1993). 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 (Giesy et al., 1994). 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.

While it is often possible to determine the concentration of each PHDH component of a complex mixture in an environmental compartment, this analytical approach has several disadvantages. Such chemical analyses can be very time consuming and expensive, chemical standards are not always readily available, and the biological effects of the mixture are nearly impossible to predict since PHDH congeners have been shown to exhibit synergism, additivity or antagonism of toxic effects (Tillitt et al., 1991). An alternative approach to the determination of toxic potency of a complex PHDH mixture in an environmental compartment is to measure the response of a biological system to the mixture as extracted from the compartment and then express the potency in terms of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEQs) (Safe, 1990; Tillitt et al., 1991). This approach utilizes the fact that the elements of the mixture are acting through the same mechanism (i.e., interaction with the AhR), and provides a measurement of an integrated biological response to the mixture which accounts for toxic interactions between congeners and bioavailability. The *in vivo* induction of CYP1A is also mediated through the AhR. Therefore, the *ex vivo* measurement of CYP1A activity in hepatic microsomes from livers of environmentally-exposed birds also provides information about the impact of many PHDH congeners which are present in the environment as a complex mixture but which act on the individual through a common mechanism (Tillitt et al., 1991).

The caffeine breath test (CBT)

A breath test has been developed to measure CYP1A activity in mammals. In that system labelled caffeine was used as a substrate (Aldridge et al., 1977; Wietholtz et al., 1981; Krüger et al., 1990; Tilson et al., 1990). The caffeine breath test has successfully detected the induction of CYP1A in human smokers (Kotake et al., 1982) and in marmoset monkeys dosed with PHDHs (Krüger et al., 1990).

In previous experiments, an avian caffeine breath test was developed for White Leghorn chickens (Feyk, 1994; Feyk et al., 1995; Feyk and Giesy, 1996). Chickens treated with TCDD N-demethylated caffeine up to ten times more rapidly than did untreated chickens when radiolabelled caffeine was used as the CBT substrate. The CBT was successfully performed several times in one individual chicken in order to observe a time course of CYP1A induction. Both tri-labelled caffeine (1,3,7-^{14}C-trimethylxanthine) and 3-methyl-^{14}C-caffeine were effective CBT substrates, while 1-methyl-^{14}C-caffeine was not. The CBT was also performed on several fish-eating bird species during a pilot study.

The basic principle on which the CBT is based is that CYP1A 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 expired breath as labelled CO_2 (Kotake et al., 1982). 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 CYP1A 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 (Figure 1).

The relative advantages of ^{14}C and ^{13}C in the CBT

Both the radioactive (^{14}C) and stable (^{13}C) isotope labelling strategies for caffeine offer unique advantages. There are situations in which each of the isotope labels is a more appropriate substrate in the CBT. Therefore, it is desirable to develop methods for both types of isotope label.

The major advantage of radioactive ^{14}C labelling is that data analysis is relatively quick, inexpensive and easy. The amount of trapped $^{14}CO_2$ from exhaled breath can be determined simply by adding an aliquant of trapping solution to scintillation cocktail and measuring the disintegrations per minute (DPM) of the solution using a scintillation counter. Scintillation counters are relatively common laboratory instruments which are easy to operate and require little analyst training, and since samples are analyzed by the counter in an automated fashion little analyst time is involved. The disadvantage of ^{14}C labelling is its radioactive nature. Radioactivity is hazardous to human health, so precautions must be taken by analysts to avoid internal exposure to the radioactive isotope. The use of radioactivity is tightly regulated. It is particularly difficult to obtain permission to perform research with radioactive tracers in a field setting. For this reason, ^{14}C labelling is more appropriate for laboratory CBT studies in facilities designed for this purpose.

Stable isotope (^{13}C) labelling is more appropriate for field CBT studies, including environmental monitoring. Since ^{13}C is a non-radioactive, non-hazardous isotope, special permits are not required for its use in any setting. Potential hazards to analyst health, health of the test subject, and to cleanliness of the test environment are eliminated. The major disadvantage of this type of label is that data analysis is more difficult. A mass spectrometer must be used to analyze the relative ratio of ^{13}C to ^{12}C in trapped CO_2 samples. This type of analysis is more difficult, time consuming and expensive than scintillation counting. Fortunately, since breath tests using ^{13}C-labelled substrates are common in human medicine there are contract laboratories available to perform $^{13}CO_2/^{12}CO_2$ ratio analyses for a reasonable charge.

Enzymes involved in caffeine and ethoxyresorufin metabolism

Several lines of evidence suggest that the 3-N-demethylation of caffeine is specifically mediated by CYP1A2 in most mammalian species. In humans this is the predominant metabolic pathway for caffeine (Berthou et al., 1991). In mammalian cell lines genetically engineered for the expression of single P450 isozymes, only cell lines expressing a CYP1A2 isozyme were able to demethylate caffeine to a significant extent (Fuhr et al., 1992). Phenacetin, a specific substrate for CYP1A2, competitively inhibited caffeine 3-N-demethylation in human liver microsomes (Berthou et al., 1991). The N-oxidation of 4-aminobiphenyl (ABP) is also specifically mediated by CYP1A2. ABP N-oxidation and caffeine 3-N-demethylation were strongly correlated in

human liver microsomes, and both reactions were inhibited by a CYP1A2-specific antibody (Butler et al., 1989b). Furthermore, caffeine 3-N-demethylation was strongly correlated with immunochemically determined CYP1A2 content in human liver microsomes (Butler et al., 1989b; Berthou et al., 1991).

A thorough review of the literature failed to disclose any studies that characterize the metabolic pathways of caffeine in birds. Caffeine that was labelled at all three methyl positions was used in our original investigations of the avian CBT in order to minimize the likelihood of overlooking a CYP1A-mediated response. Further studies with mono-labelled caffeine molecules have shown that the 3-N-demethylation of caffeine was induced in chickens following exposure to TCDD. This indicates that caffeine 3-N-demethylation may be mediated by a CYP1A enzyme in chickens as well as in humans. It is possible that there are differences in the location of maximum cytochrome CYP1A-mediated N-demethylation of the caffeine molecule among species of birds. This is known to be the case with different species of rodents (Arnaud, 1985).

Ethoxyresorufin is a highly selective substrate for cytochrome CYP1A1 in the rat (Burke et al., 1985). Other species exhibit EROD activity that is mediated by a cytochrome P450 isozyme which cross-reacts with polyclonal antibodies against rat CYP1A1, including fish such as scup (*Stenotomus chrysops*) (Stegeman and Kloepper-Sams, 1987). Fish-eating bird species were shown to possess a P450 isozyme which cross-reacted with polyclonal

antibodies against rat CYP1A1, and the degree of expression of the CYP1A1 orthologue was related to concentrations of PHDHs in the samples. Unfortunately, EROD activity was not measured in those birds (Ronis et al., 1989a). It is generally assumed that the CYP1A1 orthologue is responsible for EROD activity in birds.

In vivo caffeine N-demethylation may not be directly related to *ex vivo* hepatic EROD activity. CYP1A1 metabolizes ethoxyresorufin in most mammals, while caffeine is metabolized by CYP1A2 in humans and some rodents (Butler et al., 1989b; Berthou et al., 1992). Since both isozymes are normally induced following TCDD exposure in species which possess them, metabolism of either caffeine or ethoxyresorufin may be an appropriate substrate to use as a functional measure of TCDD exposure.

Avian CYP1A isozymes are not as well characterized as their mammalian counterparts. In some bird species, the existence of two cytochrome CYP1A isozymes is not evident (Ronis and Walker, 1989). In a study of cross reactivity with microsomes from six fish-eating bird species and polyclonal antibodies against purified rat isozymes for CYP1A1 and CYP1A2, strong reactivity was seen in all six species of birds with antibodies against rat CYP1A1. However, only one species in the study, the Great Cormorant (*Phalacrocorax carbo*), reacted with the antibodies to CYP1A2. This reactivity with CYP1A2 appeared to involve the same protein which reacted with CYP1A1, which indicated that the cormorant microsomes had a protein with shared epitopes to CYP1A1 and CYP1A2 (Ronis et al., 1989b). However, the

induction of two distinct CYP1A-like isozymes has been observed in TCDD-treated and β-naphthoflavone-treated White Leghorn chickens (Nakai et al., 1992; Kupfer et al., 1994). In these studies one of the induced proteins appeared to be a CYP1A1 analog, but the properties of other CYP1A proteins varied from study to study and their relationship to mammalian CYP1A2 was less clear.

Research Goals

The three chapters that make up this dissertation address the following research goals:

1. **Development of a stable isotope (^{13}C) CBT method for use with avian species.** Stable isotope methodology is preferable to the use of radioisotope methodology for field applications of the CBT. In Chapter 1, the development of stable isotope CBT methodology (the ^{13}C-CBT) is described. The experiment was a laboratory study with White Leghorn chickens. Methods of breath collection for the ^{13}C-CBT were altered significantly from the breath collection protocol for the ^{14}C-CBT. The instrumentation and calculations used to detect and quantitate labelled CO_2 in the breath were also quite different for the ^{13}C-CBT than those used for the ^{14}C-CBT. The comparative utility of the ^{14}C-CBT and ^{13}C-CBT methods are critiqued and discussed.

2. **Determination of dose-response relationships between PHDH exposure and rates of caffeine N-demethylation.** In previous studies of the avian CBT, chickens were treated with doses of TCDD that were known to

cause a great degree of CYP1A induction. The dose-response relationship between caffeine N-demethylation and exposure to environmentally realistic concentrations of PHDHs had not yet been examined. Therefore, it had not been determined whether the CBT was a sensitive method with which to measure intermediate levels of CYP1A induction.

In Chapter 1, the ^{13}C-CBT was performed in chickens that had been treated with intraperitoneal (i.p.) injections of one of three concentrations of 3,3',4,4',5-pentachlorobiphenyl (PCB 126), or with the corn oil vehicle. The range of PCB 126 administered encompassed a concentration thought to be minimally inductive as well as a concentration expected to produce maximal induction. In Chapter 2, a range of concentrations of PCB congeners 126 and 153 (2,2',4,4',5,5'-hexachlorobiphenyl) were administered to Common Tern hatchlings via their fish diet, and the ^{14}C-CBT was performed twice in each tern over the course of development. The smaller concentrations of PCBs administered in that experiment were also environmentally realistic. Dose-response relationships are presented and discussed for both CBT methods.

3. Assessment of the CBT as a less invasive substitute for the EROD assay as a method of measurement of CYP1A activity. The relative sensitivity and specificity of the CBT and EROD assays for the detection of PHDH-mediated enzyme induction were examined in Chapters 1 and 2. The degree of correlation of substrate metabolism during the two assays is presented and discussed. In addition, the degree of invasiveness of the CBT procedure was assessed in the Common Tern experiment presented in Chapter 2. Survival,

growth and morphological development were compared in nestlings that were fed matched diets, when some nestlings were subjected to the ^{13}C-CBT procedure and others were not.

4. Immunochemical and catalytic characterization of avian CYP1A enzymes following treatment with specific inducers and inhibitors. It is important to characterize avian CYP1A-type enzymes, because CYP1A induction is often used as a biomarker of exposure and effect of PHDHs on birds in the environment. Researchers need to be aware of any differences in CYP1A activities, inducibilities or substrate preferences that occur among avian species in order to properly interpret data from monitoring studies. The characterization of CYP1A enzymes in three avian species following the *in vivo* administration of β-naphthoflavone and/or isosafrole (mammalian CYP1A1- and CYP1A2-specific inducers, respectively) is presented in Chapter 3. Hepatic microsomes from chickens, HGs and DCCs were probed with antibodies to CYP1A1 and CYP1A2 enzymes in immunoblot experiments. Catalytic profiles of various enzyme substrates were examined, both in the presence and absence of putative CYP1A1- and CYP1A2-specific inhibitors. Differences in catalytic activities and immunoreactivities to CYP1A antibodies were observed among the three avian species, and these are discussed.

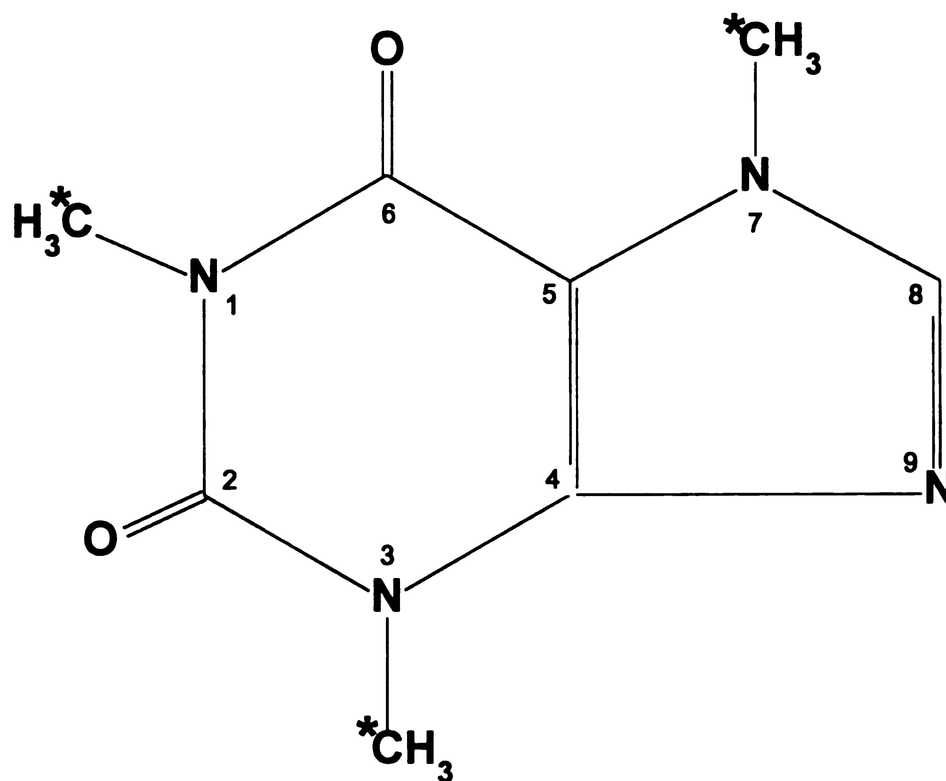


Figure 1. Caffeine. Breath test substrate is labelled in the 1-,3- and/or 7-methyl positions (*) with either ^{14}C or ^{13}C.

CHAPTER 1

Relationship between PCB 126 treatment and cytochrome P450-1A activity in chickens, as measured by *in vivo* caffeine and *ex vivo* ethoxyresorufin metabolism

(for submission in modified form to *Environmental Toxicology and Chemistry*)

INTRODUCTION

Preliminary experiments with the avian CBT were performed with radioactive 1,3,7-methyl-^{14}C-caffeine (Feyk and Giesy, 1996). This substrate, administered i.v. at 1 mg/kg body weight, effectively discriminated between control and TCDD-treated laboratory chickens during the CBT. However, it was difficult to obtain the permits needed to conduct CBTs with wild birds in the field using the radiolabelled substrate, even though radioactivity levels were trace (5 μCi/CBT). Since the ultimate goal of the avian CBT work was to develop a tool for use in field biomonitoring, it was desirable to develop an avian CBT using caffeine labelled with the stable isotope ^{13}C. A potential

advantage of a ^{13}C-CBT was that the stable isotopically-labelled compound would be inherently less hazardous to work with. All potential hazards associated with the use of radioactivity, including harm to worker or subject health and contamination of the environment, were eliminated when stable isotopes were substituted for radioisotopes. In addition, the substitution of stable isotopes for radioisotopes alleviated the need to obtain special radioactive use permits.

Preliminary CBT studies with chickens and 1,3,7-methyl-^{14}C--caffeine did not explore the dose-response relationship between PHDH treatment and the rate of *in vivo* caffeine N-demethylation (Feyk and Giesy, 1996). Chickens were dosed with quantities of TCDD which were intended to produce maximum CYP1A induction, since the primary goal of the pilot study was to determine whether the CBT method could distinguish between induced and uninduced chickens. In that experiment most chickens exhibited an "all or nothing" response; they either appeared to have greatly induced CYP1A activity or very small activity. In five chickens for which *ex vivo* EROD activity was also determined, both *ex vivo* EROD activity and *in vivo* caffeine N-demethylation exhibited the same qualitative response, although the concordance between the two assays was only moderate. In the present experiment, the dose-response relationship between PHDH treatment and the rate of caffeine N-demethylation, and the relationship between caffeine N-demethylation and *ex vivo* EROD activity in individual chickens, were examined. The polychlorinated biphenyl congener 3,3′,4,4′,5-

pentachlorobiphenyl (IUPAC #126, "PCB 126") was chosen as the inducing agent. This is a well-studied coplanar PCB congener with a potency in birds of approximately 10-fold less than that of TCDD (Bosveld et al., 1992; Kennedy et al., 1993). The same PCB congener (PCB 126) was also used in a dose-response feeding study with Common Terns that is described in Chapter 2.

The mono-labelled compound 3-methyl-^{13}C-caffeine was used in the present study. This compound is convenient for CBT use because it is commercially available, unlike the tri-labelled material. Caffeine labelled in the 3-methyl position is commonly used in human CBTs (Lambert et al., 1986, 1990), since CYP1A-mediated N-demethylation has been found to occur only at that position in humans (Berthou et al., 1991). However, there are species differences in methyl position specificity of CYP1A-mediated caffeine N-demethylation (Arnaud, 1985; Berthou et al., 1992). It is therefore important to determine the appropriate position(s) for molecule labels prior to performance of the CBT with each species. In a pilot study with chickens, 3-methyl-^{14}C-caffeine was an excellent CBT substrate which discriminated between control and TCDD-treated birds, while N-demethylation of 1-methyl-^{14}C-caffeine did not appear to be mediated by CYP1A.

The goals of the present experiment were as follows:

1) Develop methods for an avian ^{13}C-CBT.
2) Determine the optimal substrate dose of 3-methyl-^{13}C-caffeine to use in the ^{13}C-CBT.

3) Perform a dose-response study to determine the relationships between PCB 126 treatment, *in vivo* caffeine 3-N-demethylation, and *ex vivo* EROD activity in chickens.

METHODS

Animal care and treatments

Test subjects were five to seven month old White Leghorn hens of the HiSex White strain acquired from Herbrucks Poultry Ranch (Ionia MI). The chickens were individually caged, and were maintained on Purina Layena Poultry Feed and tap water (ad lib.) on a 16 h light/8 h dark cycle. Twenty hens were randomly assigned to one of four treatment groups, which were administered three intraperitoneal (i.p.) injections of either 1,5, or 50 μg/kg body weight of PCB 126 or the corn oil vehicle alone. These doses were chosen to encompass a range from slight to maximal CYP1A induction. Literature data for TCDD potency in chickens were utilized (Sawyer et al., 1986), and a 10-fold difference in potency was assumed between PCB 126 and TCDD (Bosveld et al., 1992; Kennedy et al., 1993). Induction injections were administered 96, 72 and 48 h prior to performance of the CBT. Another twelve hens were injected with either 5 μg/kg body weight PCB 126 or corn oil vehicle as described above (six per treatment group) for inclusion in a pilot study to determine the optimal dose of caffeine to use during subsequent CBTs.

^{13}C-Caffeine breath test

<u>Apparatus</u>

The breath sampling apparatus is diagrammed in Figure 2. A Coles-style endotracheal tube (size 4.5 for adult White Leghorn hens) was used (Willy Rüsch AG, Kernen, Germany). Two pieces of Clay Adams Intramedic® polyethylene tubing (I.D. 1.67 mm, O.D. 2.42 mm, Baxter, McGaw Park IL) were inserted inside the endotracheal tube. The short piece, which extended approximately 4 cm inside the tube, was connected to a miniature one-way inhalation valve (Hans Rudolph Inc., Kansas City MO). This piece of tubing should be as short as possible in order to keep breathing resistance to a minimum. The longer piece of tubing, which extended approximately 9 cm inside the tube, was connected to a miniature one-way exhalation valve (Hans Rudolph Inc., Kansas City MO). This piece of tubing was kept as long as possible in order to minimize dead space air (the sample should be obtained as close to the lungs as possible). The polyethylene tubing was fitted into a rubber septum that formed an airtight seal onto the endotracheal tube. This septum was interchangeable among endotracheal tubes of varying sizes, such that a custom fit for the trachea could be attained for each bird. The inhale/exhale valves were connected to the polyethylene tubing with plastic stubs of female Luer fittings that were epoxied into place. The exhalation valve was connected to a stopcock, which was connected to both a syringe septum and a 200 cc inflatable latex rebreathing bag (Hans Rudolph Inc., Kansas City MO). The labelled caffeine injection solution consisted of 6 mg 3-

methyl-^{13}C-caffeine/ml sterile 0.9% saline (Cambridge Isotope Laboratories, Andover, MA).

Procedure

Each breath test was performed in the following manner. Prior to beginning a breath test, the test subject was weighed and the background breathing rate was recorded (i.e., number of breaths/30 s). The topical anesthetic spray Cetacaine® (Cetylite Industries Inc., Pennsauken NJ) was applied to the glottis and trachea with a cotton-tipped applicator and re-applied as needed. An endotracheal tube/CBT apparatus was then inserted into the trachea approximately 3 cm and a background breath sample was obtained. A 50 cc sample of breath was removed from the apparatus balloon via the stopcock into a syringe, and then injected into five Exetainer® tubes (with screw-top cap and septum, Europa Scientific Inc., Cincinnati OH) in 10 cc aliquots. An injection of 3-methyl-^{13}C-labelled caffeine/kg body weight was made into the brachial vein and the timer was started. The caffeine dose used during the primary PCB 126 dose-response experiment was 3 mg caffeine/kg body weight. Breath samples were taken every 5 min for 40 min as described above. The breathing rate was monitored every 10 min during the CBT.

During the caffeine dose pilot study, caffeine doses of 1.5 and 4.5 mg/kg body weight were used during CBTs in birds that had been treated with either 5 μg/kg body weight PCB 126 or corn oil vehicle. The three caffeine doses (1.5, 3.0 and 4.5 mg/kg body weight) were compared for their ability to

discriminate between PCB-induced birds and controls.

At the end of each work day, hens that had undergone a CBT were killed by decapitation. The liver was immediately removed upon death and the gall bladder was excised from the liver without puncture. The liver was weighed, and then several 1.5 g aliquots were placed into pre-labelled 2 ml liquid nitrogen vials (Sarstedt 72.694.100, Newton NC) and immersed in liquid nitrogen until enzyme activity assays were performed. The remainder of each liver was placed in a chemically clean jar and frozen at -20°C for subsequent chemical analysis.

Data analysis

The ratio of $^{13}CO_2$ to $^{12}CO_2$ in breath samples was analyzed by use of isotope ratio mass spectroscopy (IRMS). A Europa Scientific ANCA-SL Stable Isotope Analysis System with an autosampler was utilized. Breath samples were flushed by a helium carrier, passed through a reduction furnace to remove oxygen, through a gas drying stage, and then through a gas chromatograph column to resolve CO_2 from N_2 and any trace hydrocarbons, before passing into the mass spectrometer ion source for measurement of ^{13}C enrichment. Enrichment of ^{13}C was analyzed with reference to a 5% CO_2 in N_2 working standard (1.06558 atom percent ^{13}C) (Europa Scientific Ltd., 1988), and expressed relative to the ^{13}C content of the historical calcium carbonate standard known as PDB (Boutton, 1991a).

To calculate the recovery of ^{13}C from labelled caffeine in the breath, both

the breath isotopic composition and the overall rate of CO_2 production must be known. Total CO_2 production in humans is often estimated from body weight or body surface area (Boutton, 1991b). The relationship of body weight to CO_2 production in birds was calculated with equation 1:

$$CO_2 \text{ excretion} = V_g \times P_ECO_2 / RT \quad (1)$$

where: V_g = volume of gas
P_ECO_2 = partial pressure of end expiratory CO_2
RT = gas constant times the absolute temperature

At the body temperature of chickens, 1/RT is approximately **0.051 mmol/(liter torr)** (Burger, 1980).

The partial pressure of end expiratory CO_2 in the chicken was averaged from literature values to be approximately **33 torr** (Piiper et al., 1970; McLelland and Molony, 1983; Fedde, 1986). Substitution of these values into equation 1 and simplification yields equation 2:

$$CO_2 \text{ excretion} = V_g \text{ (liter/min)} \times 33 \text{ torr} \times 0.051 \text{ mmol/liter torr)}$$
$$= 1.683 \text{ (MV) mmol/min} \quad (2)$$

where: MV = minute-volume liters/min

The value of MV may be estimated with units of ml/min by equation 3:

$$\log MV = 2.4533 + 0.77(\log BW) \text{ (Lasiewski and Calder, 1971)} \quad (3)$$

where: BW = body weight (kg)

In the following sample calculation, CO_2 excretion was calculated for a 1.6 kg chicken using equations 2 and 3.

log MV	= 2.4533 + 0.77(log 1.6)	= 2.6105 ml/min
MV	= Antilog (2.6105)	= 407.85 ml/min
MV (L/min)	= 407.85 ml/min / 1000ml/L	= 0.40785 L/min
CO_2 excretion	= 1.683 (0.40785) mmol/min	= 0.68641 mmol/min

Once this value was calculated, a series of calculations was performed to convert data from the mass spectrometer into a cumulative amount of caffeine metabolized during the CBT (Boutton, 1991a, b). Raw mass spectrometry data were expressed as a $\delta^{13}C_{PDB}$ value (percent ^{13}C in comparison to the PDB standard). The first step was to convert the $\delta^{13}C_{PDB}$ value to an R value (the absolute ratio of the sample) by use of equation 4.

$$R_{sample} = {}^{13}C/{}^{12}C = [\delta^{13}C/1000 + 1] \times R_{PDB} \tag{4}$$

where: $R_{PDB} = 0.0112372$

Next, the fractional abundance (F) of ^{13}C was calculated by use of equation 5:

$$F = \frac{R_{sample}}{R_{sample} + 1} \tag{5}$$

From this value, the atom percent excess (APE) of ^{13}C between CBT samples and background breath samples was calculated by use of equation 6:

$$APE = (F_{postdose} - F_{baseline}) \times 100 \tag{6}$$

The mmol excess ^{13}C produced per minute at each timepoint was then calculated by use of equation 7:

$$\frac{mmol\ excess\,^{13}C}{min} = \frac{APE\ breath}{100} \times \frac{mmol\ total\ CO_2}{min} \tag{7}$$

note: "APE breath" was calculated from equation 6 and "mmol total CO_2/min" was calculated from equation 2.

The mmol excess ^{13}C in the caffeine dose was calculated by use of equation 8:

$$excess^{13}C\ in\ dose = \frac{DOSE\ (mg)}{MW}\ x\ (\%label)\ x\ (\#atoms) \qquad (8)$$

where:

DOSE	= caffeine dose (mg)	=	variable
MW	= molecular weight of 3-methyl-^{13}C-caffeine	=	195.19
%label	= % of all molecules labelled	=	99%
#atom	= number of labelled atoms per molecule	=	1

The percent caffeine dose metabolized per min (PCD) could then be calculated for each time point by use of equation 9:

$$PCD = \frac{mmol\ excess^{13}C\ /\mathrm{min}}{mmol\ excess^{13}C\ in\ dose}\ x\ 100 \qquad (9)$$

Note:The numerator was calculated by use of equation 7 and the denominator was calculated by use of equation 8.

Finally, the cumulative percent of the caffeine dose recovered (CUMPCD) was calculated for each CBT time point by use of equation 10:

$$CUMPCD = [\frac{(PCD_t + PCD_{t-1})\ x\ \Delta t\ (\mathrm{min})}{2}] + CUMPCD_{t-1} \qquad (10)$$

The cumulative percent of the caffeine dose recovered over the entire 40-min CBT was used in subsequent analyses.

*Ethoxyresorufin-*o*-deethylase (EROD) assay*

A microsomal fraction was prepared from 1.0 g of liver tissue which had been stored in liquid nitrogen for about one year, following the differential centrifugation method of Bellward *et al.* (1990). All steps were performed at

4°C. Briefly, the liver sample was homogenized with Tris buffer (0.05 M Tris, 1.15% KCl, pH 7.5). A Tri-R Stir-R® (Tri-R Instruments Inc., Rockville Centre NY) with a Teflon® stir stick was used for all homogenizations and resuspensions. The initial 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). This suspension was centrifuged at 100,000 x g for 60 min. The supernatant was discarded and the microsomal pellet was resuspended in 1 ml of microsomal stabilizing buffer (20% glycerol, 0.1 M KH_2PO_4, 1 mM EDTA, 1 mM dithiothreitol, pH 7.25). The suspension was placed in Eppendorf® centrifuge tubes (Baxter, McGaw Park IL) 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 four months of the microsomal preparation.

The EROD activity and total protein concentration of microsomal samples were measured simultaneously in 96-well microtiter plates (Kennedy et al., 1993; Kennedy and Trudeau, 1994). Three replicates (wells) were prepared for each sample and resorufin standard, and one blank well without NADPH was prepared for each sample. During the EROD assay, sample wells contained 6 μl of microsomes, 30 μl of ethoxyresorufin, 30 μl of NADPH (2 mM) and 75 μl of HEPES buffer (0.05 M, pH 7.8). The ethoxyresorufin concentration was optimized for each sample (assays were conducted several

times), and ranged between 2 and 15 μM. Samples were incubated for 10 min at 37°C, and then the EROD assay was stopped by addition of 60 μl acetonitrile with 36 μg fluorescamine. The plate was then covered and allowed to sit at room temperature for 15 min, after which florescence was measured with two wavelength filter sets by use of a Cytofluor 2300 (Millipore Corp., Carlsbad CA). The fluorescent reactant of fluorescamine and protein was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm, and bovine serum albumin was used as a protein standard (6.0 to 48 μg/well). The fluorescence of the EROD product, resorufin, was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm, and resorufin was added directly to standard wells (7.5 to 180 pmol/well). Standards curves were generated on each plate and were used to quantify protein content and resorufin production in sample wells.

Chemical analysis of PCB 126 in liver

Ten gram subsamples of liver tissue were extracted and cleaned up for analysis of PCB 126 residues with use of U.S. EPA Method 1613 (U.S. EPA, 1994). Samples were blended with four times their weight of anhydrous sodium sulfate, and the resultant dried powder was extracted with 150 ml methylene chloride. The extract was concentrated to 8 ml, of which 80 μl was removed, oven-dried and weighed for a percent lipid determination. The remaining extract was purified on a column packed from bottom to top with 2 g silica gel (60-100 mesh, Davisil™ grade 635, Aldrich Chemical Co. Inc.,

Milwaukee WI), 2 g NaOH-treated silica gel (33% of 1N NaOH, w:w), and 10 g H_2SO_4-treated silica gel (44% w:w), with 2 g anhydrous sodium sulfate between each layer. The PCB fraction was eluted from the column with 200 ml hexane. This extract was concentrated to 1 ml and further cleaned up on a column of 6 g basic alumina (Activity 1, ICN Pharmaceuticals Inc., Costa Mesa CA). A waste fraction was eluted with 100 ml hexane, and then the PCB fraction was eluted with 20 ml of 1:1 methylene chloride: hexane. The final extract was reduced in volume with a gentle nitrogen stream and transferred to 1 ml iso-octane. All solvents used in extract preparation were Burdick and Jackson High Purity grade (Baxter McGaw Park, IL). Glassware and equipment used in extract preparation were rinsed with acetone and hexane before use.

Sample extracts were quantitated with use of a Perkin Elmer Autosystem gas chromatograph (GC) with electron capture detection (ECD) (Table 1). For instrument calibration purposes, 48 ng of 2,4,6-trichlorobiphenyl (IUPAC PCB #30) was added to the final extract as an internal reference standard. Five analyte concentrations were used to calculate response factors to define the operating range of the ECD. The utilized concentrations were 0.4, 2, 10, 60, and 100 ng/ml for PCB 126, and 0.8, 4, 20, 120, and 200 ng/ml for 3,3',4,4'-tetrachlorobiphenyl (IUPAC PCB #77). Sample concentrations that exceeded the range of the calibration curve were diluted 20 times with iso-octane, spiked with additional PCB 30 and reanalyzed.

Quality Assurance

GC instrumentation was calibrated prior to the analysis of each sample set of six. The means of the concentrations were best fit by a quadratic calibration curve. With the exception of the smallest analyte concentration, the coefficient of variation (CV) for the response factor at each concentration over time was less than 5%. The instrument quantitation limit was 0.4 ng/ml for PCB 126, as determined by the increased CV for calibration at that point.

To measure extraction efficiency, a nominal quantity (approximately 100 ng) of PCB 77 was added to the sodium sulfate drying column prior to extraction. Recoveries of PCB 77 from final sample extracts were compared to PCB 77 recovery from an autosampler vial that was directly spiked with the compound. Concentrations of PCB 126 in this paper have not been corrected for sample-specific extraction efficiencies, which are reported (Table 2). Mean PCB 77 extraction efficiency was 90.6%, with a standard deviation of 4.008.

The quality assurance/quality control of the overall analytical method was evaluated in several ways. Prior to analysis of chicken samples, the method was validated with pork liver spiked with 290 ng PCB 126. Three replicate analyses yielded mean recoveries of 108% and 99.4% for PCB 126 and PCB 77, with CVs of 3.74% and 1.75% respectively. Ten percent of analyzed samples were solvent blanks, which were all below 0.4 ng/ml (lower limit of calibration) for PCB 126. Ten percent of liver samples were analyzed in duplicate, and those PCB 126 determinations were less than 20% different (Table 2).

RESULTS

Caffeine substrate concentration pilot study for the ^{13}C-CBT

Mean cumulative percent dose (CUMPCD) of caffeine 3-N-demethylation during the CBT was greater in chickens treated with 5 μg PCB 126/kg body weight than in corn oil treated controls for all three caffeine substrate concentrations tested. These differences were only statistically significant when a substrate dose of 3 mg of 3-methyl-^{13}C-caffeine/ kg body weight was used in the CBT (one-tailed t-test, α = 0.10, assuming equal or unequal variance as tested by F-test, α = 0.20). Mean caffeine 3-N-demethylation was nearly twice as great in PCB-treated chickens than in control chickens when 3 mg caffeine/kg body weight was utilized (Figure 3). Therefore, a caffeine substrate concentration of 3 mg/kg body weight was used in subsequent CBTs. The rate of caffeine metabolism increased with an increase in caffeine substrate concentration in both control and PCB-treated birds (0.335, 0.763, and 0.837 μg caffeine/min/kg body weight in PCB-dosed chickens when 1.5, 3.0 or 4.5 mg caffeine/kg body weight was used as CBT substrate, respectively) (Figure 3).

Dose-response study with PCB 126

Mean CUMPCD caffeine 3-N-demethylation was greater in chickens treated with medium and high doses of PCB 126 (5 and 50 μg/kg body weight) than in chickens treated with corn oil vehicle or a low PCB 126 dose (1 μg/kg

body weight) (Table 2, Figure 4a). Rates of caffeine 3-N-demethylation were not significantly different between control chickens and chickens from any PCB 126 treatment group (Dunnett's test, $\alpha = 0.05$). Variance among individuals in caffeine 3-N-demethylation was greater in the control and 50 μg PCB 126/kg body weight dose groups than in the 1- or 5- μg PCB 126/kg body weight dose groups (Figure 4a). The slope of the linear correlation between CUMPCD caffeine 3-N-demethylation and the $\log_{10}$ of the measured hepatic concentration of PCB 126 was not significantly different from zero ($\alpha = 0.10$), and the coefficient of determination was small ($R^2 = 0.2057$, Figure 5a).

EROD activity was significantly correlated with the $\log_{10}$ of the measured hepatic concentration of PCB 126 ($R^2 = 0.8754$, $p \leq 0.001$, Figure 5b). Variation of CYP1A activities among individual corn oil treated control chickens was less when measured by the EROD assay than when measured by the ^{13}C-CBT (Figure 4, Table 2). Differences in EROD activity were great between the treatment groups. EROD activity was significantly different between control chickens and chickens from the medium and high dose groups (Dunnett's test, $\alpha = 0.05$, Figure 4b). Absolute values of resorufin production during the EROD assay spanned three orders of magnitude among treatments (Table 2).

The relationship between CUMPCD caffeine 3-N-demethylation and EROD activity was not strong ($R^2 = 0.2024$, $p \leq 0.09$, Figure 6). However, with the exception of one chicken (CK 13, a control hen with a CUMPCD of 1.03), all birds with a CUMPCD of greater than 0.75 CUMPCD exhibited moderate to great EROD activity (i.e., greater than 200 pmol resorufin/min/mg

protein).

There was a strong linear relationship between the dose of PCB 126 injected i.p. and the measured hepatic PCB 126 concentration in the chickens ($R^2 = 0.8223$, $p \leq 0.001$, Figure 7). An average of 1.1% of total administered PCB 126 was recovered in the liver tissue.

DISCUSSION

Caffeine substrate concentration pilot study for the ^{13}C-CBT

The CBT caffeine substrate dose which was chosen for use in this study, 3 mg/kg body weight, is the same that has been used in human studies (Kotake et al., 1982; Lambert et al., 1986, 1990, 1992). Of the three caffeine substrate doses tested in this study, 3 mg/kg body weight provided the greatest discrimination between PCB-treated chickens and controls. When caffeine substrate doses of 1, 3 and 5 mg/kg body weight were tested in humans, substrate saturation was found to occur at a dose of approximately 3 mg/kg body weight (Kotake et al., 1982). In that study, the rate of caffeine 3-N-demethylation did not increase when the substrate dose was increased from 3 to 5 mg/kg body weight. In contrast, in this study the rate of caffeine 3-N-demethylation did increase when the caffeine substrate dose was increased from 3 to 4.5 mg/kg body weight. This increase was great in control chickens (from 0.396 to 0.657 μg/min/kg body weight), but moderate in PCB-treated chickens (from 0.763 to 0.837 μg/min/kg body weight). These results

imply that the caffeine substrate in this study was not available in excess, and that the reported caffeine 3-N-demethylation rate was not a maximum rate. When measuring *in vivo* caffeine metabolism during the ^{13}C-CBT in birds, it may not be possible to administer caffeine at a saturating dose without confounding the CBT results. This is because great doses of caffeine can increase the metabolic rate and the relative utilization of fats and carbohydrates (Acheson et al., 1980), which could influence CO_2 production and breath $^{13}C/^{12}C$ ratios (Schoeller et al., 1980; Lambert et al., 1986). This would confound the changes in $^{13}C/^{12}C$ ratios which result from CYP1A-mediated 3-N-demethylation. Therefore, it is desirable to administer a caffeine substrate dose which is small enough to minimize such physiological effects, while being great enough to produce sufficient ^{13}C enrichment for detection in control birds. The effect of caffeine administration on avian physiology has not been adequately studied, and would be a fruitful topic for future CBT research.

Comparison of ^{13}C-CBT and EROD activity

The *in vivo* ^{13}C-CBT and *ex vivo* EROD assay each displayed relative advantages and disadvantages during this study. The EROD assay seemed to provide a more effective, sensitive measurement of CYP1A activity than the ^{13}C-CBT. Differences in EROD activity were dramatic between chickens from various PCB 126 dose groups, and resorufin production spanned three orders of magnitude over the range of PCB 126 doses administered. In contrast, PCB 126 treatment resulted in a mere doubling of caffeine 3-N-demethylation during

the ^{13}C-CBT, and due to great among-individual variation few treatment differences were statistically significant. The comparative individual variation in CYP1A activity among control chickens as measured by the two assays was especially interesting. In the EROD assay, resorufin production from control chicken microsomes was always small, and barely above the detection limit of the assay (approximately 1 pmol/min/mg protein). In contrast, caffeine 3-N-demethylation in control chickens as measured with the ^{13}C-CBT exhibited great variability among individuals (from below detection to 1.03 CUMPCD, the sixth greatest degree of caffeine 3-N-demethylation by an individual chicken in this study). There are several possible explanations for this finding: either constitutive enzymes were involved in caffeine 3-N-demethylation but not ethoxyresorufin-*O*-deethylation, or the measurement of *in vivo* caffeine 3-N-demethylation in this study was confounded by changes in $^{13}C/^{12}C$ ratios and/or CO_2 production which were not related to CYP1A activity.

The primary advantage of the ^{13}C-CBT is that the method is less invasive than the EROD assay. No mortality has yet been associated with the ^{13}C-CBT assay. In contrast, the *ex vivo* EROD assay requires the analysis of liver tissue, which can be obtained by biopsy or more commonly by killing the test subject. The biopsy procedure is only an option for highly trained professionals (usually veterinarians), and is only useful with relatively large animals that can tolerate the removal of 1 g of liver tissue. It is often unacceptable to kill test subjects for the procurement of liver tissue, particularly if the animal is an endangered species or if multiple measurements of CYP1A activity are desired.

Comparison of the ^{13}C-CBT and the ^{14}C-CBT

The avian ^{13}C-CBT developed during this study displayed advantages and disadvantages relative to the previously developed ^{14}C-CBT (Feyk and Giesy, 1996). Both assays measure *in vivo* caffeine 3-N-demethylation, which is thought to be mediated by CYP1A. The difference between the assays is in the nature of the isotopic label on the carbon atom of the methyl group, which is detected in breath CO_2 following CYP1A-mediated N-demethylation. The ^{13}C label used in the present study was a stable isotope, while the ^{14}C label used in the previous study was radioactive.

There were several relative advantages to the use of stable isotopically-labelled caffeine. The less hazardous nature of stable isotopes in comparison to radioactive isotopes has been discussed. Another advantage of the stable isotope method was that the breath collection procedure was found to be less invasive than that used during the ^{14}C-CBT assay. Breath collection during the ^{14}C-CBT involved continuous collection of all breath over a 40-min breath test while the bird wore a collection mask attached to the breath test apparatus. The ^{14}C-CBT method was associated with about a 10% mortality rate, which was attributed to stress or to a possible interference with free breathing. In contrast, breath collection during the ^{13}C-CBT consisted of spot samples taken every 5 min for 40-min by use of an endotracheal tube. This method was well tolerated by test subjects, and no mortalities have yet been attributed to the method.

The differences in breath collection methodology were necessitated by

differences in the isotopes involved and the analytical methods for their detection. Since the natural relative abundance of ^{14}C is small, all measurable ^{14}C activity in a radioactive breath sample can be attributed to caffeine metabolism. If a large amount of ambient air CO_2 is also collected, as occurs with the ^{14}C-CBT assay, it is of no concern because the ambient air will not contain an appreciable amount of ^{14}C. When all breath CO_2 is continuously collected throughout the breath test period, the cumulative caffeine N-demethylation during the ^{14}C-CBT can be accurately calculated from scintillation counts. In contrast, about 1.1% of carbon in ambient air is in the ^{13}C form. Consequently, a relatively pure breath sample must be obtained during the ^{13}C-CBT assay. Inclusion of ambient air must be kept to a minimum, both to minimize a confounding effect on the breath $^{13}C/^{12}C$ ratio and to assure an adequate amount of total CO_2 in the test sample (the concentration of CO_2 in the exhaled breath is much higher than that in the ambient air). For this reason, exhaled breath is collected directly from the trachea during the ^{13}C-CBT assay.

Data acquisition and analysis is more complex and difficult for the ^{13}C-CBT than the ^{14}C-CBT. The stable isotope method, isotope ratio mass spectroscopy (IRMS), does not directly provide an absolute amount of labelled carbon from metabolized caffeine, as was obtained from the radioactive method. Instead, IRMS provides a ratio of ^{13}C to ^{12}C in the sample, and a series of calculations must be performed in order to determine the amount of caffeine metabolized by each bird. Inaccuracies and false assumptions are

likely throughout these calculations, as discussed in the following section, and are probably responsible for the great variability in calculated caffeine metabolism which was observed in this experiment. Also, an isotope ratio mass spectrometer is more expensive than a scintillation counter, and is available in far fewer laboratories. We found the IRMS method to be less sensitive at detecting a signal from CO_2-labelled molecules from caffeine N-demethylation than was scintillation counting. During the ^{14}C-CBT experiment only 1 mg caffeine was used per kg body weight, and only a small fraction of those caffeine molecules were labelled with ^{14}C, and yet the scintillation signal was easily detected. In contrast, the ^{13}C-CBT required a dose of 3 mg caffeine per kg body weight when 99% of caffeine molecules were labelled with ^{13}C, and yet the detected enrichment of ^{13}C was very small. For example, in the chicken with the greatest CUMPCD caffeine metabolism, the difference between the background $\delta^{13}C_{PDB}$ and the $\delta^{13}C_{PDB}$ of the most ^{13}C-enriched sample (t=40 min) was only 2.38‰. The minimum detectable signal in $^{13}CO_2$ breath tests has been calculated to be 1.4‰, which is 2 times the standard deviation of 0.72‰ for the normal variability of the baseline $^{13}CO_2/^{12}CO_2$ ratio of exhaled CO_2 (Schoeller et al., 1977).

The ^{14}C-CBT was more effective than the ^{13}C-CBT at measuring differences in *in vivo* caffeine N-demethylation between control and PHDH-treated chickens. Differences in caffeine N-demethylation between control and PHDH-treated chickens approached an order of magnitude when measured with the ^{14}C-CBT (Feyk and Giesy, 1996), while they were only doubled during this

^{13}C-CBT experiment. Variation in N-demethylation among control chickens was less during the ^{14}C-CBT. This was likely due to the more robust methodology of the ^{14}C-CBT versus the ^{13}C-CBT, as regarded breath collection (cumulative versus spot-checking), sensitivity of signal detection (scintillation versus ^{13}C enrichment), and accuracy of data analysis (direct attribution of scintillation to the caffeine label versus complex calculations involving many assumptions).

Due to the sizable advantages of the radioactive CBT method, including ease of data analysis and interpretation, it is recommended that the ^{14}C-CBT method be used whenever possible during laboratory experiments. However, due to the difficulties in obtaining radioactive use permits for field studies it is recommended that the stable isotope CBT method be implemented for field CBT work.

Factors that can influence ^{13}C-CBT results

The most significant factor affecting the sensitivity and precision of $^{13}CO_2$ breath tests is the normal variability of the baseline $^{13}CO_2/^{12}CO_2$ ratio of exhaled CO_2 (Schoeller et al., 1977). A number of factors can affect this baseline ratio. Most of these factors have been well characterized in humans, and breath test protocols have been altered to keep their influence to a minimum. Unfortunately, some of these factors are much less controllable in avian subjects than in humans.

Diet can influence the baseline $^{13}CO_2/^{12}CO_2$ ratio (Schoeller et al., 1980). Various dietary constituents have different isotopic ratios, and as they are

oxidized these differences are reflected in the isotopic ratios of respiratory CO_2. Differences in isotopic ratios originate from differences in the photosynthetic processes which fix carbon dioxide into carbohydrate in plants. Some plants fix CO_2 into a three-carbon intermediate through the Calvin-Benson reaction, and other plants have a four-carbon intermediate through the Hatch-Slack photosynthetic pathway. These two pathways differ in their isotopic discrimination against ^{13}C in atmospheric CO_2, such that C_4 plants contain a greater ^{13}C abundance than do C_3 plants. A further discrimination against ^{13}C occurs in the transformation of carbohydrate to protein and lipid, such that lipids have the lowest ^{13}C abundance within a given food chain. In human breath tests, subjects are often asked to fast for 8 h prior to the CBT in order to minimize baseline shifts (Lambert et al., 1990). After such a fast, the subject is usually metabolizing well-mixed endogenous fuel sources in constant proportion (Schoeller et al., 1980). Special snacks that minimize baseline shifts have been developed for those situations when fasting is inappropriate. Such precautions are not possible for field CBT use, nor were they taken during this study, as the primary goal of the present research was to develop a tool for field use. It is likely that failure to control food consumption prior to the CBT led to decreased CBT sensitivity in the present experiment.

Another factor which may affect ^{13}C-CBT results is stress. Mild exercise has been found to increase the magnitude of $^{13}CO_2/^{12}CO_2$ fluctuations in human exhaled breath (Schoeller et al., 1977). Exercise increases both the percentage of CO_2 arising from skeletal muscle mass and the mobilization of stored

nutrients. This changes the percentage of carbohydrate, protein and lipid being oxidized to CO_2 and alters the $^{13}CO_2/^{12}CO_2$ ratio of exhaled breath. This effect is minimized during CBTs with humans by asking the patient to sit calmly for 15 min prior to the CBT, and then throughout the CBT procedure. It is quite another matter to calm a bird during the CBT procedure. We attempted to minimize stress by covering each chicken's eyes between measurement time points, but disturbances at 5 min intervals were an inherent part of the protocol and most birds were undoubtedly stressed. There was a great deal of variability between individuals regarding their propensity to struggle or to passively cooperate during the CBT.

The rate of CO_2 production was also an important factor that influenced breath test results. The overall production of CO_2 is an important component of ^{13}C-CBT calculations, and in this study it was estimated from literature values. Unfortunately, stress probably had an impact on CO_2 production. The breathing rate increased over time during the CBT in about half the test subjects; often this breathing was rapid and shallow in comparison to normal background breathing. If the total volume of metabolic CO_2 production is increased, it can dampen the ^{13}C signal produced by substrate metabolism (Arnaud et al., 1980).

Tracheal dead space must be considered when using the ^{13}C-CBT. This refers to the volume of air which fills the trachea upon inhalation and then is immediately exhaled without gas exchange having taken place. When performing the breath test in humans, the first part of each exhaled breath is

discarded in order to avoid the inclusion of dead space air in the sample. The avian ^{13}C-CBT method involves sampling the entire contents of many expired breaths, including the dead space air. The tracheal dead space volume of the domestic chicken is approximately 4.56 ml (Hinds and Calder, 1971), out of a total tidal volume of 33.0 ml (Fedde, 1986). Since the concentration of CO_2 in the exhaled breath is much greater than that in the ambient air/dead space, the effect of dead space air is probably negligible on the $^{13}CO_2/^{12}CO_2$ isotopic ratio. Also, this is an error which should be constant from bird to bird within the same species and size. Tracheal dead space must be considered when applying the ^{13}C-CBT to different bird species, however. Results could be particularly affected in birds with elongated necks such as herons, or with tracheae elongated into convolutions such as some cranes and swans. Tracheal volumes for many avian species have been determined (Hinds and Calder, 1971).

Posture can affect avian respiration in several ways (King and Payne, 1964). When chickens are placed on their back, amplitude of breathing is reduced, frequency of breathing is increased and the minute volume is reduced in comparison to erect chickens. Since breathing rates influence the CBT, it is important that test subjects remain in an erect, "natural" position during the test.

Recommendations for future research

In order to improve the utility of the avian ^{13}C-CBT, the test will need to be optimized to decrease baseline $^{13}CO_2/^{12}CO_2$ ratio variability and to enhance the sensitivity of the method. In order to increase the detection of ^{13}C from caffeine N-demethylation, it may be possible to use caffeine labelled in all three methyl positions with ^{13}C. Tri-labelled 1,3,7-^{14}C-caffeine was used in earlier chicken CBT work, and in that experiment differences in N-demethylation rates between control and TCDD-treated birds were successfully detected (Feyk and Giesy, 1996). The tri-labelling strategy would only increase ^{13}C-CBT signal detection if N-demethylation of caffeine is at least partially mediated by CYP1A at multiple methyl positions . While this dissertation begins to characterize the enzymes involved in caffeine N-demethylation, more work is needed on this topic. Changes to the CBT protocol, such as pre-test fasting and direct measurement of CO_2 production, may improve researcher control over breath baseline $^{13}CO_2/^{12}CO_2$ ratio variability. A stronger ^{13}C signal would probably be measured in larger birds, which could be administered a greater absolute caffeine dose. However, a great change in $\delta^{13}C_{PDB}$ values has been observed during breath tests with rats (Guilluy et al., 1991), illustrating that successful breath tests can be performed with small animals as well. There are significant differences in the rat and chicken CBT protocols: rats were maintained in metabolic cages which were directly linked to an IRMS, and rats were administered a much greater caffeine substrate dose (10 mg tri-labelled 1,3,7-^{13}C-caffeine/kg body weight, i.p.). Perhaps a greater ^{13}C signal could be

detected during the chicken ^{13}C-CBT if greater caffeine substrate concentrations were utilized. However, in order to implement this strategy it would first be necessary to study the physiological effects of caffeine on the birds.

In conclusion, the avian ^{13}C-CBT is a technique with potential utility for laboratory studies and wildlife monitoring. In its present form, there is a great amount of variability associated with baseline $^{13}CO_2/^{12}CO_2$ ratios, which reduces the sensitivity of the method. Further research will be required to refine the ^{13}C-CBT in order to increase its sensitivity and utility.

Table 1. Instrument parameters of Perkin Elmer Autosystem gas chromatograph.

Injector	splitless at 240°C, initial purge off, set to open 1 min, auto inject 1 μl
Oven Program	50°C hold 1 min, ramp 10°/min to 120°C hold 2 min, ramp 2°/min to 260°C, ramp 20°/min to 280° hold 15 min
Column	DB-5 30m x 0.25mm id. with 0.25 μm film, head-pressure 20 psi He
Detector	Electron Capture, temperature 350°C, attenuation 8, A/D range 1Volt maximum

Table 2. Liver chemical analysis, hepatic ethoxyresorufin-*O*-deethylase activity and cumulative percent caffeine dose metabolized during 40-min ^{13}C-CBT by chickens treated with PCB 126

Bird I.D.	PCB 126 dose ($\mu g/kg$)[1]	Hepatic PCB 126 conc. (ppb)[2]	Extraction efficiency (%)[3]	% lipid[4]	Cumulative % caffeine[5]	EROD[6]
1	0	b.d.[7]	91	9.12	0.37	1.34
2	1	1.87	90	5.34	0.47	486
3	5	9.01	89	5.05	1.17	1158
4	50	40.8	96	4.67	0.85	1684
5	0	b.d.	91	6.11	n.d.[8]	<1
6	1	1.19	93	10.8	0.21	2.48
7[9]	5	17.7 15.0	93 90	4.07 4.69	0.76	933
8	50	82.9	93	4.76	1.35	1640
9	0	b.d.	93	5.62	b.d.	1.57
10	1	0.89	94	4.66	0.66	50.3
11	5	1.66	93	4.94	1.06	395
12	50	83.2	86	8.79	1.47	1169
13	0	b.d.	79	12.3	1.03	1.79
14	1	0.32	88	6.48	0.55	7.90
15	5	7.56	91	6.86	0.77	691
16	50	40.8	93	3.54	0.45	1305
17	0	b.d.	95	5.04	0.71	<1
18	1	0.89	90	6.20	0.46	4.45
19[9]	5	1.46 1.53	83 88	5.81 6.75	1.33	229
20	50	45.5	94	4.82	0.69	1020

1 = dose ($\mu g/kg$ body weight) administered three times i.p.
2 = as measured by chemical analysis of liver tissue
3 = as measured by recovery of PCB 77 spike
4 = percent lipid in liver tissue (wet weight)
5 = cumulative percent of caffeine dose 3-N-demethylated during 40 min CBT
6 = hepatic microsome ethyoxyresorufin-*o*-deethylase activity (pmol resorufin/min/mg protein)
7 = below detection
8 = not determined (insufficient carbon in breath samples)
9 = duplicate chemical analysis performed

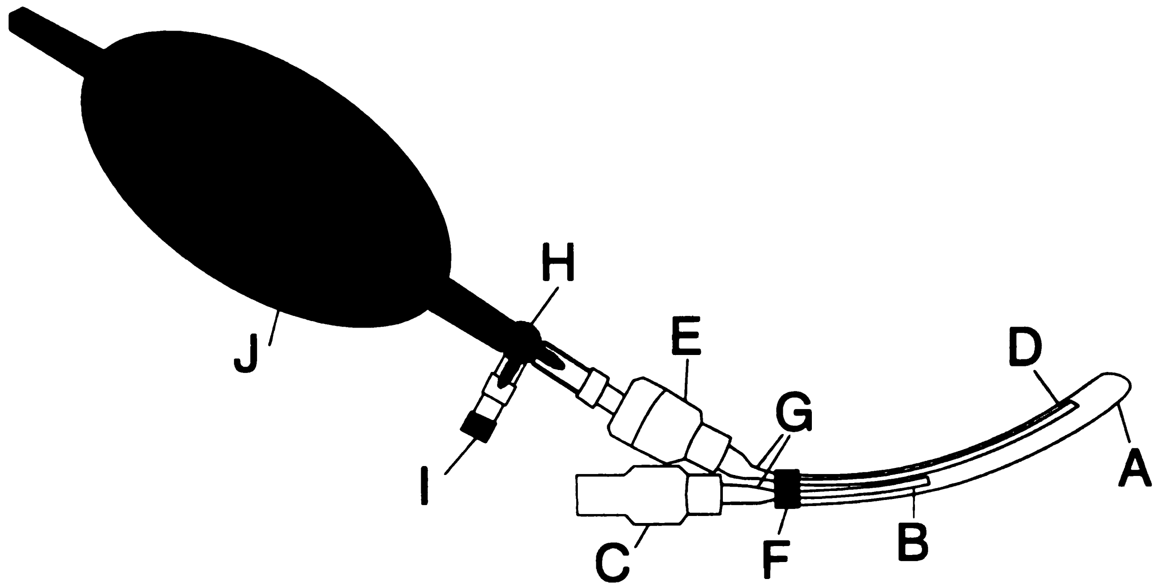


Figure 2. Apparatus for the avian caffeine breath test with stable isotopically (^{13}C) labelled substrate. A = endotracheal tube; B = tubing for inhalation valve; C = one-way inhalation valve; D = tubing for exhalation valve; E = one-way exhalation valve; F = rubber septum; G = female Luer fittings; H = stopcock; I = syringe septum; J = latex rebreathing bag.

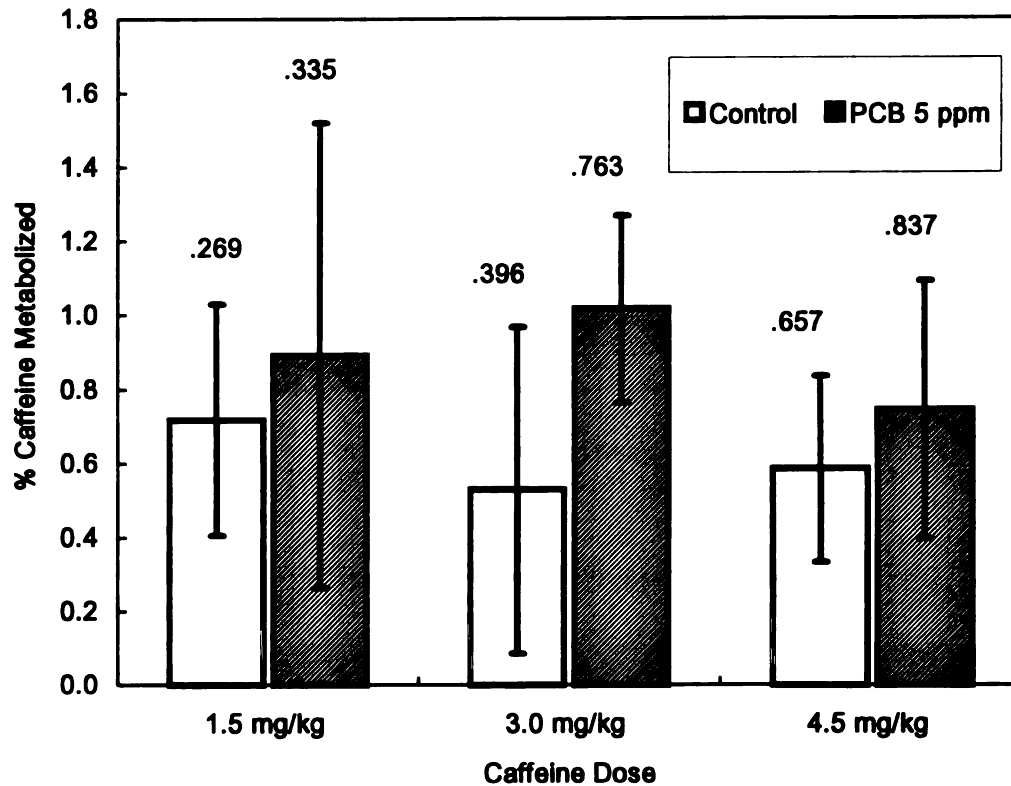


Figure 3. Mean ± 1 s.d. cumulative percent dose of 3-methyl-^{13}C-caffeine N-demethylated during 40-min CBT in chickens. Open bars represent corn oil treated controls; shaded bars represent chickens treated with three i.p. injections of 5 μg PCB 126/kg body weight 96, 72 and 48 h prior to the CBT. X axis indicates 3-methyl-^{13}C-caffeine substrate dose utilized during CBT. The number above each bar represents the mean rate of 3-methyl-^{13}C-caffeine N-demethylation in μg caffeine/min/kg body weight. n = 3 for each treatment, except n = 4 and 5 for control and PCB-treated chickens at 3.0 mg caffeine/kg body weight, respectively.

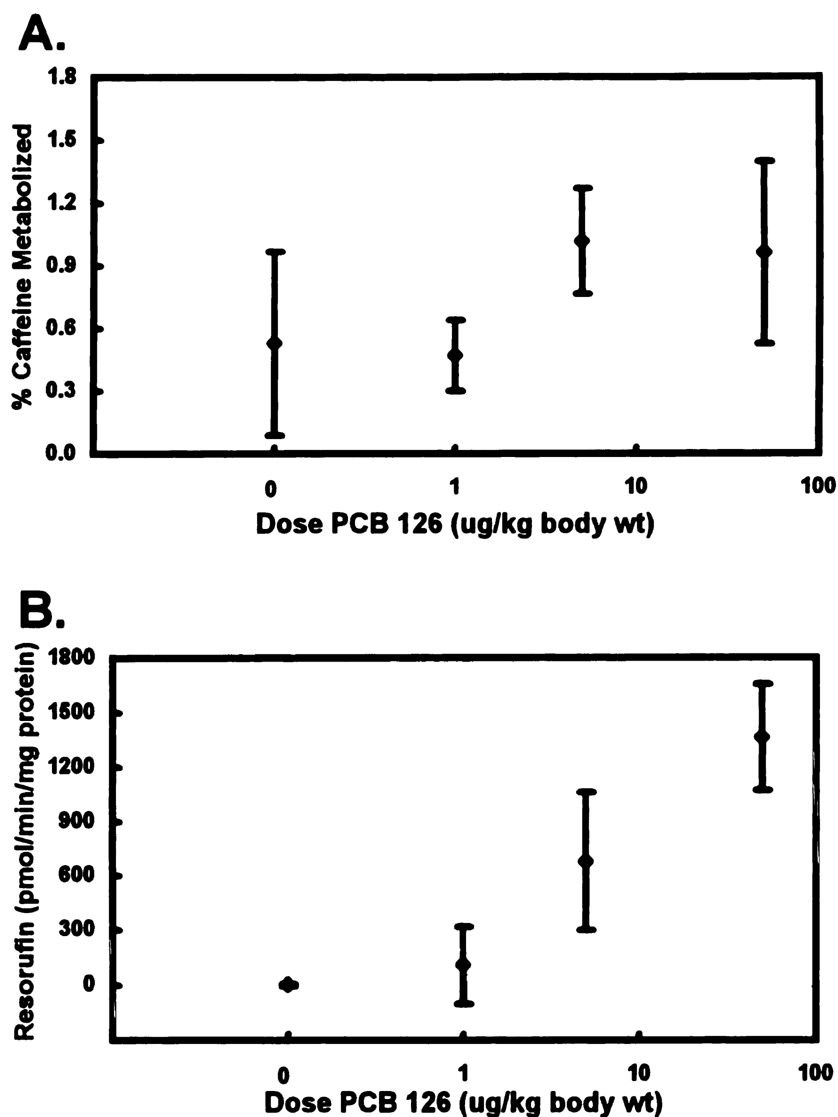


Figure 4. Mean ± 1 s.d. (a) cumulative percent dose of 3-methyl-^{13}C-caffeine N-demethylated during 40-min CBT in chickens (caffeine substrate dose was 3 mg/kg body weight), and (b) rate of resorufin production in ethoxyresorufin-*O*-deethylase (EROD) assay with hepatic microsomes (pmol/min/mg microsomal protein). X axis indicates dose of PCB 126 injected i.p. three times (96, 72 and 48 h prior to the CBT). n=5 for each treatment, except n=4 for CBT data for control treatment group.

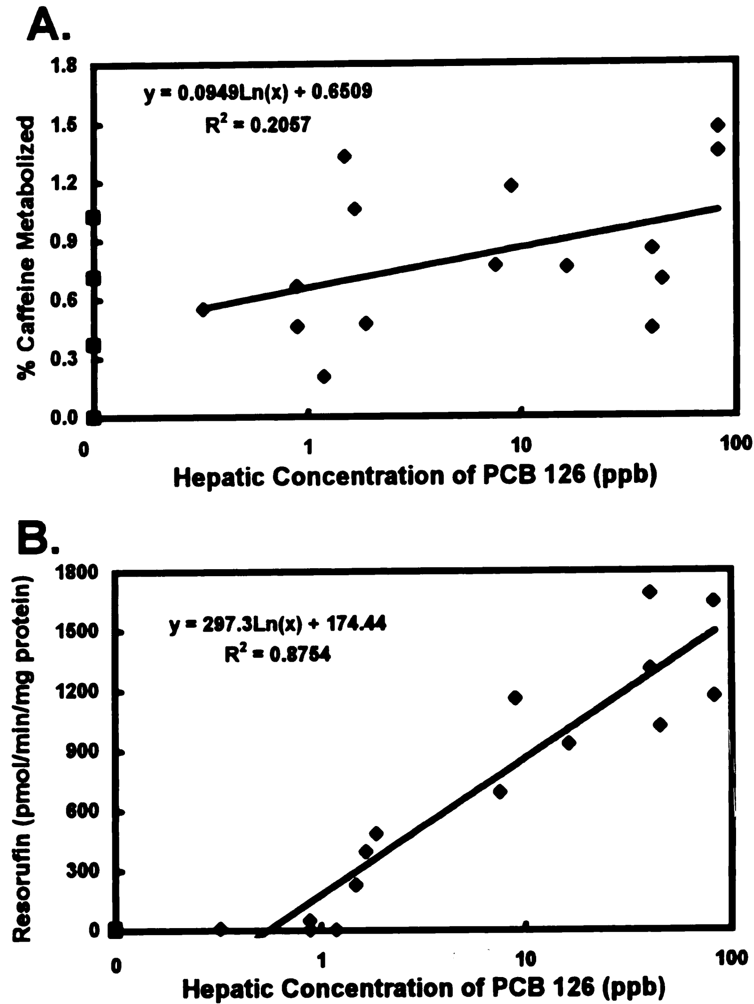


Figure 5. Measured hepatic concentration of PCB 126 (ppb) in relation to (a) cumulative percent dose of 3-methyl-^{13}C-caffeine N-demethylated during 40-min CBT (caffeine substrate dose was 3 mg/kg body weight) and (b) rate of resorufin production in ethoxyresorufin-*O*-deethylase (EROD) assay with hepatic microsomes (pmol/min/mg microsomal protein) in chickens.

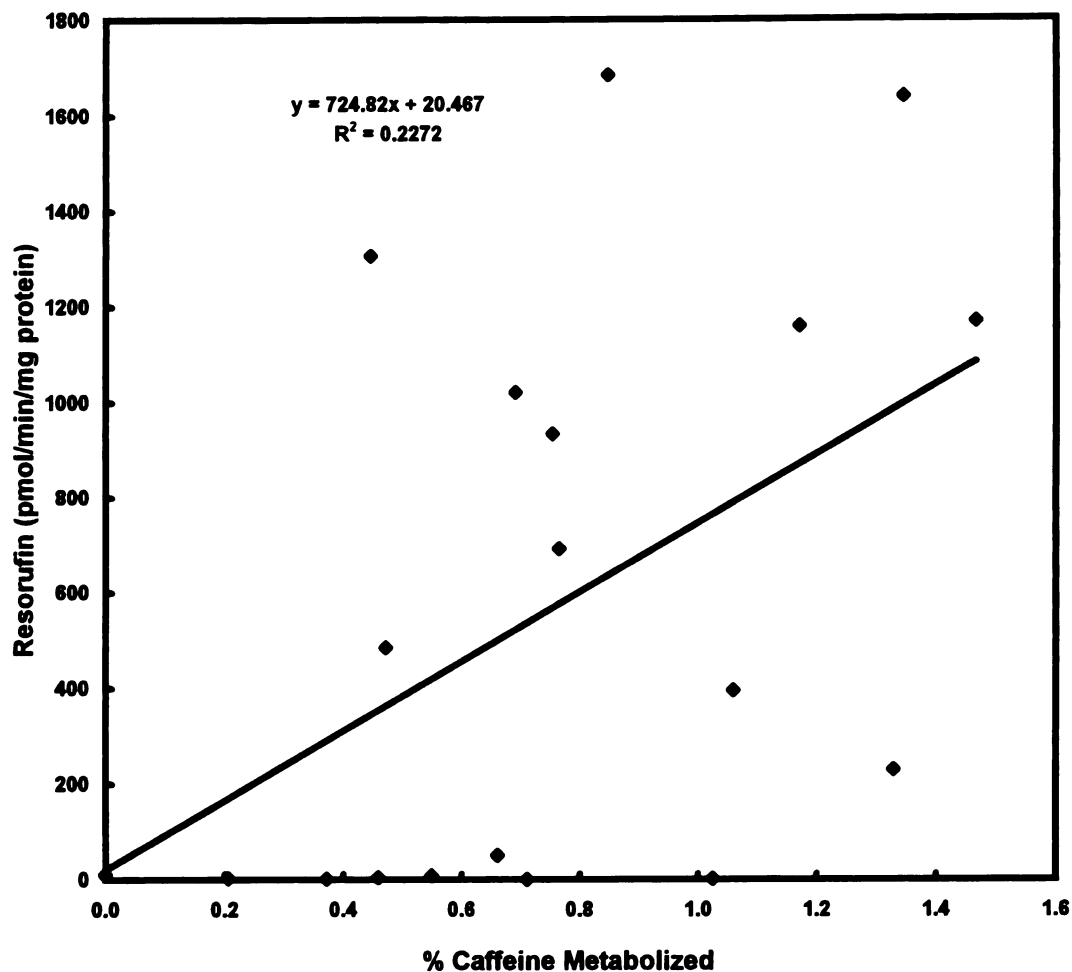


Figure 6. Rate of resorufin production in ethoxyresorufin-*O*-deethylase (EROD) assay with hepatic microsomes (pmol/min/mg microsomal protein) from individual chickens in relation to cumulative percent dose of 3-methyl-^{13}C-caffeine N-demethylated during 40-min CBT in the same birds. Caffeine substrate dose was 3 mg/kg body weight.

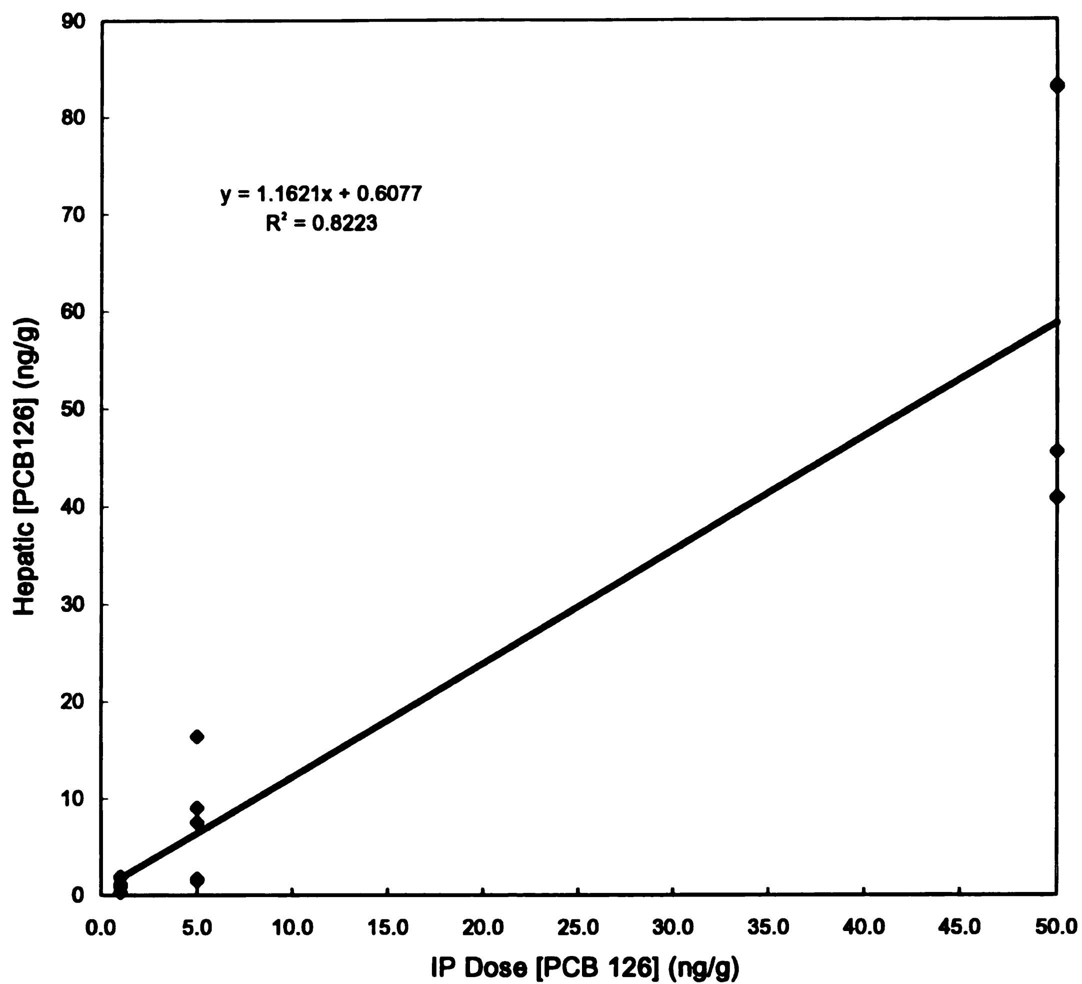


Figure 7. Hepatic concentration of PCB 126 (ppb) in chickens in relation to dose of PCB 126 administered three times by i.p. injection 96, 72 and 48 h prior to death (n = 15).

CHAPTER 2

Changes in cytochrome P450-1A activity during development in Common Tern chicks fed polychlorinated biphenyls, as measured by an *in vivo* caffeine breath test

(for submission in modified form to *Environmental Toxicology and Pharmacology*)

INTRODUCTION

Preliminary development of the avian CBT involved use of a surrogate species (the domestic chicken) and PHDH exposure regime (i.p. injections of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or "TCDD") that were somewhat unrepresentative of the environmental situation of concern (Feyk and Giesy, 1996). While pilot studies were also performed with several piscivorous avian species (Feyk et al., 1995; Feyk and Giesy, 1996), the dose-response relationship between PHDH exposure and *in vivo* caffeine N-demethylation had not yet been determined in a piscivorous avian species. The present study was designed to address these issues, and was part of a larger collaborative project

(Bosveld et al., 1995a). This project was a laboratory feeding study in which a range of PCBs were introduced to hatchling Common Terns (*Sterna hirundu*) through their fish diet. Two PCB congeners, the highly toxic coplanar congener PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl) and the environmentally abundant di-ortho congener PCB 153 (2,2′,3,3′,4,4′-hexachlorobiphenyl), were added to the diet in a ratio commonly observed in the environment (Bosveld et al., 1995b). The CBT was performed twice in the terns during the course of nestling development, with use of radiolabelled ^{14}C-caffeine substrate. Only those treatment groups with relevance to the CBT experiment are mentioned here, although the original group treatment names are retained for ease of comparison between manuscripts.

There were several goals for this CBT experiment. The first goal was to observe the dose-response relationship between an environmentally realistic PCB exposure and caffeine N-demethylation in hatchling Common Terns. The degree of correlation was observed between *in vivo* caffeine N-demethylation and terminal *ex vivo* ethoxyresorufin *o*-deethylase (EROD) activity, in order to determine whether the CBT might be a suitable substitute for the more commonly used EROD assay. The usefulness of the CBT as a tool to make multiple CYP1A activity measurements was also assessed. We observed the manner in which enzyme activity changed in individual terns during development in response to dietary PCBs. A final goal of the research was to determine whether the CBT procedure had any influence on nestling survival, growth or morphological development, in order to gain insight into whether the

method was truly "non-invasive" in a laboratory setting.

METHODS

Experimental design

For the purposes of the CBT experiment, thirty Common Tern hatchlings were divided into six treatment groups of five birds each. Two treatment groups were assigned to each of three dietary PCB dose levels (Table 3). Hatchlings from the CBT groups were subjected to the CBT procedure, while hatchlings from the G groups were not. This allowed a comparison of growth, survival, enzyme activity and morphological parameters in CBT and no-CBT (G) individuals, in order to assess any possible effects of the CBT procedure itself on the tern nestlings. The CBT was performed twice in each tern assigned to the CBT treatment groups, at one and two weeks of age, in order to assess how caffeine metabolism changed in each individual over time in response to PCB exposure and to development.

Animal care and treatments

Common Tern eggs were collected in May 1994 from a breeding colony located on Huizerhoef, a small (about 1 km^2) island on Lake Gooimeer in the Netherlands. The eggs were incubated in the laboratory (Research Institute of Toxicology, University of Utrecht) at 37.5°C and 55% humidity, and were automatically tipped every hour. Pipping embryos were transferred to

separated cages in an incubator at 37.5°C and 80% humidity. After hatching, tern chicks were divided into six treatment groups of five birds each. Each group was housed in a separate 90x60x60 cm cage, which was kept at constant temperature with a 100 watt ceramic heating bulb.

Hatchlings had free access to drinking water, and were hand-fed fish every 2 to 3 h from 7 AM to 9 PM each day. The fish diet varied during the experiment due to age-dependent preferences in fish size and because there was a limited supply of favored fish. For the first four days post-hatch, tern chicks were fed sprat (*Sprattus sprattus*) from the Wadden Sea and sparling (*Osmerus eperlanus*) from the Baltic Sea. From day 5 until the end of the experiment, the tern nestlings were fed anchovy (*Engraulis encrasicolus*) from the Atlantic near Spain and sandeel (*Ammodytus lancea*) from Lake Ijsselmeer, The Netherlands. The fish were frozen at -20°C and thawed prior to feeding. Immediately prior to feeding each tern, every fish was individually weighed and the proper amount of a corn oil solution containing PCB 126 and PCB 153 was injected into the fish gastrointestinal tract using a Rainin® pipette (Table 3).

Hatchling body weight was monitored at the beginning and end of each day. The birds were killed on day 21 post-hatch following anesthesia with diethylether, and morphological measurements were made at necropsy. Livers were removed immediately upon death and divided into subfractions. Each liver portion was snap-frozen in liquid nitrogen and stored at -70°C until biochemical analyses were performed. The thyroid and thymus were dissected and weighed, and skeletal sizes and weights were measured using the isolated

and cleaned bones. The tibia, femur, ulna and primaries were measured.

Growth and morphological parameters were compared between CBT terns and terns from the G groups that received the same dietary treatments. The morphology at day-21 of laboratory Common Terns from the CBT-0 (control) group was also compared to the morphology of five Common Terns that were naturally raised to 21 d of age on Slijkplaat Island (Lake Haringvliet, The Netherlands). That colony was visited to mark and fence a cluster of one day old hatchlings, and then revisited on day-21 to collect five of the chicks. A two-sample F test was performed for each contrast in order to assess equality of variance using a one-tailed p value of 0.20. The t test was then performed, assuming equal or unequal variance as appropriate, with significance placed at $\alpha = 0.10$.

Cytochrome P450-1A (CYP1A) activity assays

In vivo *Caffeine Breath Test (CBT)*

The CBT was performed twice with each assigned individual, once on day 7 or 8 post-hatch and again at day 15 or 16. CBT methods are as described in detail elsewhere (Feyk et al., 1995; Feyk and Giesy, 1996). At the start of the CBT each bird received an i.v. injection of 1 mg caffeine/kg body weight containing 5 μCi of ^{14}C radioactivity. Due to limited substrate availability, two different caffeine substrates were utilized. Nine birds received tri-labelled 1,3,7-[^{14}C]trimethylxanthine (caffeine) that had been synthesized in our laboratory (Feyk and Giesy, 1996), while five birds received mono-labelled

3-methyl-[^{14}C]caffeine (Moravek Biochemicals, Brea CA). These substrates have produced similar results in previous CBT experiments (Feyk and Giesy, 1996). The same caffeine substrate was used during weeks 1 and 2 for each bird. Following caffeine injection, CO_2 from expired breath was collected in trapping solution at 10 min intervals for a total of 40 min. The ^{14}C activity from expired $^{14}CO_2$ in the trapping solution was determined by liquid scintillation with use of "Ultima Gold XR" cocktail (Packard, Meriden CT).

The total mass of caffeine metabolized during the 40 min CBT was calculated by summing the activities (μCi) of $^{14}CO_2$ expired and then converting the total activity to mass of caffeine (pmol) using the specific activity of the caffeine. The total pmol caffeine metabolized was then divided by the quantity of caffeine originally administered and expressed as the percent administered dose metabolized (PADM) during the CBT. Within each week, mean PADM for the CBT-3 and CBT-4 groups were contrasted to the mean PADM of the control CBT-0 group using a one-sided Bonferroni t statistic (α = 0.05).

Ex vivo *hepatic ethoxyresorufin-O-deethylase (EROD) assay*

Hepatic microsomes were prepared by differential centrifugation as described previously (Bosveld et al., 1995b), and microsomal suspensions were stored at -70°C until the EROD assay was performed. Microsomal protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules CA). The assay was performed in 96-well microtiter plates (Costar Inc, Cambridge MA), and absorption at 595 nm was

measured with a Bio-Rad 3550 microplate reader (Bio-Rad Laboratories, Hercules CA). Bovine serum albumin (BSA) was used as a protein standard (Sigma Chemical Co., St. Louis MO).

Microsomal EROD activities were analyzed fluorimetrically using 24-well microtiter plates (Greiner GmbH, Frichenhausen, Germany). Microsomal suspensions were diluted with sample buffer (0.1 M $Na_2HPO_4 \bullet 12H_2O/KH_2PO_4$, 1.15% KCl, pH 7.4). Sample wells contained 375 μl TRIS buffer (0.1 M TRIS, 0.1 M NaCl, pH 8.0), 25 μl microsomal dilutions, 50 μl BSA (Sigma, 10 mg/ml) and 25 μl 7-ethoxyresorufin (Boehringer, 50 μM in methanol/TRIS, 1:9 v:v). Plates were pre-incubated at 37°C for 5 min, and then the reaction was started by addition of 25 μl NADPH (Boehringer, 4 mg/ml in TRIS) while shaking. Reactions were stopped after 10 min by addition of 1 ml ice-cold methanol. Fluorescence was measured on a Cytofluor 2300 (Millipore Intertech, Malborough, MA) with excitation and emission wavelenghts of 530 and 590 nm, respectively. Resorufin production in sample wells was quantified by comparison to a resorufin (Kodak, San Diego CA) standard curve and expressed as pmol resorufin/min/mg microsomal protein.

Residue analysis

Polychlorinated -biphenyl (PCB), -dibenzodioxin (PCDD) and -dibenzofuran (PCDF) concentrations were measured in the diet, and in tern livers from groups G-0, G-3 and G-4. Diet analyses were performed in triplicate on samples of approximately 20 g pooled anchovy. The extraction,

clean-up and analysis of diet and liver samples were performed as previously described (Bosveld et al., 1993, 1995b). Di-ortho PCB congeners were measured using gas chromatography (GC) with electron capture detection (ECD), while planar PCB, PCDD and PCDF congeners were measured using GC with mass spectrometry (MS). Positive electron impact was used for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and octachlorodibenzodioxin, while negative chemical ionization was used for the detection of other planar congeners.

The toxicity of each congener was related to the toxicity of TCDD (the most potent PHDH) by use of TCDD equivalency factors (TEFs) as proposed by Safe (1990, 1994). The congeners were then summed to calculate the overall TCDD equivalents (TEQs) in the samples as an estimate of dioxin-like potency.

RESULTS

Concentrations of PHDHs in the background diet and in tern liver

Several PHDH congeners were detected in the background anchovy diet, which was the main component of the diet over the three week feeding period. The mean and s.d. of the TEQs in the background diet was 22 ± 11 pg TEQ/g anchovy, wet weight. The major contributor to this total TEQ value was PCB congener 77, which was responsible for 53% of the TEQ_{total}, or 11.7 pg TEQ/g anchovy. Other significant contributors to the TEQ_{total} were PCB 126 (16%), the mono-ortho PCBs (15%), the di-ortho PCBs (8%) and the polychlorinated

dibenzofurans (3%).

The pattern observed in the terminal PHDH liver concentrations reflected both the dietary background exposure and the exposure to spiked PCBs 126 and 153. Liver concentrations of PCB 126 were increased 3 or 23 times above the control group (G-0) in groups G-3 and G-4, respectively, while PCB 153 was increased 12 or 105 times for these two treatments respectively. Total TEQs in the tern livers were influenced by PCB 77 from the background diet. As a result, the total TEQs in the livers from terns in group G-3 were not greater than those from the control group G-0 (Figure 8). However, total TEQs in the livers of terns from group G-4 were significantly greater than those of the G-0 group (greater than 4-fold), due principally to the experimentally introduced PCB 126.

The Caffeine Breath Test (CBT) and EROD assay

Comparison between PCB treatment groups

The relationship between PCB treatment and caffeine N-demethylation was not constant throughout this experiment (Table 4). When the CBT was performed on the tern chicks at one week of age, the rate of caffeine N-demethylation was significantly greater in chicks from the CBT-4 group than in those from the CBT-0 group (one-tailed Bonferroni t-test, $\alpha = 0.01$). Caffeine N-demethylation was not significantly greater in chicks from the CBT-3 group than in those from the CBT-0 group at week 1 (one-tailed Bonferroni t-test, $\alpha = 0.05$). PCB-related treatment differences were less apparent when

the CBT was repeated at two weeks of age. Although mean caffeine N-demethylation was still greater in the CBT-4 group than in the CBT-0 group, the difference was no longer statistically significant at week two (one-tailed Bonferroni t-test, $\alpha = 0.05$).

Caffeine N-demethylation did not change in a predictable manner over time in individual terns. The terns doubled their body weight during this time (from about 50 to about 100 g), and consequently the amount of caffeine administered during the breath test also doubled. The absolute quantity of caffeine metabolized during the CBT was increased proportionately. However, when expressed as percent administered dose metabolized, approximately half the terns exhibited a greater amount of caffeine N-demethylation at week-1 than week-2, while half the terns exhibited a lesser amount (Figure 9). This pattern appeared to be unrelated to PCB dose.

Terminal hepatic EROD activity at 21 days of age exhibited differences among treatment groups. EROD activity was significantly greater in chicks from the CBT-4 group than in those from the CBT-0 group (one-tailed Bonferroni t-test, $\alpha = 0.01$). EROD activity was not significantly greater in chicks from the CBT-3 group than in those from the CBT-0 group (one-tailed Bonferroni t-test, $\alpha = 0.05$)

The relationship between week-2 caffeine N-demethylation and terminal EROD activity at week-3 varied among the PCB dose groups. In the CBT-0 and CBT-3 groups, there was no apparent correlation between enzyme activities as measured by the two assays (Figure 10). However, in the greatest PCB dose

group (CBT-4), there appeared to be a linear relationship between caffeine N-demethylation in the CBT and resorufin production in the EROD assay. This relationship was not tested for significance due to the small sample size.

Effect on growth, survival and morphological parameters

The CBT did not have any detectable effects on growth, survival or morphological development in tern nestlings when compared to their paired no-CBT (G) counterparts. Only one chick died during the course of this experiment. The death occurred in a tern from the CBT-4 group on day 4, which was prior to any CBT testing. Growth was similar among terns in all six laboratory groups (Figure 11), and terminal body weight did not vary significantly among terns in the laboratory groups (Table 5). None of the morphological parameters measured were significantly different between the paired CBT and no-CBT laboratory groups (two-tailed t-test, $\alpha = 0.10$, assuming equal or unequal variance as tested by F-test, $\alpha = 0.20$), including skeletal lengths, skeletal weights or organ weights (Table 5). There were few statistically significant differences in morphological parameters between CBT-0 terns and naturally raised chicks from the wild (Hf). Body weight at 21 d of age was not significantly different between those two groups. Mean liver weight was less in CBT-0 chicks than Hf chicks, and that difference was statistically significant ($p < 0.05$). The only other significantly different morphological parameter was tibia weight, which was greater in CBT-0 terns than in Hf terns ($p < 0.10$).

DISCUSSION

The anchovy diet utilized in this experiment was contaminated with PHDHs that contributed significantly to the terminal liver TEQ concentration in all dose groups. As a result, this study did not have an ideal control group that was unexposed to PCBs. The anchovy diet was acquired from the environment and resembled a degree of contamination that wild Common Terns might realistically encounter. This study confirms the difficulty of acquisition of unexposed control reference populations or prey bases with which to conduct experiments that involve the effect of contaminants on free-ranging or wild-caught animals.

The CBT was successful at detecting PCB treatment differences, particularly during week 1. It was not surprising that there was no difference in caffeine N-demethylation between the CBT-0 and CBT-3 groups during week 1, because there was apparently no difference in total TEQ exposure between the two groups. (Although terminal PCB concentrations were not measured in the CBT terns, it is assumed that they were comparable to their G counterparts that received the same diets.) A four-fold increase in terminal liver TEQ concentrations in the G-4 group corresponded to a doubled rate of caffeine N-demethylation in the CBT-4 group compared to the CBT-0 and CBT-3 groups during week 1. The effect of PCB treatment on caffeine N-demethylation rates was less apparent at two weeks of age, and treatment related differences were no longer statistically significant. A rapidly changing body composition may

have been responsible for these results.

Caffeine N-demethylation did not increase from week-1 to week-2 in a dose-dependent manner despite a continued exposure to relatively great concentrations of PCBs in the diet of the highest dose group. Enzyme induction in response to dietary exposure may have been dampened by the dynamics of growth, body composition and energy sources during development. In a study with nestling Herring Gulls, the residue level of organochlorine (OC) contaminants rose rapidly in the embryo as the yolk was absorbed, and then plummeted as the chicks switched from a dependence on yolk to external food sources (Peakall et al., 1986). In the absence of OC contaminant exposure from food sources, residue concentrations in nestlings are further decreased by dilution during this period of rapid body growth. In addition, the body composition of pre-fledged nestlings changes toward increased fat and decreased water content throughout the growing period in several fish-eating bird species (Dunn, 1975; Dunn and Brisbin, 1980). Some of the dietary PCBs administered to the terns in this study may have partitioned to peripheral adipose tissue such that they were not available in the liver to stimulate enzyme induction, and liver PCBs concentrations may have been greater during week-1 than week-2 post-hatch.

The pipping embryo is the preferred life stage at which to measure EROD activity in biomonitoring studies (Rattner et al., 1996). Pipping embryos exhibit great levels of induction but relatively small variabilities in enzyme activities as compared to adult birds or nestlings during the rapid post-hatch growing period

(Rattner et al., 1996). Unfortunately, the CBT cannot be performed during the pipping embryo stage because the birds are too small and weak for the procedure. The present study confirms the idea that avian CYP1A activity should ideally be measured as early in post-hatch life as possible, because PCB-related treatment differences were significant at week-1 but not at week-2.

Methods are needed to assess the influence of endogenous factors on mixed-function oxygenase (MFO) activities in wildlife species. MFO activities are currently used in biomonitoring programs to assess the potential impact of environmental contaminants on wildlife populations, and it is important to understand the confounding variables that can influence MFO activities in addition to contaminants. For example, the changes of MFO activity that might occur in birds at different life stages (Peakall et al., 1986) or seasons (Fossi et al., 1990) have important implications for bioeffects monitoring. Non-invasive procedures such as the CBT provide researchers with a method to directly measure changing MFO activities in an individual bird in response to various treatments. In this study, the CBT was successfully used to examine the combined influences of development and dietary exposure to PCBs on MFO activity.

Terminal EROD activity provided a more reliable indication of PCB exposure than did *in vivo* caffeine N-demethylation at two weeks of age. Hepatic EROD activities were consistently small in terns from the CBT-0 and CBT-3 dose groups, while caffeine N-demethylation ranged from relatively small to great rates in those terns. However, although the sample size of this

group was too small to make definitive statements, caffeine N-demethylation and EROD activities appeared to be strongly correlated in terns from the CBT-4 group that received relatively great PCB exposures. Similar results were also observed in chickens treated with TCDD, in which caffeine N-demethylation and EROD activities were roughly comparable (Feyk and Giesy, 1996). The week-long delay between measurement of week-2 caffeine N-demethylation and terminal EROD activity at 21 days of age might also be partly responsible for the lack of correspondence between the two assays that was observed in this experiment.

Constitutive hepatic EROD activity appears to be smaller than constitutive caffeine N-demethylation in birds. Hepatic EROD activity is thought to be catalyzed primarily by CYP1A1, which evidently has only a small constitutive activity in fish-eating birds. The fact that caffeine N-demethylation increases similarly to EROD activity in PCB-exposed birds indicates that it is probably also catalyzed by inducible CYP1A enzymes, particularly since this pathway is well characterized in mammalian systems (Berthou et al., 1992; Fuhr et al., 1992). However, caffeine N-demethylation appears to have significant constitutive activity in some relatively unexposed birds. This constitutive activity implies that other enzymes in addition to CYP1A1 may be responsible for caffeine N-demethylation in the Common Tern. The possible non-specificity of caffeine N-demethylation for CYP1A enzyme(s) with low constitutive activity may reduce the utility of the CBT as a monitoring tool for PHDH-related exposure and effects in birds, and warrants additional research.

The lack of impact of the CBT on tern nestling growth, survival or morphological development was a positive aspect of the CBT method. No significant differences in growth, survival or morphology could be attributed to the CBT procedure in laboratory-raised terns, and few morphological parameters were significantly different between naturally raised terns and laboratory-raised CBT-0 terns. The relatively large livers observed in the naturally-raised Hf terns were probably related to some factor at the site where the eggs were laid. In the collaborative study, laboratory-raised chicks hatched from eggs acquired at the Lake Haringvliet colony had relatively larger livers than the laboratory-raised G terns from the Lake Gooimeer colony (Bosveld et al., 1995a). The present study confirmed that the CBT is a relatively non-invasive method with which to measure *in vivo* MFO activity, particularly in a laboratory setting. Additional factors would need to be considered in field studies, however. The mere physical presence of a researcher on a bird island can have negative repercussions on subsequent nestling survival (American Ornithologists' Union, 1988). In addition, the terns in this study were habituated to humans and may have experienced less stress during the CBT than a field-caught nestling would. Nevertheless, the CBT method is certainly less invasive than *ex vivo* techniques that require a liver sample, such as the EROD assay.

Table 3. PCBs added to the diet for each dose group. Note that background PHDH concentrations are not included in the concentrations or TEQs shown.

Group	PCB 126 ng/g fish	PCB 153 ng/g fish	TEQ[1] pg/g fish
G-0/CBT-0	0	0	0
G-3/CBT-3	1	1000	100
G-4/CBT-4	10	10,000	1000

1 = TCDD equivalents from Safe (1990, 1994).

Table 4. Cumulative percent caffeine dose metabolized during 40-min ^{14}C-CBT ("Week 1 or Week 2 CBT"), or terminal hepatic EROD activity ("Week 3 EROD") expressed as pmol resorufin/min/mg microsomal protein, in Common Terns. Mean ± s.d., n=5 for CBT-0 and CBT-3, n=4 for CBT-4.

	CBT-0	CBT-3	CBT-4
Week 1 CBT	0.032±0.014	0.031±0.014	0.056±0.014[a]
Week 2 CBT	0.040±0.022	0.030±0.015	0.049±0.029
Week 3 EROD	18.2±2.91	17.7±5.70	71.9±22.0[a]

a= significantly greater than CBT-0, α=.01, one-tailed Bonferroni t-test.

Table 5. Terminal morphometry. Skeletal weights and lengths, and weights of whole body and organs.

Data expressed as means, with s.d. in parentheses. n = 5 unless otherwise noted.

	body weight (g)	liver (g)	thymus (mg)	thyroid (right) (mg)	tibia (cm)	tibia (g)	femur (cm)	femur (mg)	ulna (cm)	primaries (cm)
CBT-0	106 (7)	3.43 (0.53)	408 (126)	6.4 (2.7)	4.24 (0.21)	2.25 (0.14)	2.38 (0.18)	158 (25)	7.01 (0.36)	15.81 (0.27)
G-0 (n = 6)	109 (7)	3.42 (0.52)	441 (85)	6.4 (2.4)	4.21 (0.08)	2.16 (0.10)	2.48 (0.08)	157 (19)	6.79 (0.14)	15.97 (0.44)
CBT-3	102 (3)	3.14 (0.31)	406 (136)	6.3 (3.6)	4.12 (0.10)	2.34 (0.15)	2.42 (0.04)	147 (20)	6.86 (0.15)	15.83 (0.66)
G-3	106 (7)	3.33 (0.71)	504 (96)	5.4 (2.2)	4.17 (0.16)	2.28 (0.38)	2.46 (0.04)	152 (15)	6.91 (0.28)	15.86 (0.46)
CBT-4 (n = 4)	104 (12)	3.04 (0.39)	437 (196)	4.2 (0.1)	4.23 (0.10)	2.40 (0.45)	2.43 (0.10)	148 (25)	6.84 (0.17)	15.90 (0.42)
G-4	105 (4)	3.18 (0.15)	483 (174)	4.2 (3.8)	4.25 (0.16)	2.35 (0.26)	2.47 (0.06)	155 (12)	6.98 (0.20)	16.06 (0.30)
Hf	111 (15)	4.4[a] (0.4)	584 (252)	3.8 (2.3)	4.10 (0.03)	2.08[b] (0.09)	2.47 (0.07)	152 (15)	6.79 (0.23)	15.14 (1.19)

a = significantly different from CBT-0, $p < 0.05$, two-tailed t-test assuming equal variance
b = significantly different from CBT-0, $p < 0.10$, two-tailed t-test assuming unequal variance

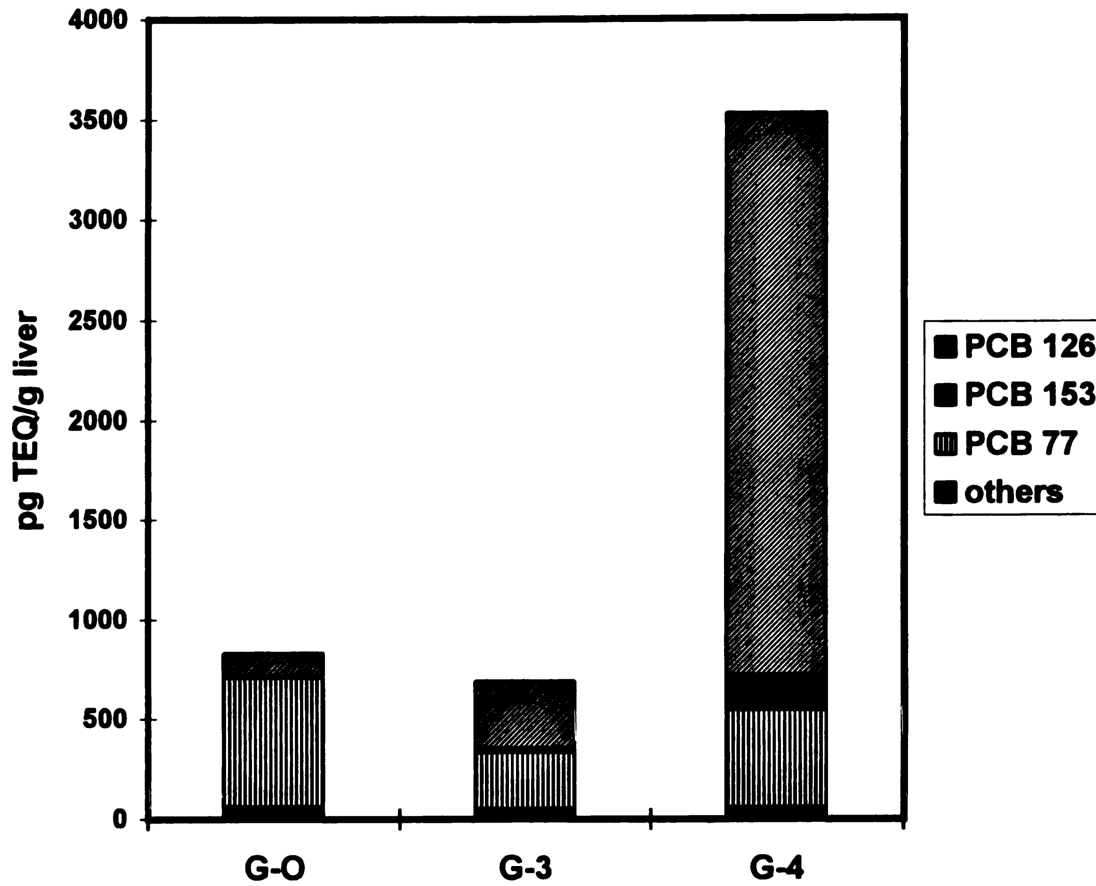


Figure 8. Contribution of several PCB congeners towards the total TEQs measured in Common Tern livers. Values are means of five individuals for treatment groups G-3 and G-4, and six individuals for G-0.

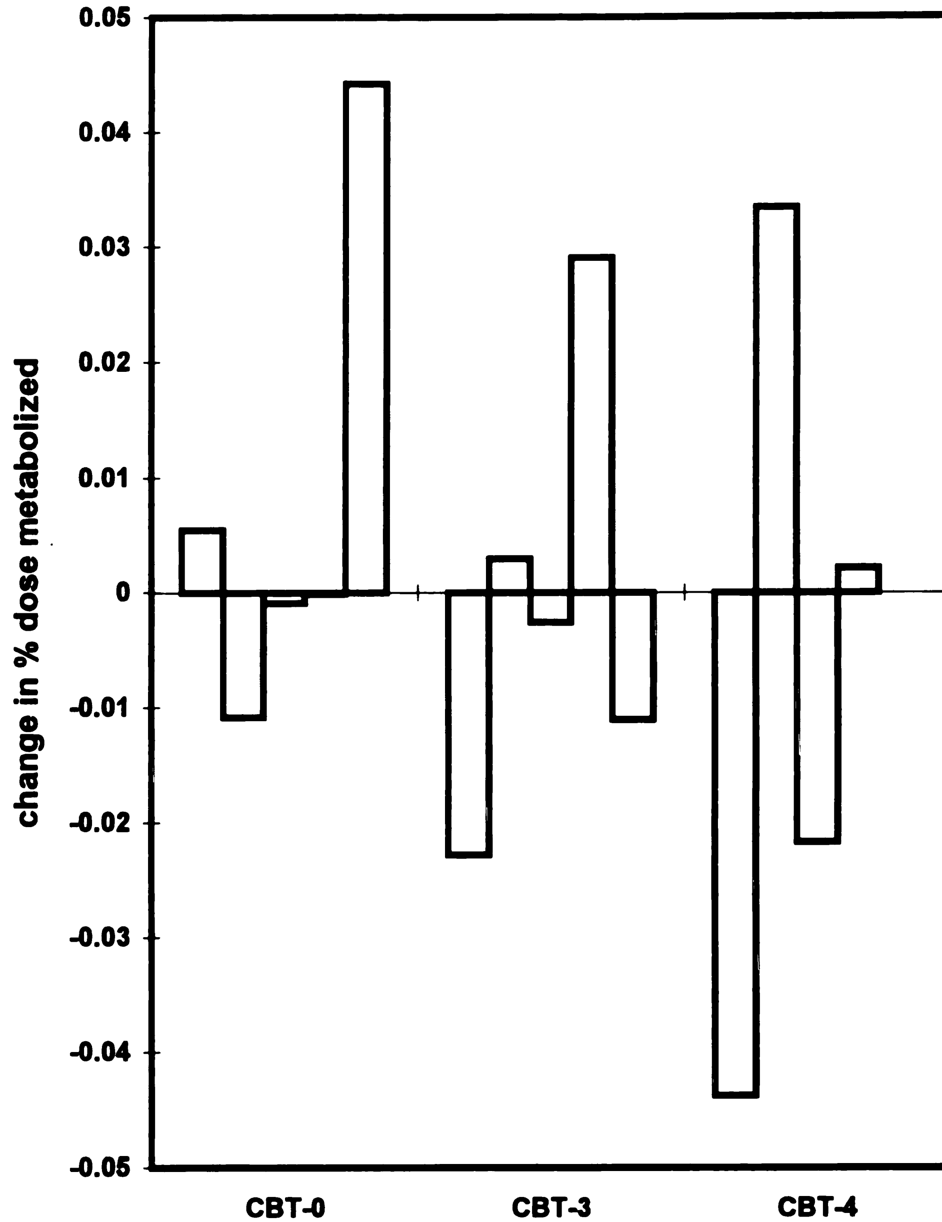


Figure 9. Relative change in the percent caffeine dose metabolized during the 40-min CBT from week-1 to week-2 in individual Common Terns.

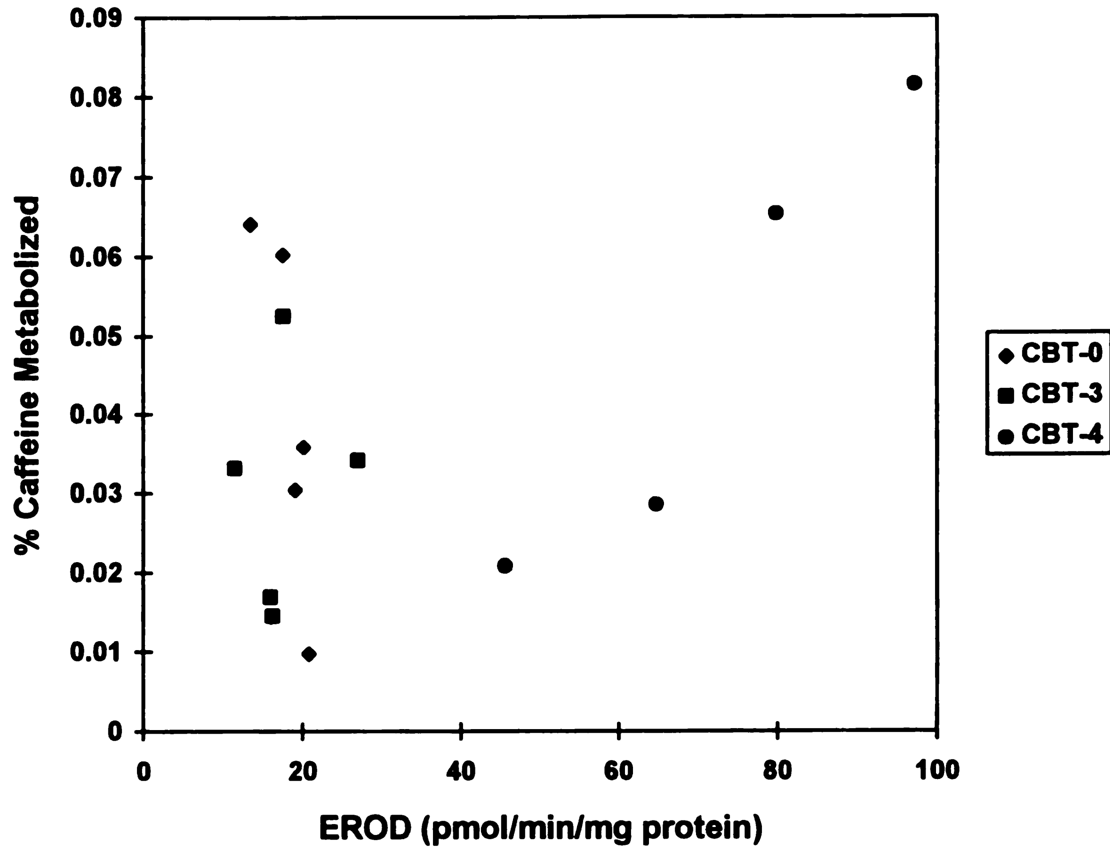


Figure 10. Percent caffeine dose metabolized during the week-2 CBT and terminal hepatic EROD activity at 21 days of age (pmol resorufin/min/mg protein) in individual Common Terns.

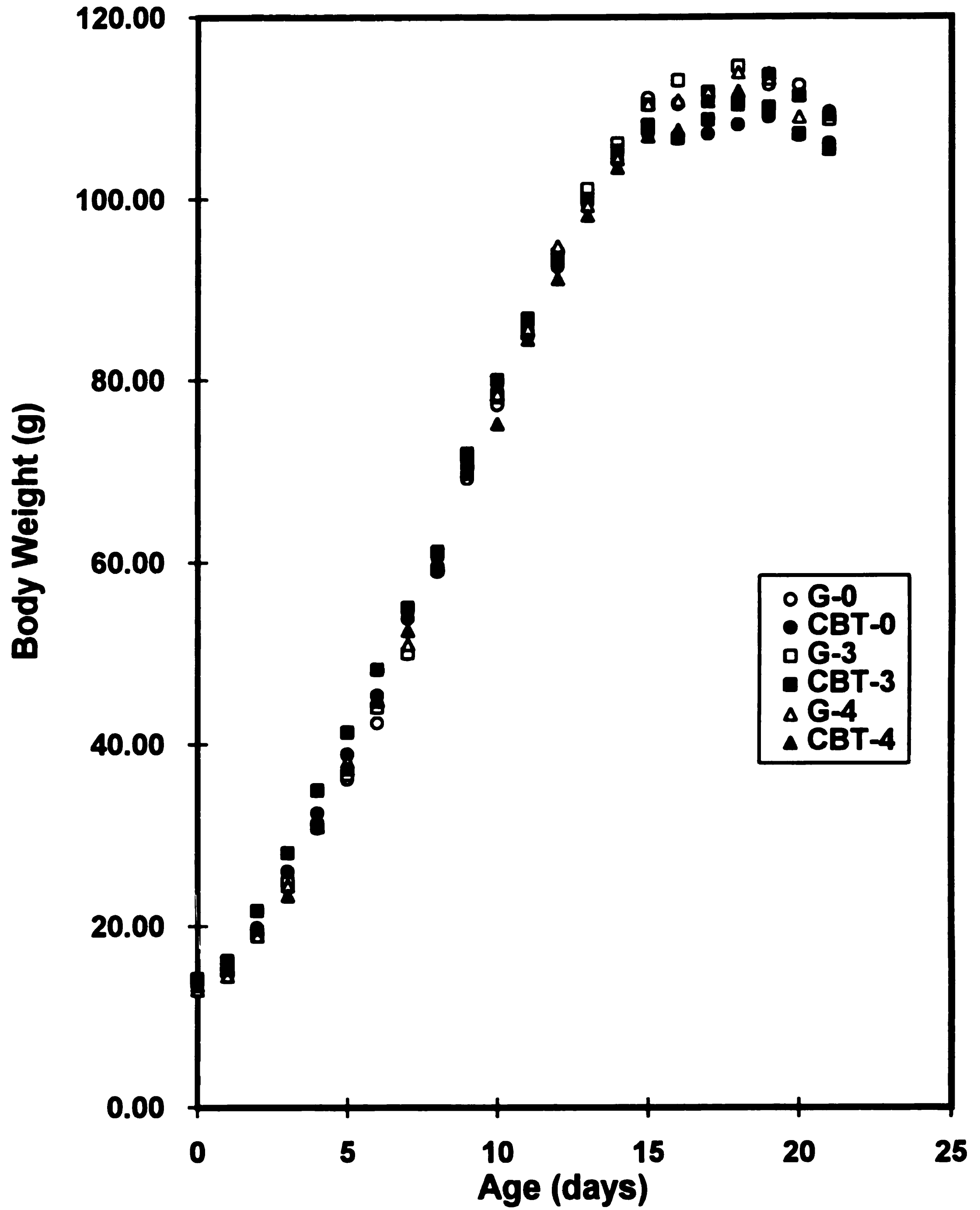


Figure 11. Daily mean body weight in Common Tern nestlings as measured at 7 AM. n = 5, except in G-0 (n=6) and CBT-4 (n=4).

CHAPTER 3

Alkoxyresorufin-*O*-dealkylase activity and immunochemical properties of hepatic cytochrome P450-1A in three avian species treated with *β*-naphthoflavone or isosafrole

(for submission in modified form to *Comparative Biochemistry and Physiology*)

INTRODUCTION

While CYP1A enzymes have been well characterized in several mammalian (Thomas et al., 1984; Rodrigues and Prough, 1991; Tsyrlov et al., 1993) and fish (Stegeman, 1989; Morrison et al., 1995) species, less is known about CYP1A enzymes in birds (Walker and Ronis, 1989). CYP1A induction is sometimes used as a biomarker of exposure to PHDHs in fish-eating bird populations (Peakall et al., 1986), and an enhanced understanding of avian CYP1A could lead to improvements in sampling design and data interpretation in biomonitoring programs (for example, see Fossi et al., 1990). Also,

knowledge of avian CYP1A enzymes and their substrate specificities would aid in the assessment of the suitability of proposed alternate CYP1A substrates such as caffeine (Feyk and Giesy, 1996). Information regarding differences in specific aspects of CYP1A induction may provide important new clues about the differences in sensitivity to PHDH toxicity that have been observed among avian species (Brunström and Reutergårdh, 1986; Brunström, 1988). Induction of CYP1A enzymes can cause a beneficial enhancement of metabolism and excretion of PHDHs (Walker, 1978), but CYP1A induction can also have adverse consequences such as the production of mutagenic metabolites from polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (Butler et al., 1989a). Characterization of avian CYP1A enzymes could also contribute to the study of the evolution of CYP1A genes, and the phylogenetic relationships among avian, fish and mammalian CYP1As (Nebert et al., 1989; Morrison et al., 1995).

The catalytic activities of various cytochrome P-450 enzymes towards their specific substrates are often used to quantify enzyme induction levels. Alkoxyresorufin compounds have been particularly useful substrates in these assays for several reasons (Burke et al., 1985). The specificity of the alkoxyresorufin-*o*-dealkylase (alk-ROD) reaction is dependant on the length of the alkyl side chain, such that different isozymes exhibit specificities for the various alk-ROD reactions. In addition, the induction efficacies of alk-ROD activities following treatment with prototypical P450 inducers are great. The product of the alk-ROD reaction is resorufin, a fluorescent compound that can

be conveniently measured in multi-well plates (Kennedy et al., 1993). Alk-ROD reactions have been performed in rats (Burke et al., 1985; Lubet et al., 1990a; Nakajima et al., 1990) and mice (Nerurkar et al., 1993) using hepatic microsomes or purified cytochrome P-450 (CYP) enzymes from rats treated with inducers, in the presence and absence of antibodies to specific CYP enzymes. This has provided a wealth of information about inducer specificities for particular isozymes and the specificities between CYP enzymes and alkyl side chains in these mammalian species. While changes in alk-ROD activities following treatment with classical inducers have also been studied in fish (Ankley et al., 1987) and birds such as the Mallard Duck (Leffin and Riviere, 1992) and Japanese Quail (Lubet et al., 1990b), comparatively less is known about alk-ROD profiles and specificities in non-mammalian species. Others have recognized the need for alk-ROD profile data in avian species, and additional information regarding avian alk-ROD profiles has recently emerged (Melancon, 1996).

Several inducers and inhibitors are known to affect mammalian CYP1A. While neither of the inducers used in this experiment exert their effect exclusively on one of the two CYP1A isozymes, β-naphthoflavone (BNF) primarily induces CYP1A1 while isosafrole primarily induces CYP1A2 in most mammals (Ryan et al., 1980; Thomas et al., 1984). Isosafrole can also inhibit CYP1A2 through the formation of a stable complex between the heme of cytochrome P-450 and an isosafrole metabolite, but this complex can be displaced by a variety of agents in order to observe the concurrent induction

(Ryan et al., 1980). Ellipticine is a potent inhibitor of CYP1A activity, and does not appear to have specificity for one of the two CYP1A enzymes (Murray and Reidy, 1990; Tassaneeyakul et al., 1993). In contrast, furafylline has been found to be a specific inhibitor of CYP1A2 (and not CYP1A1) in humans and rats (Sesardic et al., 1990a).

Immunoreactivity of proteins with antibodies toward purified CYP enzymes is important complementary information to enzyme catalytic activities, because it provides a qualitative or quantitative assessment of the amount of immunoreactive protein. Immunoblotting can aid in identification of CYP proteins, and can elucidate relationships among CYP proteins from different species. Also, at times large amounts of immunoreactive but functionally inactive CYP apoproteins can be present (Guengerich et al., 1982; Seidel et al., 1984). In one study alk-ROD activity was induced by lesser, but inhibited by greater PHDH treatment concentrations, while immunoreactive CYP1A protein concentration was directly proportional to PHDH concentration (Hahn et al., 1993).

The primary goal of this project was to examine the characteristics of CYP1A forms in three avian species using catalytic and immunochemical techniques. Specifically, changes in alk-ROD profiles were determined following treatment with isosafrole and/or BNF in chickens (*Gallus domesticus*), Herring Gulls (*Larus argentatus*) (HGs) and Double-crested Cormorants (*Phalacrocorax auritus*) (DCCs). The degree of inhibition of alk-ROD activities by ellipticine or furafylline was also determined. The immunoreactivity of

hepatic microsomal proteins from the birds with monoclonal and polyclonal antibodies against CYP1A proteins was investigated.

METHODS

Induction injection solutions

All solutions except β-naphthoflavone (BNF) were prepared such that target doses could be administered in a volume of less than 1 ml per kg body weight. Corn oil was used as the vehicle for PCB 126, BNF and isosafrole, and physiological saline was used as the vehicle for phenobarbital. It was necessary to prepare BNF solutions at concentrations of 80 mg/ml corn oil or less in order to obtain uniform suspensions that were free-flowing enough to pass through 20 gauge needles. BNF (Sigma Chemical Co., St. Louis MO) was dissolved in corn oil by heating the suspension in a 170°C oil bath. After cooling, BNF crystallized to form a thick gel that was stirred to produce a uniform BNF suspension.

Animal care and treatments

Laboratory chickens were acquired, fed and housed as described in Chapter 1. Hepatic microsomes from chickens treated with corn oil or 50 μg/kg body weight PCB 126 (Chapter 1) were included in the present experiment. In addition, twelve chickens were assigned to one of four alternate treatment groups. Three chickens were administered two i.p.

injections of BNF (80 mg/kg body weight) 96 h and 48 h prior to being killed by decapitation. Three chickens received i.p. injections of isosafrole (Aldrich, Milwaukee WI) (150 mg/kg body weight) for three consecutive days; the birds were killed 24 h after the third induction injection. Another three chickens were injected with both BNF and isosafrole as described above. Finally, three chickens were treated daily for three consecutive days with an i.p. injection of 80 mg sodium phenobarbital/kg body weight (Sigma Chemical Co., St. Louis MO); the birds were killed 24 h after the third induction injection. Upon death, livers were immediately removed from the chickens, placed in cryovials in 1 g aliquots, and stored in liquid nitrogen until microsomes were prepared (less than one year).

Great Lakes fish-eating birds were studied on Gull Island, Lake Huron in 1995. An enclosure was erected around 19 HG nests and 29 DCC ground nests on May 18, shortly prior to hatch. Enclosures were constructed with flexible plastic fencing (rectangular mesh, 0.75" x 0.85") and metal conduit (0.5" i.d.). These enclosures allowed the nestling's parents to fly unimpeded in and out of the area, but prevented escape of the nestlings by foot. The enclosure was subsequently removed from the DCC colony when it was observed to be unnecessary, as the altricial nestlings did not leave their nests following manipulation. From June 10-26, 14 HG and DCC nestlings were treated with BNF and/or isosafrole (seven of each species). Dosing protocols were the same as those described for chickens. Treated chicks were banded and weighed upon capture, and were returned to their nests immediately

following each i.p. induction injection. No chicks were treated with corn oil vehicle alone due to logistical difficulties. Instead, "controls" were untreated chicks of comparable size to the experimental nestlings that were randomly caught from outside the enclosures. The liver of each bird was removed immediately upon death. Several 1 g aliquants of liver were placed in cryovials and stored on dry ice for a maximum of 4 d prior to transfer to liquid nitrogen. Microsomes were prepared within 8 months of liver collection.

Hepatic microsomal fractions from chickens, HGs and DCCs were prepared with use of differential centrifugation (Bellward et al., 1990). In addition, some microsomes from isosafrole-treated birds were prepared with a cyclohexane incubation step as described by Sesardic et al. (1990c) in order to dissociate a possible isosafrole metabolite-CYP1A complex. Microsomal pellets were suspended in 1 ml of microsomal stabilizing buffer (20% glycerol, 0.1 M KH_2PO_4, 1 mM EDTA, 1 mM dithiothreitol, pH 7.25) and stored in 100 μl aliquots at -80°C. Enzyme activity assays and immunoblots were performed within 4 months of microsomal preparations.

Antibodies and immunoblotting

Several antibodies were used to quantify CYP1A enzyme(s) in avian hepatic microsomes. The monoclonal antibody (MAb) 1-12-3 (passage 6) was raised in mouse ascites fluid against cytochrome P450E from the marine fish *Stenotomus chrysops* (scup) (Park et al., 1986), and recognizes mammalian CYP1A1 forms but not CYP1A2 forms (however, see Kloepper-Sams et al.,

1987). Two polyclonal antibodies were also used (from Dr. Peter Sinclair, Veterans Administration Hospital, White River Junction, VT). "PEG" was raised against mouse CYP1A1 and binds to mammalian CYP1A1 and CYP1A2 forms equally well. "RYE" was raised against mouse CYP1A2 and binds to mammalian CYP1A2 forms strongly and CYP1A1 forms weakly (Stegeman et al., 1995).

Western blot analysis (Towbin et al., 1979) was performed on hepatic microsomal samples from 38 birds using MAb 1-12-3. Microsomal proteins (30 or 60 μg protein for samples with comparatively greater or lesser EROD activity, respectively) and a range of standards (scup pool #92-150, 0.05 to 1.0 pmol CYP1A1) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel. The separated proteins were electrophoretically transferred to a Nytran nylon membrane (Schleicher & Schuell, Keene NH). The Nytran membrane was incubated with blocker buffer (Rad-Free, Schleicher & Schuell, Keene NH; 1% in Tris-buffered saline), followed by primary antibody (10 μg/ml blocking buffer), followed by secondary antibody (goat anti-mouse IgG linked to alkaline phosphatase, Schleicher & Schuell, Keene NH; 1:5000 v:v in blocking buffer). Immunoreactive proteins were detected with chemiluminescence with use of the substituted 1,2-dioxetane-phosphate CSPD (Tropix, Dedham MA); film was exposed to the membrane in CSPD solution for 3 min. Light intensities of immunoreactive protein bands were quantified by video-imaging densitometry (Kodak DCS 200 digital camera system and NIH Image 1.55 software). Values

for scup-equivalent CYP1A from MAb 1-12-3 cross-reactive proteins in avian hepatic microsomes were determined from the experimental scup CYP1A1 standard curve. Comparisons of absolute quantities of CYP1A should not be made between species, since the relative reactivities of the CYP1A from scup and each avian species to MAb 1-12-3 are not known.

Western blots of selected avian microsome samples using PEG and RYE antibodies were performed as described above, with the following modifications. Samples with greater or lesser EROD activity were loaded at 20 and 60 μg protein per lane, respectively. Secondary antibody was goat anti-rabbit IgG linked to alkaline phosphatase (1:10,000 v:v, Schleicher & Schuell, Keene NH). Standard lanes were loaded with 5 μg microsomal protein from a rat treated *in vivo* with 500 μM 3,3',4,4'-tetrachlorobiphenyl (PCB 77). Since the CYP1A content of the rat microsomal standard has not been quantitated, only relative comparisons between band intensities of samples could be made.

Alkoxyresorufin-O-dealkylase (Alk-ROD) activity assays

Eth- (EROD), meth- (MROD), pent- (PROD), and benzyl- (BROD) oxyresorufin-*O*-dealkylase activities and total protein concentration of microsomes were measured simultaneously in 96-well microtiter plates (Kennedy et al., 1993; Kennedy and Trudeau, 1994). Six replicates (wells) were prepared for each sample with each substrate; two of these were in the absence of inhibitor, and two each were in the presence of 10 μM ellipticine or 200 μM furafylline. Three replicates were prepared for each standard

concentration, and one blank well without NADPH was prepared for each sample and substrate. The ethoxyresorufin (ER) stock solution was prepared in methanol; all other substrate stocks were prepared in dimethylsulfoxide (DMSO). Substrate concentrations were optimized empirically; 15 μM was used for EROD, PROD and BROD activities, while 15 μM and 5 μM were used for MROD determinations of relatively small and great activity samples respectively. Pent- (PR) and benzyl- (BR) oxyresorufin working solutions were prepared in glass test tubes with added bovine serum albumin (BSA, final well concentration 0.044%). BSA enhanced substrate solubility in the assay buffer (HEPES, 0.05 M, pH 7.8). Microsomes, substrate, inhibitor and NADPH (0.425 mM) were incubated for 10 min at 37°C, and then alk-ROD activity was stopped by addition of 60 μl acetonitrile with 36 μg fluorescamine. The plate was then covered and allowed to sit at room temperature for 15 min, after which fluorescence was measured with two wavelength filter sets by use of a Cytofluor 2300 (Millipore Corp., Carlsbad CA). The fluorescent reactant of fluorescamine with protein was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm, and BSA was used as a protein standard (6.0 to 48 μg/well). The fluorescence of the alk-ROD product (resorufin) was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Resorufin was added directly to standard wells (7.5 to 180 pmol/well). Protein values determined in 12 replicate wells during EROD and MROD assays were averaged and used for normalization of PROD and BROD activities, in order to eliminate interference from substrate

BSA added in the PROD and BROD assays and to enhance assay-to-assay consistency.

RESULTS

Immunoreactivity of antibodies with CYP1A enzymes

Immunoreactivity of hepatic microsomes with MAb 1-12-3 varied among avian species and among treatments (Figure 12). After a 3 min film development, no bands were detected in microsome samples from any untreated birds (Figures 12A, 12B, 12C). When the constitutive amount of CYP1A immunoreactive protein in microsomes from a few control birds was investigated on a separate gel with a 30 min film development, immunoreactive CYP1A was not detectable in chicken microsomes, while DCC and HG samples contained 0.20 and 0.14 pmol scup-equivalent CYP1A/mg microsomal protein respectively (data not shown). Treatment with BNF resulted in the induction of a single band immunoreactive with MAb 1-12-3 in all three avian species (Figures 12A, 12B and 12D), as did treatment of chickens with PCB 126 (Figure 12C). No band immunoreactive with MAb 1-12-3 was observed in microsomes from phenobarbital-treated chickens (Figure 12C). Isosafrole treatment of DCCs and HGs induced a single band immunoreactive with MAb 1-12-3 (Figures 12A and 12B), but no immunoreactive bands were observed in microsomes from isosafrole-treated chickens (Figure 12D). In fact, treatment of chickens with both BNF and isosafrole resulted in less

immunoreactive CYP1A protein than treatment with BNF alone (Figure 12D). This suggests that isosafrole inhibited the induction of immunoreactive CYP1A protein by BNF in that species.

Mirror-image immunoblots were used to compare the immunoreactivity of a few microsome samples with three antibodies to CYP 1A protein on the same gel (Figure 13). In the rat microsome standards, two immunoreactive bands of approximately equal intensity were observed with the polyclonal antibody PEG, while only one band was readily apparent with the polyclonal antibody RYE. Bands of immunoreactivity between the avian microsomal samples and PEG were much fainter than those produced with MAb 1-12-3. No bands were observed in untreated birds of any species with the PEG antibody after a 30 min film development. An immunoreactive band with PEG was observed in a BNF-treated chicken but not an isosafrole-treated chicken, while a very faint band of immunoreactivity with MAb 1-12-3 was observed in the isosafrole chicken following a 30 min film development (Figure 13A). In two representative HGs, single bands of immunoreactivity with PEG were of about equal intensities in the BNF- and isosafrole-treated birds, while the band produced with MAb 1-12-3 in the isosafrole-treated HG was more intense (Figure 13B). Immunoreactivities in two representative DCCs were similar to those of HGs, except that the band of immunoreactivity between PEG and the BNF-treated DCC was quite wide and might consist of two poorly separated bands (Figure 13C). None of the avian microsomal samples in the mirror image gels produced detectable immunoreactive bands with the RYE antibody. When

microsomes from 24 birds representing each species and treatment were immunoblotted with the polyclonal RYE antibody, an immunoreactive band was only observed with one individual (the HG treated with both BNF and isosafrole, data not shown).

Alkoxyresorufin-O-dealkylase activities

<u>Profiles of relative alk-ROD activities; Effect of inducers</u>

Profiles of relative EROD, PROD, MROD and BROD activities are shown for each species and treatment group (Figure 14). The limit of detection of alk-ROD assays was 1 pmol/min/mg protein. In corn-oil treated control chickens, there was no measurable alk-ROD activity (Figure 14A). Alk-ROD activities in untreated Great Lakes fish-eating birds were generally greater in DCC than HG chicks (Figures 14B and 14C). A notable difference in BROD activity was observed between untreated DCC and HG chicks. BROD activity was not detectable in HG chicks but was nearly as great as EROD activity in DCC chicks (68 pmol/min/mg protein).

The profile of isosafrole induction varied greatly among the three avian species. In isosafrole-treated chickens, there was no measurable alk-ROD activity (Figure 14D). Isosafrole induced all four measured alk-ROD activities in both HG and DCC chicks (Figures 14E and 14F). The greatest isosafrole-mediated induction was a 67-fold increase in MROD activity in HG chicks (Table 6). Maximum alk-ROD activity in isosafrole-treated birds was greater in DCC chicks than in HG chicks (560 pmol/min/mg protein EROD versus 284

pmol/min/mg protein MROD, respectively). In no instance did microsomal incubation with cyclohexane result in increased alk-ROD activities (data not shown).

There were also species differences in the profile of BNF induction. In particular, HGs were comparatively refractory to BROD induction and exhibited greater PROD induction than chickens or DCCs (Figures 14G, 14H and 14I, Table 6). Maximum alk-ROD activities were greatest in chickens, followed by DCCs and HGs (1040 pmol/min/mg protein EROD, 599 pmol/min/mg protein EROD and 387 pmol/min/mg protein MROD, respectively).

The three species also exhibited different alk-ROD profiles in response to a combined treatment of BNF and isosafrole. In regards to both maximum activities and relative alk-ROD profiles, chickens and DCCs treated with both BNF and isosafrole displayed activities very similar to those treated with BNF alone (Table 6). In HGs, the profile of alk-ROD activities was similar among isosafrole, BNF or combined treatments. EROD and MROD activities were always greater than PROD or BROD activities. However, maximum alk-ROD activity was greater in HGs receiving combined treatment rather than those receiving treatment of either inducer alone (557 pmol/min/mg protein EROD with combined treatment versus 284 pmol/min/mg protein MROD with isosafrole treatment or 387 pmol/min/mg protein MROD with BNF treatment).

The profiles of alk-ROD activities were similar in chickens treated with PCB 126 and BNF (Figures 14G and 14M), while phenobarbital treatment produced little inductive effect. Induction of EROD, MROD and BROD activities

were all great in PCB 126-treated chickens, while induction of PROD was small (Table 6). Treatment of chickens with PCB 126 resulted in the overall greatest maximum alk-ROD activity of this experiment (1264 pmol/min/mg protein EROD). Phenobarbital treatment caused a slight induction of EROD activity in chickens (from below detection in controls to an average of 7.6 pmol/min/mg protein in phenobarbital-treated chickens). All other alk-ROD activities were not detected in phenobarbital-treated chickens.

Effect of inhibitors

Ellipticine was a potent and non-selective alk-ROD inhibitor in this experiment. Incorporation of 10 μM ellipticine in the reaction mixture resulted in at least 82% inhibition of all alk-ROD activities, and most activities were inhibited by greater than 96% (data not shown).

The effect of 200 μM furafylline on alk-ROD activities varied more among species than among treatments (Figure 15). In HGs, BROD was most dramatically inhibited (> 86%) in birds treated with inducers, while PROD inhibition in HGs was approximately 23%. EROD inhibition by furafylline was greatest in HGs treated with both BNF and isosafrole (41%). MROD was slightly inhibited in BNF and isosafrole-treated HGs (around 10%), but was not inhibited in HGs treated with both inducers. In DCCs, PROD was most dramatically inhibited (> 83%) in birds treated with inducers, while other alk-ROD activities were reduced by 50 to 60%. Profiles of inhibition in chickens were similar among treatments; BROD was inhibited most (usually less than

60% inhibition), followed by a similar inhibition of EROD and MROD (usually less than 40% inhibition). Furafylline also inhibited EROD activity in phenobarbital-treated chickens (mean activity reduced from 7.6 to 0.8 pmol/min/mg protein).

Relationship between EROD activity and immunoreactive CYP1A protein

Species- and treatment-related differences in the relationship between EROD activity and scup-equivalent CYP1A protein, as measured by immunoreactivity with MAb 1-12-3, were observed (Figure 16). In HGs and DCCs, there was no apparent clustering of treatment groups (isosafrole, BNF or combined). The greatest difference between the two fish-eating bird species was in the slope of the relationship between CYP1A protein and EROD activity; DCCs appeared to have a greater increase in EROD activity per increase in immunoreactive CYP1A protein than did HGs. In contrast, four treatment groups seemed to cluster in chickens. Chickens treated with PCB 126 exhibited a great increase in both EROD activity and CYP1A protein, while chickens treated with BNF exhibited a great induction in EROD activity but a lesser apparent induction of CYP1A protein. Chickens treated with isosafrole did not exhibit detectable EROD activity or CYP1A protein. Interestingly, chickens treated with both BNF and isosafrole exhibited greatly increased EROD activity, but relatively small increases in immunoreactive CYP1A protein.

DISCUSSION

Alk-ROD profiles in untreated birds

Patterns of alk-ROD activity in untreated individuals varied among the three avian species. Alk-ROD activities may have been greater in untreated HGs and DCCs relative to chickens because they were acquired from the wild. The HGs and DCCs were therefore exposed to ambient levels of environmental contaminants and were not true controls. BROD activity was not detected in untreated HGs but was nearly as great as EROD activity in untreated DCCs. The relative degree of BROD and MROD activity in untreated birds varied greatly among species in one comprehensive study (Melancon, 1996). Many avian species, including the Mallard Duck, Lesser Scaup, Sandhill Crane, Red-winged Blackbird, European Starling and Screech Owl, resembled the HGs in this study in that greater MROD than BROD activities were observed in untreated individuals. A few avian species such as the Black-necked Stilt, Barn Swallow and Northern Bobwhite resembled DCCs in that the activity of BROD was greater than that of MROD in untreated birds. Similar to the present study with nestlings, those researchers also observed that BROD activity was greater than MROD activity in hepatic microsomes from untreated pipping DCC embryos. However, alk-ROD profiles may sometimes change within a species at different life stages. In the American Kestrel, MROD activity was greater than BROD activity in adults, while the reverse was true in 10-day-old nestlings (Melancon, 1996). The biological mechanisms behind these differences in

catalytic activities would be a fruitful area for continued research.

Effect of inducers on avian CYP1A protein and alk-ROD activities

BNF was an effective inducer of alk-ROD activity and CYP1A-immunoreactive protein in all three avian species studied. It was interesting that the concentration of CYP1A1-like protein (as measured by immunoreactivity with MAb 1-12-3) in hepatic microsomes seemed to be induced by BNF to a similar extent in all three species, while the degree of induction of alk-ROD activities varied significantly among species. These discrepancies may have been caused by differing affinities of CYP1A1-like protein for MAb 1-12-3 among the three avian species, such that the quantity of CYP1A1-like protein was underestimated in species with lesser affinities. Alternatively, the CYP1A1-like protein may have had a greater specific activity in some avian species relative to others.

Induction of chicken EROD activity by BNF was greater than 1000-fold, which is greater than that typically measured in mammals. BNF can induce EROD activity up to 100-fold in rats (Haaparanta et al., 1983; Burke et al., 1985; Lubet et al., 1990a) and mice (Lubet et al., 1990b), and rainbow trout EROD activity was induced 28-fold by BNF (Celander and Förlin, 1991). The induction of EROD activity in chickens by BNF observed in this study was greater than that previously reported in chickens (40-fold; Gupta et al., 1990), Japanese Quail (20-fold; Lubet et al., 1990b) and Mallards (5-fold; Leffin and Riviere, 1992). In mammals, constitutive hepatic EROD activity is catalyzed

by CYP1A2, while induced EROD activity is predominately catalyzed by CYP1A1 (Nerurkar et al., 1993). The induction of EROD activity by BNF in this study, along with the induction of a protein with which MAb 1-12-3 was immunoreactive, suggests the presence of an inducible CYP1A1-like protein in all three avian species studied.

MROD activity was also induced by BNF in all three species studied here, and the relative order of MROD induction efficacy among species was similar to that of EROD (i.e., chicken>HG>DCC). MROD is considered to be primarily catalyzed by CYP1A2 in the rat (Namkung et al., 1988) and mouse (Tsyrlov et al., 1993). However, MROD was not induced in rats treated with isosafrole (Burke et al., 1985), which is considered to be a CYP1A2-specific inducer in mammals (Sesardic et al., 1990b). The degree of MROD induction by BNF was greater in chickens and HGs in this experiment (660 and 91-fold, respectively) than that observed in mammals treated with BNF (29, 32 and 45-fold in rats, hamsters and mice respectively; Lubet et al., 1990b), or in chickens (33-fold; Gupta et al., 1990) or Japanese Quail (36-fold; Lubet et al., 1990b). The HGs resembled hamsters (Lubet et al., 1990b) in that MROD activity was greater than EROD actitivity in BNF-treated individuals.

The induction of PROD activity by BNF is normally only 2- to 8-fold in mammals (Burke et al., 1985; Lubet et al., 1990b), and PROD was induced by BNF treatment 6-fold in Mallard Duck (Leffin and Riviere, 1992) and 4-fold in Japanese Quail (Lubet et al., 1990b). While the induction of chicken PROD by BNF treatment in this experiment was also small (1.5-fold), PROD activity was

more greatly induced in BNF-treated DCCs and HGs (13- and 53-fold, respectively). The isozyme specificity of PROD activity can be very different between birds and mammals. In mammals, PROD activity is greatly induced by phenobarbital (Burke et al., 1985; Lubet et al., 1990b), and is mediated by P450s in the 2B family (Namkung et al., 1988). In contrast, avian PROD activity is often not induced by phenobarbital treatment (Lubet et al., 1990b; Leffin and Riviere, 1992; Rattner et al., 1993). The present results are in agreement with these observations, since PROD activity was not detected in phenobarbital-treated chickens. The degree of induction by phenobarbital of other monooxygenase activities, and of CYP2B cross-reactive proteins, varies greatly among avian species (Ronis and Walker, 1989; Melancon, 1996). A significant correlation was observed between EROD and PROD activities in Great Blue Herons that were environmentally exposed to dioxins from pulp mill effluents (Bellward et al., 1990), and a significant correlation was observed between PROD and aromatic hydrocarbon hydroxylase (AHH) activities (but *not* EROD) in Black-crowned Night Herons that were environmentally exposed to organochlorine contaminants (Rattner et al., 1993). The combined results imply that PR may be metabolized by CYP1A-like enzymes in some avian species.

Like PROD, BROD is usually only minimally induced in mammals by BNF treatment (2-fold in rat, hamster and mouse; Lubet et al., 1990b). In the rat, BROD activity can be induced by a variety of agents. In one study, rat BROD activity was induced 95-fold by phenobarbital, 43-fold by isosafrole, and 17-

fold by BNF (Burke et al., 1985). Not surprisingly, in the rat BROD activity was found to be non-specific to a particular isozyme; both CYP1A1 and CYP2B1/2 exhibited significant activity (Namkung et al., 1988). Phenobarbital is a significant inducer of BROD activity in other mammals as well, including the mouse and rabbit (Lubet et al., 1990b). The relative inducibility of BROD seems to vary significantly among avian species. A 13-fold induction of BROD activity by BNF in Japanese Quail (Lubet et al., 1990b) is similar to the 15-fold induction of BROD activity in HG observed in this study. The induction of BROD activity by BNF was comparatively less in DCCs (5-fold), but greater in chickens (490-fold). Phenobarbital had a small effect on BROD induction in birds, including a 4-fold induction in Japanese Quail (Lubet et al., 1990b), a 3-fold induction in Black-crowned Night Herons (Rattner et al., 1993), and a lack of induction in chickens in this study. BROD activity may be a better biomarker of PHDH exposure than EROD activity in Bald Eagles. Bald Eagles living near pulp mills in British Columbia exhibited low absolute EROD activity (2 - 9 pmol/min/mg protein), while BROD activity was greater (6 to 56 pmol/min/mg protein) and was induced 6- to 8-fold at PHDH-contaminated sites relative to control sites (Elliott et al., 1996). In the channel catfish, BROD activity was increased 33-fold by the prototypical CYP1A inducer 3-methylcholanthrene, but BROD was not induced by phenobarbital (Ankley et al., 1987). In general, CYP1A may play a greater role in non-mammalian than mammalian BROD activity.

Differences in response to isosafrole treatment were observed among

the three avian species. Isosafrole treatment caused induction of alk-ROD activities and immunoreactive CYP1A protein in both HGs and DCCs, and the relative protein and alk-ROD induction patterns were similar to those caused by BNF. In contrast, neither alk-ROD activity nor MAb 1-12-3-reactive CYP1A protein were detectable in hepatic microsomes from isosafrole-treated chickens. Sinclair (1989) also did not observe detectable EROD activity in microsomes from chicken liver cells dosed *in vitro* with isosafrole, although cytochrome P450 content was induced. In those microsomes, a faint band of immunoreactivity of a 57 kDa protein was observed with a polyclonal antibody to chicken P-450_{MC}, a likely CYP1A1 protein. In our study, co-administration of BNF and isosafrole in chickens resulted in a suppression of immunoreactive CYP1A protein, but not EROD activity, relative to BNF treatment alone. It is possible that in the chicken, isosafrole could be bound to or blocking the MAb 1-12-3 epitope on the CYP1A1-like protein without affecting enzyme activity. This is somewhat doubtful, because the MAb 1-12-3 antibody reacts with CYP1A protein of a variety of taxa (Stegeman, 1989). Therefore, the epitope is likely to be a highly conserved sequence that is critical to enzyme function. An alternate possibility is that more than one cytochrome P450 isozyme could be induced in the chicken by BNF, and both could be immunoreactive with MAb 1-12-3. Both would need to have similar electrophoretic properties, since only one immunoreactive band was observed in BNF-treated chickens. If only one of the two isozymes exhibited EROD activity, binding of isosafrole to the non-EROD isozyme might somehow cause a loss of immunoreactive protein

without a loss of EROD activity. Interestingly, in mammals a metabolic intermediate of isosafrole generates a stable inhibitory complex *in vivo* with the heme of CYP1A2 (Ryan et al., 1980) but not CYP1A1 (Murray and Reidy, 1990).

Patterns of relative alk-ROD activities were useful indicators of inducer influence in both species. In untreated HGs, EROD activity was greater than MROD activity, whereas in isosafrole- or BNF-treated HGs MROD activity was greater than EROD activity. In untreated DCCs, BROD activity was greater than MROD activity, whereas in BNF- or isosafrole-treated DCCs MROD activity was greater than BROD activity. Similar results were obtained with pipping DCC embryos treated with 3-MC, which exhibited greater MROD than BROD activity (463 and 161 pmole/min/mg protein respectively) (Melancon, 1996). The profile of alk-ROD activities in BNF- or isosafrole-treated HGs resembled that of Common Terns dosed with PCB 126 and PCB 153 in the diet. In that experiment, Common Tern EROD and MROD activities were nearly equal and were induced, while PROD and BROD activities were small (Bosveld et al., 1995a).

Effect of inhibitors on alk-ROD activities

Ellipticine (10 μM) was a potent and non-specific inhibitor of all alk-ROD activities in all three avian species in this experiment. These results are similar to those observed in human hepatic microsomes, where 5 μM ellipticine non-selectively abolished both CYP1A1- and CYP1A2-mediated phenacetin-*O*-

deethylase activity (Tassaneeyakul et al., 1993). However, alk-ROD inhibition in Common Tern hepatic microsomes by ellipticine was quite different (Bosveld et al., 1995a). While PROD was 99% inhibited by 0.1 μM ellipticine, EROD and MROD activities were only 90% and 57% inhibited, respectively, by ellipticine concentrations ranging from 10 μM to 200 μM in the assay medium.

Furafylline is a selective inhibitor of CYP1A2 (and *not* CYP1A1) in several mammalian species, including mice (Tsyrlov et al., 1993), rats and humans (Sesardic et al., 1990a). However, the potency of furafylline as a CYP1A2 inhibitor varies dramatically among species. Human CYP1A2 is exquisitely sensitive to inhibition by furafylline (Sesardic et al., 1990a), since the IC_{50} for *in vitro* CYP1A2 inhibition was more than 1000-fold greater in the rat than the human. The present results show partial inhibition of some avian alk-ROD activities by furafylline, which is in marked contrast to the complete lack of EROD, MROD or PROD inhibition by furafylline observed by other workers in Common Tern hepatic microsomes at assay concentrations as great as 500 μM (Bosveld et al., 1995a). Differences in relative patterns of alk-ROD inhibition by furafylline were observed among avian species in this experiment. In chickens treated with BNF, PCB 126 or isosafrole and BNF, no differences among treatments were observed in the degree of furafylline inhibition of EROD, BROD and MROD, which were all very induced. This result, coupled with the same pattern of alk-ROD induction in chickens by these three treatments, may indicate that all three treatments were causing induction of the same alk-ROD-active isozyme(s) by the same mechanism. The fact that

EROD and MROD were very similarly inhibited by furafylline in chickens may indicate that ER and MR are metabolized by the same isozyme(s); the consistently greater EROD than MROD activity may indicate that the isozyme(s) has a slighly greater affinity for ER than MR. The fact that BROD activity was lower than EROD or MROD activity in induced chickens, coupled with the greater susceptibility of BROD to furafylline inhibition, might also be due to a lesser affinity of the isozyme(s) for BR relative to ER or MR. DCCs resembled chickens in that EROD, MROD and BROD were all induced similarly among several treatments (BNF, isosafrole, or BNF and isosafrole), and they were all inhibited to a similar extent by furafylline. This may mean that all treatments induced the same alk-ROD-active isozyme(s) in a similar manner in DCCs. However, "constitutive" MROD activity in untreated DCCs was not inhibited by furafylline. It is therefore possible that constitutive MROD may involve a different isozyme than that involved in induced DCCs, and that the constitutive isozyme is not affected by furafylline. In both DCCs and HGs, the alk-RODs that were most dramatically inhibited by furafylline in induced birds had small activities prior to furafylline treatment (i.e., PROD in DCCs, and BROD and PROD in HGs). Although EROD and MROD activities were similar and induced in HGs from all treatments (BNF, isosafrole, or BNF and isosafrole), the degree of furafylline inhibition was not similar between EROD and MROD. In particular, MROD activity was only minimally inhibited by furafylline in HGs. This may indicate that MROD and EROD activities are not mediated by identical isozyme(s) in the same proportions in HGs, as might have been surmised after

examining the induction data alone.

CYP1A isozymes in birds

My observation of reactivity of a single protein band in avian hepatic microsomes with MAb 1-12-3 following BNF treatment provides support to many other reports of a CYP1A1-like protein in birds. Black-crowned Night Herons treated with 3-methylcholanthrene (3-MC) also showed induction of a single protein band with cross-reactivity to MAb 1-12-3 (Rattner et al., 1993), as did pigeons treated with Aroclor 1254 (Borlakoglu et al., 1991). Bald Eagles (Elliott et al., 1996) and chickens (Stegeman, 1989) have also exhibited immunoreactivity of a single protein band in hepatic microsomes with MAb 1-12-3. One protein band has also been detected in hepatic microsomes of several fish-eating bird species using various polyclonal antibodies to rat CYP1A1 protein (Ronis et al., 1989a, b; Murk et al., 1993; Sanderson et al., 1994). The observation of a single band of immunoreactivity with MAb 1-12-3 in avian microsomes does not prove that there was only one immunoreactive protein in the microsomes. There is considerable evidence for the existence in birds of at least two CYP1A-like proteins with similar electrophoretic properties, which often appear as a single wide band on gels following electrophoresis (Sundstrom et al., 1989; Rifkind et al., 1994).

The relatively weak cross-reactivity of avian hepatic microsomes with polyclonal PEG and RYE antibodies probably indicates that the epitopes to mouse CYP1A2 were not recognized. The weak immunoreactivity of PEG with

microsomes from BNF-treated birds, and isosafrole-treated HGs and DCCs, was probably due to recognition of mouse CYP1A1. This would explain why the microsomes from almost all of the birds did not exhibit detectable immunoreactivity with RYE, which recognizes mammalian CYP1A2 forms strongly but has very weak immunoreactivity with mammalian CYP1A1. The faint band detected with RYE and one HG was probably due to CYP1A1 recognition. That individual had a greater concentration of microsomal scup-equivalent CYP1A protein (measured with MAb 1-12-3) than any other bird in the study. In a related study that involved six species of fish-eating birds, a polyclonal antibody against rat CYP1A2 recognized a protein in only one species (Ronis et al., 1989b). Microsomes from the Great Cormorant contained a single protein band that was recognized by antibodies to rat CYP1A2. Since that band was of the same molecular weight as an anti-CYP1A1 protein band observed in the cormorant, the authors concluded that there was only a single CYP1A protein in the cormorant that had shared epitopes to CYP1A1 and CYP1A2.

Several studies present evidence for the existence of multiple CYP1A forms in birds. In all of these studies there was strong evidence for the existence of an avian CYP1A1-like protein, due to immunoreactivity with antibodies to CYP1A1 proteins from other species, inducibility with classic CYP1A1 inducers such as TCDD, BNF and 3-MC, and characteristic catalytic activities. However, the properties of other avian CYP1A proteins vary from study to study, and their relationship to mammalian CYP1A2 is less clear. In

this study, treatment of birds with isosafrole and BNF, considered to be selective inducers of mammalian CYP1A2 and CYP1A1 respectively, did not appear to cause selective induction of avian analogs. Instead, in the HG and DCC isosafrole seemed to cause induction of the CYP1A1-like protein, in a manner similar to BNF. In isosafrole-treated chickens neither MAb 1-12-3-reactive protein nor EROD activity were detected. EROD activity could not be reconstituted by microsomal treatment with cyclohexane in those chickens, but the induction of P-450s other than the CYP1A1 analog may have occurred. Two bands of immunoreactivity against polyclonal anti-rat CYP1A1 were observed in guillemots, but neither of these electrophoretically separated proteins were recognized by polyclonal anti-rat CYP1A2 on Western blots (Ronis et al., 1989b). Following electrophoresis and protein staining, several protein bands were observed in Aroclor-treated pigeons with molecular weights in the CYP1A range. The identity of one of the proteins was established as CYP1A1-like based on immunoreactivity with MAb 1-12-3, but the identity of the other protein was assumed without further characterization to correspond to a CYP1A2-like protein (Borlakoglu et al., 1991).

Studies that have focused on the purification and characterization of cytochrome P-450 forms in chickens treated with classical CYP1A inducers have provided some insight into avian CYP1A forms. Purified P450s designated "$TCDD_{AHH}$" (Rifkind et al., 1994), "BNF-C" (Gupta et al., 1990) and "P450MC" (Sinclair et al., 1989) may all be the same protein, based on N-terminal amino acid sequences, and the purified P450 "P448L" (Hokama et al.,

1988) also has similar catalytic and spectral properties. Based upon catalytic and immunochemical properties, this protein appears to be an analog of mammalian CYP1A1. A second P-450 with a similar molecular weight as CYP1A has also been detected in chickens following treatment with classic CYP1A-type inducers, but it appears to be unrelated to CYP1A. Since they all have similar molecular weights, do not catalyze the EROD reaction, and have a high spin signal, "P448H" (Hokama et al., 1988), "BNF-A" (Gupta et al., 1990) and "FA" (Rifkind et al., 1994) may be the same protein. The protein FA was not related to CYP1A based upon catalytic, sequence or immunochemical characteristics, and had a sequence similar to BNF-A. Another P450 form designated $TCDD_{AA}$ has been purified from hepatic microsomes from TCDD-treated chickens, and was characterized as being CYP1A2-like (Rifkind et al., 1994). This protein showed cross-reactivity with a polyclonal antibody against rat CYP1A2, and shared some sequence and catalytic properties with mammalian CYP1A2. Another protein that was designated "BNF-B" has been purified from BNF-treated chickens (Gupta et al., 1990). The BNF-B protein shared some common properties with $TCDD_{AA}$, but their sequences did not match. It is evident that several proteins in the same molecular weight range as the CYP1A1 analog can be induced by classical CYP1A inducers in birds. Some of these proteins may be CYP1A analogs, while others appear to be unrelated to CYP1A. The results of the isosafrole treatment in this study indicate that the chicken CYP1A "system" may be different from that of the HG or DCC. Isosafrole may cause induction of a

CYP1A2-like enzyme with no EROD activity in chickens, while in the DCC and HG isosafrole may simply induce the CYP1A1-like enzyme. Enzyme purification studies with avian species other than chickens following treatment with PHDHs would therefore be of considerable interest. The further characterization of these avian P450s and the availability of avian CYP1A-specific antibodies would aid greatly in the study of CYP1A induction in fish-eating birds that are environmentally exposed to Ah-active environmental contaminants.

Table 6. Alkoxyresorufin-*O*-dealkylase (alk-ROD) activity and induction by BNF and/or isosafrole or PCB 126. "Greatest activity" refers to the alk-ROD of greatest relative activity expressed as pmol resorufin/min/mg microsomal protein. Fold-induction above constitutive activity is indicated for each treatment, species and alk-ROD activity. All values are means. Cleaved side chains in alk-ROD reactions: E = eth, P = pent, M = meth, and B = benzyl.

Inducer		Untreated		Isosafrole		BNF			Isosafrole + BNF			PCB
Species		HG	DCC	HG	DCC	CK[a]	HG	DCC	CK	HG	DCC	CK
n		3	3	3	3	3	3	2	3	1	2	4
Greatest ALK-ROD		E	E	M	E	E	M	E	E	E	E	E
Activity		15.2	78.8	284	560	1040	387	599	1041	557	601	1264
fold-induction	E	-	-	17	7.1	1040*	19.3	7.6	1041*	36.5	7.6	1264*
	P	-	-	11*	12*	1.5*	52.6*	13.4*	0	84.7*	11.7*	4.8*
	M	-	-	67	16	660*	90.8	19.5	608*	126	19.5	978*
	B	-	-	5.2*	4.3	490*	15.4*	4.8	473*	64.7*	5.4	882*

a = White Leghorn chicken (*Gallus domesticus*)

*value is actual activity in pmol resorufin/min/mg protein, since activity in untreated birds was below the assay detection limit of 1 pmol/min/mg protein.

Figure 12. Immunoreactivity of DCC (A), HG (B), and chicken (C and D) hepatic microsomes with MAb 1-12-3. Treatments are indicated below each band ("cont" = control, "iso" = isosafrole, "BNF" = β-naphthoflavone, "B + I" = β-naphthoflavone and isosafrole, "PCB" = PCB 126, "PB" = phenobarbital). Lane 10 is pmol of scup CYP1A1 standard.

A. Double-crested Cormorant

cont cont iso iso iso BNF BNF B+I B+I 0.25 stnd

B. Herring Gull

cont cont cont iso iso iso BNF BNF BNF 0.50 stnd

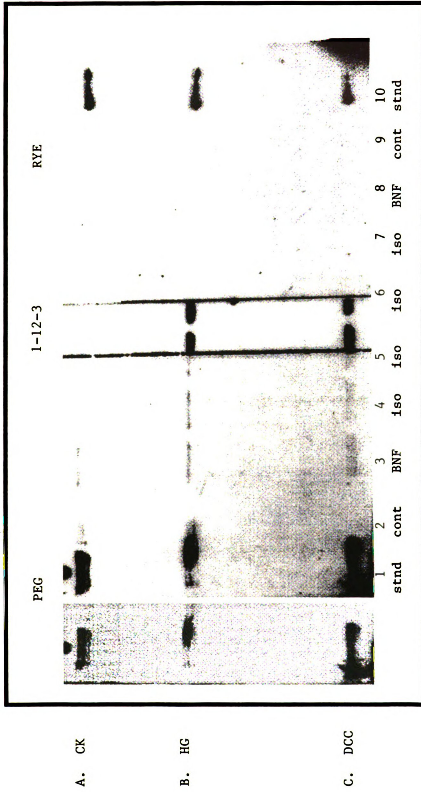
C. Chicken

cont cont cont PCB PCB PCB PB PB PB 0.75 stnd

D. Chicken

BNF BNF BNF iso iso iso B+I B+I B+I 1.0 stnd

Figure 13. Mirror image immunoblots of chicken (A), HG (B) and DCC (C) hepatic microsomes with three antibodies against CYP1As. Within each gel, lanes 1 and 10 are microsomes from a rat treated with PCB 77 used as a positive control. Lanes 2 and 9 are microsomes from the same control birds, while lanes 3 and 8 are microsomes from the same β-naphthoflavone-treated birds. Lanes 4 through 7 are microsomes from the same isosafrole-treated birds. Lanes 1 - 5 were incubated with the PEG antibody, while lanes 6 - 10 were incubated with the RYE antibody. Lanes 5 and 6 were split, and the middle section of each was incubated with MAb 1-12-3.



A. CK

B. HG

C. DCC

Figure 14. Mean and s.d. of hepatic alk-ROD activities (pmol resorufin/min/mg microsomal protein). Dark bars represent constitutive activities in control birds; open bars represent induced activities in treated birds. Cleaved side chains in alkoxyresorufin *O*-dealkylase reactions: E = eth, P = pent, M = meth, and B = benzyl. Note the different values on the y axes of the graphs.

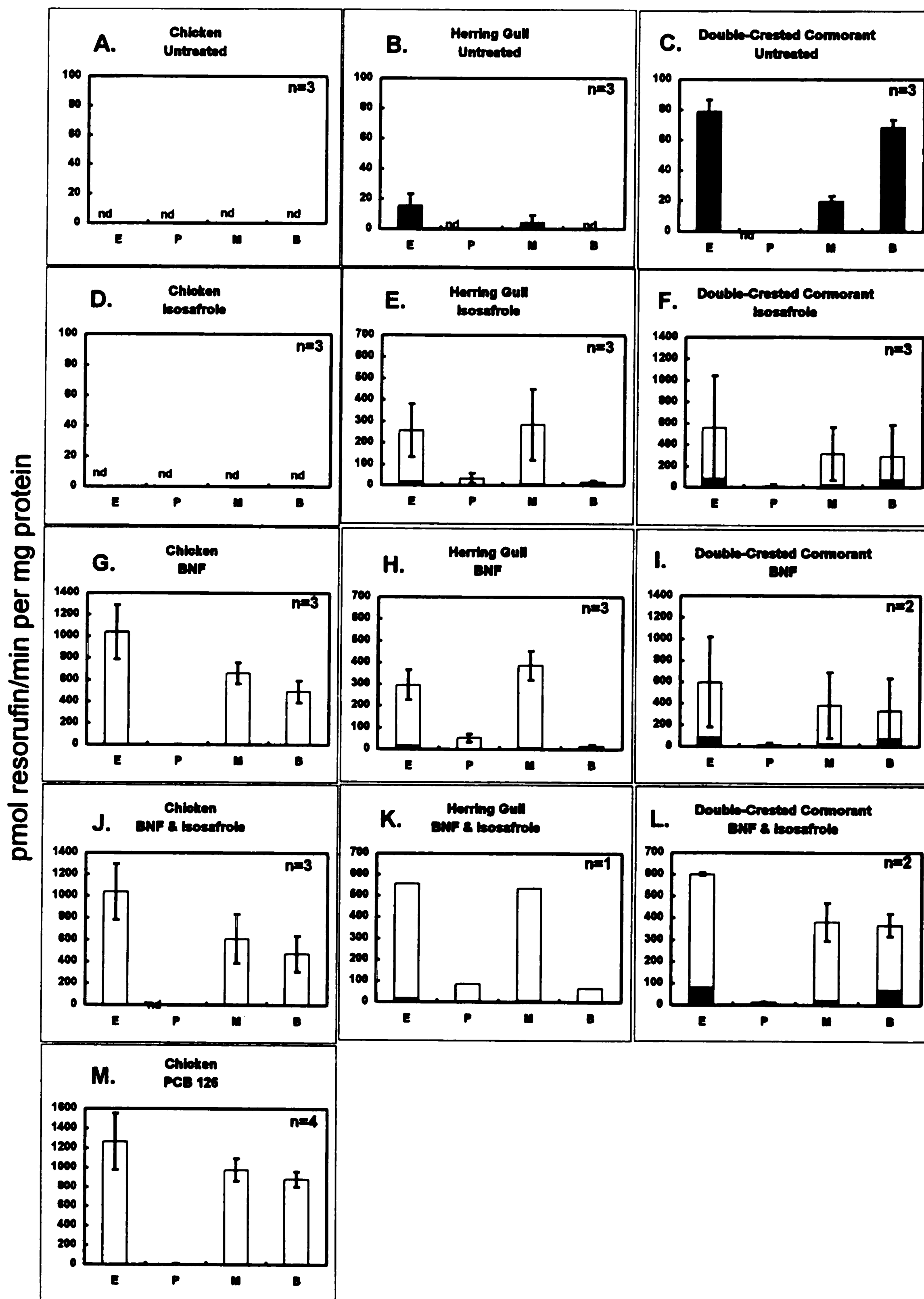
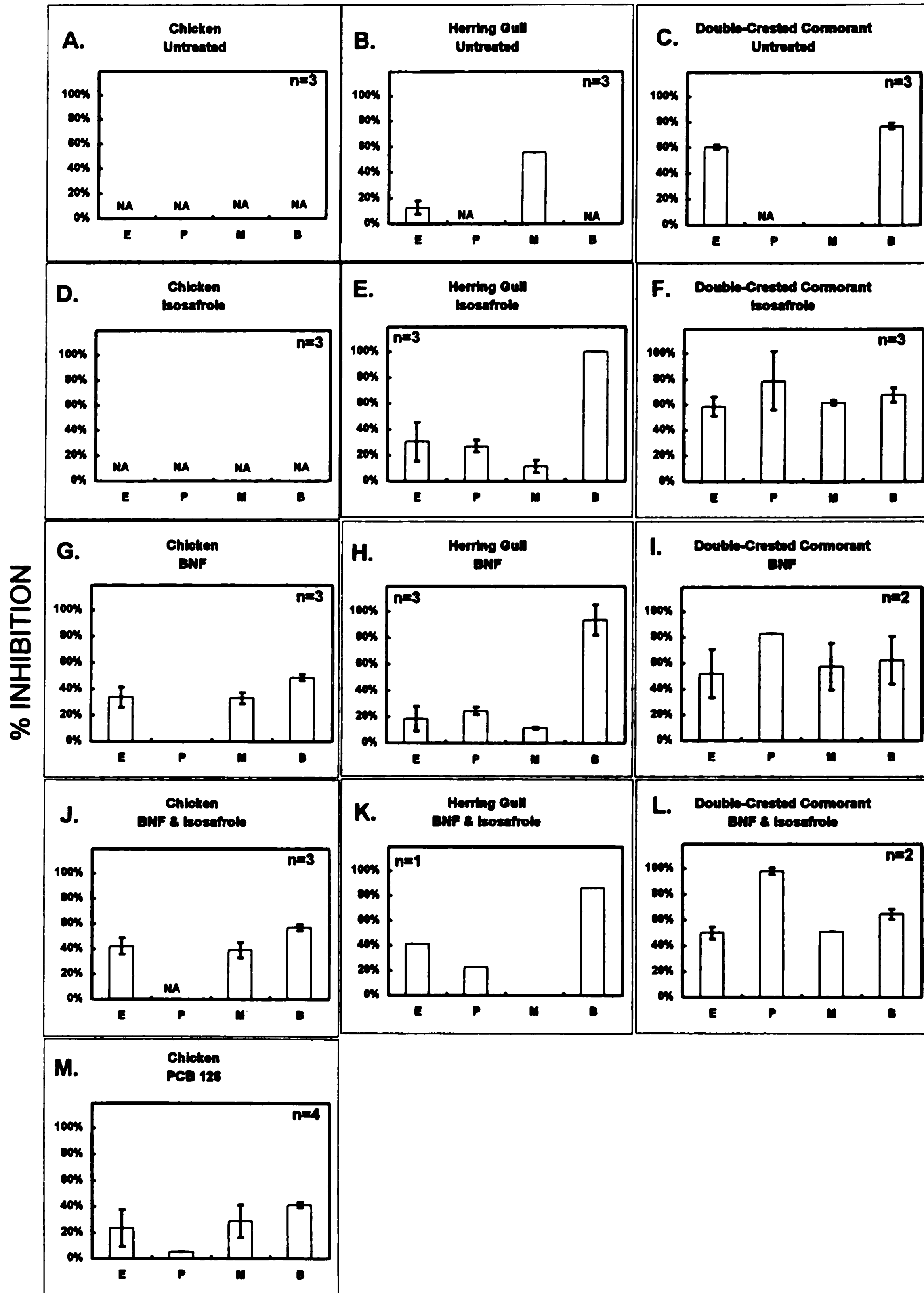

pmol resorufin/min per mg protein
A. Chicken Untreated
n=3
B. Herring Gull Untreated
n=3
C. Double-Crested Cormorant Untreated
n=3
D. Chicken Isosafrole
n=3
E. Herring Gull Isosafrole
n=3
F. Double-Crested Cormorant Isosafrole
n=3
G. Chicken BNF
n=3
H. Herring Gull BNF
n=3
I. Double-Crested Cormorant BNF
n=2
J. Chicken BNF & Isosafrole
n=3
K. Herring Gull BNF & Isosafrole
n=1
L. Double-Crested Cormorant BNF & Isosafrole
n=2
M. Chicken PCB 126
n=4
nd
E P M B

Figure 15. Percent inhibition of hepatic alk-ROD activities by 200 μM furafylline (mean ± s.d.). Cleaved side chains in alkoxyresorufin *O*-dealkylase reactions: E = eth, P = pent, M = meth, and B = benzyl.


% INHIBITION
A.
Chicken
Untreated
n=3
B.
Herring Gull
Untreated
n=3
C.
Double-Crested Cormorant
Untreated
n=3
D.
Chicken
Isosafrole
n=3
E.
Herring Gull
Isosafrole
n=3
F.
Double-Crested Cormorant
Isosafrole
n=3
G.
Chicken
BNF
n=3
H.
Herring Gull
BNF
n=3
I.
Double-Crested Cormorant
BNF
n=2
J.
Chicken
BNF & Isosafrole
n=3
K.
Herring Gull
BNF & Isosafrole
n=1
L.
Double-Crested Cormorant
BNF & Isosafrole
n=2
M.
Chicken
PCB 126
n=4
0%
20%
40%
60%
80%
100%
NA
E
P
M
B

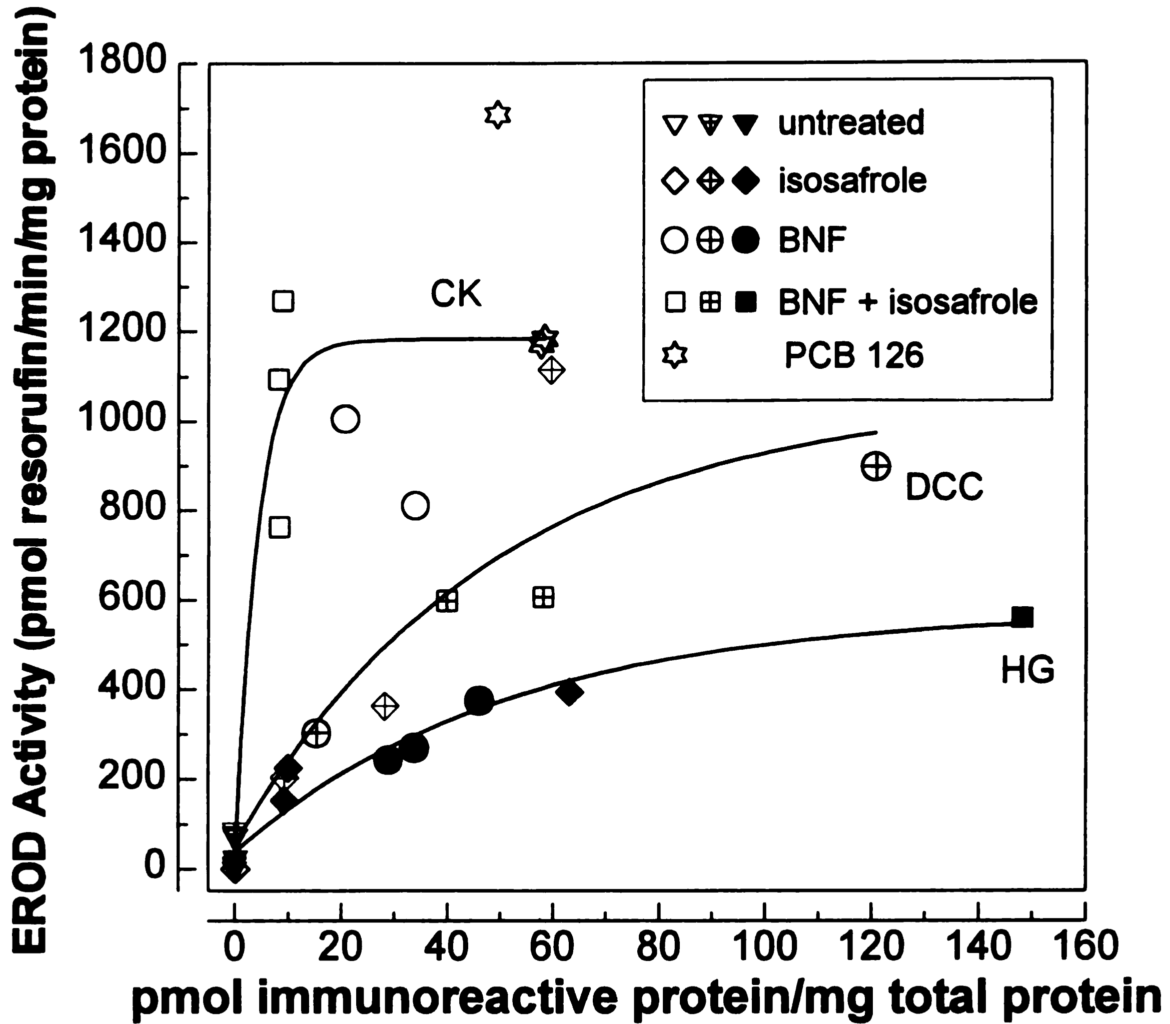


Figure 16. Ethoxyresorufin-*O*-deethylase (EROD) activity (pmol resorufin/min/mg microsomal protein) and immunoreactive CYP1A protein (pmol scup-equivalents to MAb 1-12-3) in livers from individual birds that were treated *in vivo* with the indicated inducers. Symbol representations: open = chicken, hatched = Double-crested Cormorant, closed = Herring Gull.

SUMMARY AND CONCLUSIONS

The CBT was not as sensitive or specific as the EROD assay as an indicator of PHDH exposure and effect in birds. EROD activity was characterized by a consistently small constitutive activity in untreated birds. Caffeine N-demethylation rates were considerably more variable than EROD activity rates in untreated birds. Many untreated birds exhibited significant constitutive caffeine N-demethylation, regardless of the CBT method utilized. In addition, the degree of induction of EROD activity was greater than the degree of induction of caffeine N-demethylation. In chickens treated with great concentrations of PHDHs, EROD activity was up to three orders of magnitude greater than that of untreated chickens. In contrast, caffeine N-demethylation rates merely doubled in treated chickens as compared to controls, as measured by the ^{13}C-CBT. In previous studies with TCDD and chickens, the maximal degree of caffeine N-demethylation induction observed was one order of magnitude as measured by the ^{14}C-CBT.

The CBT does not have much potential utility as a biomonitoring tool for exposure to PHDH compounds in birds, because it would be difficult to distinguish PHDH-related enzyme induction from constitutively great rates of caffeine N-demethylation. Present environmental exposures of birds to PHDHs

are generally far below the levels that cause maximum CYP1A induction, and biomonitoring tools must be sensitive to detect the small biological changes that occur. Another reason the CBT is a less sensitive method than the EROD assay is due to the life stages at which enzyme activities can or cannot be measured. The pipping embryo is the preferred life stage at which to measure EROD activity in biomonitoring studies (Rattner et al., 1996), but the CBT cannot be performed during the pipping embryo stage because the birds are too small and weak for the procedure.

The ^{14}C-CBT was more effective than the ^{13}C-CBT at measuring differences in *in vivo* caffeine N-demethylation between control chickens and chickens treated with great concentrations of PHDHs (Feyk and Giesy, 1996). The ^{14}C-CBT method produced less variable and more robust estimations of enzyme activity than did the ^{13}C-CBT, due to differences in methods of breath collection (cumulative versus spot-checking), sensitivity of signal detection (scintillation versus ^{13}C enrichment), and accuracy of data analysis (direct attribution of scintillation to the caffeine label versus complex calculations involving many assumptions).

The ^{14}C-CBT had utility as a laboratory tool with which to measure PHDH-mediated enzyme induction over time in individual birds. The ^{14}C-CBT was performed multiple times in Common Tern nestlings over the course of their development, and changes in enzyme activity were observed. The ^{14}C-CBT method was not invasive, as no alterations of survival, growth or morphological development were observed in the CBT test subjects relative to

paired controls. The ^{14}C-CBT could effectively be used to measure relatively great alterations in enzyme activities that occur over time in response to endogenous factors, environmental conditions or chemical exposures.

Both similarities and differences were observed in inducibilities and enzyme activity patterns among the three avian species examined. Chicken CYP1A was not induced by isosafrole, although HGs and DCCs were strongly induced by the compound. All three species exhibited the induction of a CYP1A1-like protein following treatment with BNF. The patterns of alk-ROD metabolism varied significantly among species. In particular, "constitutive" BROD activity was much greater in DCCs than in HGs. The induction of MROD activity by BNF or isosafrole was great in the HGs and DCCs, and the relative patterns of EROD, MROD and BROD activities were useful as indicators of PHDH exposure in those species.

Evidence suggests that CYP1A is involved in caffeine N-demethylation in birds. Caffeine N-demethylation is induced in concert with EROD activity following exposure to PHDHs, and is probably partially mediated by CYP1A1. However, the great constitutive activities of caffeine N-demethylation observed both in the chicken ^{13}C-CBT study and the Common Tern ^{14}C-CBT study indicate that other enzymes in addition to CYP1A1 are probably also involved.

Recommendations for Wildlife Researchers and Managers

While the CBT shows promise as a method with which to measure avian CYP1A activity, this research illustrates the need for further investigations of

caffeine as an avian CYP1A substrate. The avian CBT should not be adapted as a standard method until the enzyme(s) involved in caffeine N-demethylation are more definitively determined for each species to be studied. Further characterization of avian caffeine N-demethylation should focus on enzyme purification in each species of interest following treatment with specific inducing agents, followed by the acquisition of amino acid sequence information and the production of antibodies to the enzymes. The antibodies could then be used as specific inhibitors in *in vitro* caffeine assays with avian hepatic microsomes.

Following a determination of the specificity of caffeine N-demethylation for CYP1A in an avian species, the ^{14}C-CBT should be used to learn more about the details of CYP1A induction. Since the CBT can be performed multiple times in an individual bird, it is a potentially valuable method with which to investigate the influence of various factors on CYP1A activity. The influence of age, season, reproductive status, diet and temperature could be investigated. Also, information could be obtained regarding the onset and duration of CYP1A induction following chemical exposure. This would provide wildlife managers with valuable information with which to design improved CYP1A biomonitoring programs and to properly interpret the significance of CYP1A activity data.

Finally, managers should support the continued development and improvement of non-invasive methods with which to biomonitor the effects of environmental contaminants on wildlife. Future research could focus on

attempts to improve the ^{13}C-CBT method in order to reduce measurement variability. In particular, the accuracy of data calculations could be improved for the ^{13}C-CBT. It would be best to measure actual CO_2 expiration rather than to calculate an estimated quantity. Alternatively, the estimation of total expired CO_2 might be improved by sedation of the bird prior to the ^{13}C-CBT. Sedation would produce a more uniform breathing rate by the bird throughout the ^{13}C-CBT. However, it would first be necessary to ascertain that the administered sedative would not affect CYP1A activity. Accuracy of background $^{13}CO_2/^{12}CO_2$ ratios might be improved by the performance of multiple measurements of background breath. Another research tactic might be to avoid the complications of avian respiration and the one-carbon metabolic pool by measurement of caffeine metabolites in the blood. This would best be accomplished by the administration of caffeine radiolabelled within the xanthine backbone rather than on a methyl group. Radiolabelled metabolites could then be extracted from plasma and quantified by high pressure liquid chromatography with radiometric detection.

The avian CBT is not a suitable replacement for the EROD assay in biomonitoring programs at this time. However, this research suggests that biomonitoring programs could be improved by the measurement of several *ex vivo* alk-ROD activities in addition to EROD activity. The relative patterns of EROD, MROD and BROD activities were different in untreated HGs and DCCs relative to PHDH-treated HGs and DCCs, and were useful as indicators of PHDH exposure. Therefore, it is recommended that alk-ROD activity profiles

of these three substrates be examined in future avian biomonitoring studies, because valuable information would be obtained for little added time or cost above that required for the measurement of EROD activity alone.

APPENDICES:

STANDARD OPERATING PROCEDURES AND LABORATORY PROTOCOLS

AQUATIC TOXICOLOGY LABORATORY

DEPARTMENT OF FISHERIES AND WILDLIFE
PESTICIDE RESEARCH CENTER
INSTITUTE FOR ENVIRONMENTAL TOXICOLOGY
MICHIGAN STATE UNIVERSITY

Draft Release 1.0

Prepared by Lori A. Feyk

April 9, 1995

Method Standard Operating Procedure Check List:

TITLE: **THE AVIAN CAFFEINE BREATH TEST (CBT) WITH STABLE ISOTOPICALLY (^{13}C) LABELLED SUBSTRATE**

SCOPE: This protocol describes the procedure used to perform the caffeine breath test in birds using caffeine labelled with the stable isotope ^{13}C. This method allows for an *in vivo* assessment of the subject's cytochrome P-450-1A activity.

REFERENCED DOCUMENTS:

A description of the methods for the avian CBT using caffeine labelled with the radioactive isotope ^{14}C can be found in the accompanying SOP entitled "The avian caffeine breath test (CBT) using radioactive ^{14}C-labelled caffeine".

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SUMMARY OF METHOD:

The bird is weighed and the caffeine injection volume is calculated for a dose of 3 mg caffeine/kg body weight. The bird's background breathing rate is recorded (i.e., number of breaths/30 sec). The local anesthetic Cetacaine® is applied to the glottis and trachea with a Q-tip and re-applied as needed. An endotracheal tube/CBT apparatus is inserted into the trachea approximately 3 cm and a background breath sample is obtained. A 50 cc sample of breath is removed from the apparatus balloon into a syringe, and then injected into five Exetainer tubes in 10 cc aliquots. An injection of 3 mg of ^{13}C-labelled caffeine/kg body weight is made into the brachial vein and the timer is started. Breath samples are taken every 5 min for 40 min as described above. The breathing rate is monitored every 10 min during the CBT. The $^{13}C/^{12}C$ ratio in the breath samples is analyzed with an Isotope Ratio Mass Spectrometer (IRMS).

SIGNIFICANCE AND USE:

The induction of cytochrome P450-1A (CYP1A) activity is an important biomarker of exposure of birds to polyhalogenated aromatic hydrocarbons (PHDHs) such as some PCB and dibenzodioxin congeners. These are environmental contaminants of concern in some areas of the world, including the Great Lakes of North America. CYP1A activity is traditionally measured *ex vivo* in liver tissue, and test subjects are usually killed to obtain the liver sample. The CBT method is a less invasive *in vivo* method to measure CYP1A activity which obviates the need to kill the test bird. Since the method is non-destructive, it can be used to measure changes in CYP1A activity over time in response to changing PHDH exposure, to changing environmental factors such as temperature, or to internal changes such as fluctuating levels of sex hormones. In addition, the less invasive nature of the breath test may enable the monitoring of the CYP1A induction status of threatened or endangered bird species in PHDH-contaminated areas.

Relationship to similar assay; comparative advantages of each

This assay is similar to the CBT for birds using radioactive ^{14}C-labelled caffeine. Both assays measure *in vivo* CYP1A activity. The assay described in this document involves caffeine labelled with the stable isotope ^{13}C. The relative advantage of using the stable-isotopically labelled compound is that it is inherently less hazardous to work with. All hazards associated with the use of radioactivity, including harm to worker or subject health and contamination of the environment, are eliminated when stable isotopes are substituted for radioisotopes. In addition, the substitution of stable isotopes for radioisotopes alleviates the need to obtain special radioactive use permits, which are extremely difficult to obtain for field work.

The major disadvantage to the use of stable isotopically-labelled caffeine is that the samples are more difficult and expensive to analyze, and require the use of a relatively rare and expensive analytical instrument. Stable isotope breath samples must be analyzed with IRMS, while radioactive breath samples are analyzed with a common scintillation counter. In addition, the type of data which is obtained from the stable isotope method is less straightforward to interpret. Stable isotope data are expressed as ratios of ^{13}C to ^{12}C, and it is not straightforward to relate this information back to the amount of caffeine metabolized. Since the natural abundance of ^{14}C is very low, all measurable ^{14}C activity in a radioactive breath sample can be attributed to caffeine

metabolism. It is a simple matter to calculate the amount of caffeine metabolized based on the scintillation counts. In contrast, approximately 1.1% of carbon in the environment is in the form of the stable isotope ^{13}C. The exact ratio of abundance can be different in various environmental compartments, including the levels of the food chain. This makes data interpretation of stable isotope data more difficult, as background metabolic processes need to be taken into account.

Due to the sizable advantages of the radioactive CBT method, including ease of data analysis and interpretation, it is recommended that that method be used whenever possible during laboratory experiments. However, due to the difficulties in obtaining radioactive use permits for field studies it is recommended that the stable isotope CBT method outlined in this SOP be implemented for field CBT work.

INTERFERENCES:

1. The bird's breathing rate can effect the ratio of $^{13}C/^{12}C$ in the breath. The theory behind this problem is that while the amount of ^{13}C is the breath during the CBT is essentially fixed and stems directly from the rate of caffeine metabolism, an increase in regular $^{12}CO_2$ can occur if breathing becomes more rapid. This would result in a dampening of the caffeine metabolism signal. For this reason it is desirable to maintain the same breathing rate in the bird throughout the test, so that the background breath ratio is as directly comparable to the CBT breath ratios as possible.

   The implication of this is that stress to the bird must be minimized however possible. Covering the bird's eyes often helps. In addition, every effort must be made to control the ambient temperature in order to minimize panting; this may be difficult in a field setting. The stress response is observed to be highly variable from individual to individual, and this problem may be a largely uncontrollable variable which adds to the overall variability of the method.

2. Posture can effect avian respiration in several ways (King and Payne, 1964). When chickens are placed on their back, amplitude of breathing is reduced, frequency of breathing is increased and the minute volume is reduced in comparison to erect chickens. Since breathing rates influence the CBT (see section 1), it is important that test subjects remain in an erect, "natural" position during the test.

3. Tracheal dead space must be considered when using this method. This refers to the volume of air which fills the trachea upon inhalation and then is immediately

exhaled without gas exchange having taken place. When performing the breath test in humans, the first part of each exhaled breath is discarded in order to avoid the inclusion of dead space air in the sample. The method described in this SOP for the CBT in birds involves sampling the entire contents of many breaths, including the dead space air. The tracheal dead space volume of the domestic chicken is approximately 4.56 ml, out of a total tidal volume of 33 ml. Since the concentration of CO_2 in the exhaled breath is much greater than that in the ambient air/dead space, the effect of dead space air is probably negligible on the $^{13}CO_2/^{12}CO_2$ isotopic ratio. Also, this is an error which is constant from bird to bird within the same species and size. This factor should be considered when applying the CBT to a different bird species, however. Results could be particularly affected in birds with elongated necks such as herons, or with tracheae elongated into convolutions such as some cranes and swans. Tracheal volumes for many species are given by Hinds and Calder (1971).

APPARATUS:

The breath sampling apparatus is diagrammed in dissertation Figure 2. A Coles-style endotracheal tube (size 4.5 for adult White Leghorn hens) is used. Two pieces of Clay Adams Intramedic® polyethylene tubing (I.D. 1.67 mm, O.D. 2.42 mm) are inserted inside the endotracheal tube. The short piece, which extends approximately 4 cm inside the tube, is connected to a miniature one-way inhalation valve (Hans Rudolph, Kansas City MO). This piece of tubing should be as short as possible in order to keep breathing resistance to a minimum. The longer piece of tubing, which extends approximately 9 cm inside the tube, is connected to a miniature one-way exhalation valve (Hans Rudolph, Kansas City MO). Its longer length is an attempt to obtain a sample as close to the lungs as possible in order to minimize dead space air. The polyethylene tubing is connected to the endotracheal tube and the inhale/exhale valves with plastic stubs of female Luer fittings which are epoxied into place. The exhalation valve is connected to a stopcock, which is connected to both a syringe septum and a 200 cc inflatable latex rebreathing bag (Hans Rudolph, Kansas City MO). During breath sampling, the stopcock is open between the exhalation valve and the rebreathing bag. Then, in order to withdraw the breath from the bag into a syringe the stopcock is opened between those two ports.

MATERIALS:

A. Instruments

1. Isotope Ratio Mass Spectrometer
2. Scales to weigh bird and liver (laboratory or portable for field)

B. Work supplies

1. test procedures, laboratory or field notebook
2. Cetacaine® local anesthetic
3. long-stemmed Q-tips
4. 1 ml Tuberculin syringes
5. 25-gauge needles
6. alcohol swabs
7. caffeine injection solution
8. CBT apparatus as described above
9. timer
10. 13 ml Exetainer® tubes with screw-top cap with septum (Europa Scientific, Cincinnati OH), 45 per CBT
11. styrofoam test tube holder/shipper (Polyfoam Packers Corp., Wheeling IL), 1 per CBT
12. 60 cc syringe
13. adjustable cat harness
14. vet wrap (Coban™ 1583 Self-Adherent Wrap, 3M Medical-Surgical Div., St. Paul MN)
15. labels for Exetainer tubes (Avery style 5267)

C. Supplies for removing the liver (if desired)

1. liquid nitrogen-safe 2 ml vials with caps
2. paper
3. filet knife
4. small sharp scissors
5. garden shears
6. liquid nitrogen
7. cloth labels for liquid nitrogen vials
8. tweezers

D. Additional supplies to pack for field work

1. dry ice or liquid nitrogen dewar
2. cooler for carcass storage
3. 1 liter distilled water
4. ziplock, whirlpack and alligator bags
5. sharpies (fine black)
6. water/weatherproof lab notebook and pen
7. pencil and ballpoint pen
8. lab tape and duct tape
9. garbage bags
10. kim wipes
11. sharps container
12. compass
13. temperature/humidity gauge
14. 2-ml vial holder
15. toilet paper
16. screen house
17. hammer
18. aspirin & misc first aid
19. chemically-cleaned jars and labels
20. sun screen
21. bug spray
22. gloves
23. pillowcases
24. spring scale
25. slide film & camera
26. "wet ones"
27. waders or knee boots
28. banding gear (optional)
29. permits
30. baseball caps
31. two extra styrofoam test-tube holders (Polyfoam Packers)

HAZARDS AND PRECAUTIONS:

Birds may have been exposed to toxic chemicals, and trace amounts of these biohazards may be present in the bird's tissues and waste. Therefore, caution should be exercised when handling the birds, and laboratory coats and gloves should be worn at all times in the lab.

PREPARATION OF APPARATUS:

1. **Preparation of caffeine injection solution**

   Prepare the injection solution to contain 3 mg ^{13}C-labelled caffeine/kg body weight, with a maximum injection volume of 1 ml. Proceed with the following steps:

   a. Determine the number of doses to be prepared in a tube, and the maximum weight of the planned test subjects. Calculate the amount of labelled caffeine to weigh and the solution volume from these values.

   b. Weigh out the desired amount of ^{13}C-labelled caffeine and transfer it into an empty, sterile Vacutainer® tube. A common source and type of caffeine which is used is 3-methyl-^{13}C-caffeine from Cambridge Isotope Labs, Andover MA.

   c. Add the necessary volume of sterile 0.9% saline (1 ml per dose). Vortex to mix.

2. **Maintenance of apparatus**

   The endotracheal tube should be carefully cleaned with a Q-Tip and water between birds (do not immerse the tube or apparatus in water). The polyethylene tubing is connected to the endotracheal tube and the inhale/exhale valves with plastic stubs of female Luer fittings which are epoxied into place. Epoxy does not ideally bind the plastic components, and must be maintained periodically. In addition, the exhalation valve accumulates condensation and must be periodically cleaned. This can be accomplished by carefully disassembling the valve (it is very delicate!), soaking the pieces in distilled water, and then leaving it to dry thoroughly. Consult manufacturers directions if a more thorough cleaning is desired.

3. Prior to the day of the test, use a laser printer to make labels for the breath test tubes (Avery® 5267) . Label each tube with the CBT date, subject ID, sample time point and subsample ID, and last name/institution affiliation of researcher. If liver samples will be obtained, label the cryovials appropriately. Prepare the caffeine injection solution

in advance.

PROCEDURE:

1. Place vet wrap around the bird's legs, tying them together. Weigh the bird.
2. Calculate the volume of caffeine injection solution to administer, so that the bird will receive a 3 mg caffeine/kg body weight dose.
3. Set the bird in a natural, erect position in the assistant's lap. Allow some time to go by, for the bird to calm down (approximately 5 min). Count the number of breaths/30 sec.
4. Spray the local anesthetic Cetacaine® onto a Q-tip. Apply it to the glottis and trachea opening. Wait one minute for the anesthetic to have maximum effect.
5. Insert the endotracheal tube/CBT apparatus into the trachea approximately 3 cm, and wait for the sampling balloon to fill (approximately 30 sec). This is most easily accomplished by having an assistant pry and hold the mouth open, while a second person inserts the tube. This is a background breath sample.
6. Remove the tube from the bird; close the stopcock. When ready, turn the stopcock so that it is open between the balloon and the syringe septum. Withdraw 50 cc of breath from the balloon into the syringe (using a 25 gauge needle on the syringe). Inject this in 10 cc aliquots into each of 5 Exetainer tubes.
7. Remove the stopcock from the exhalation valve. Open the stopcock between the balloon and the ambient air, and press the balloon to evacuate all remaining air from the sampling balloon. Reattach the stopcock to the exhalation valve.
8. Inject 3 mg ^{13}C-labelled caffeine/kg body weight into the brachial vein of the bird. Start the timer.
9. Gently place the cat harness around the body of the bird, and fasten it so that the bird is lightly restrained but the harness is not snug. Return the bird to the assistant's lap; keep it in an upright position.
10. Collect breath samples starting at 5 min post-injection, and then every 5 min for 40 min, as described in steps 14.5 through 14.7. To do this, begin 30 sec before each time point to pry the mouth open.
11. Re-apply Cetacaine® as needed, as evidenced by signs of discomfort or distress during the procedure. Reapplication is generally advised every 10 min. For birds which appear to dislike Cetacaine® application as much as the endotracheal tube itself, stress can be minimized by applying Cetacaine® directly to the endotracheal tube. This is not recommended for every case, since it results in a prolonged contact with the local

anesthetic which could have adverse consequences such as dehydration of the epithelium, an escharotic effect, or a local allergic reaction.

12. Monitor the respiration rate every 10 min. It is desired that the breathing rate remain constant throughout the CBT...although not much can be done about it if it doesn't.
13. Send the samples off for IRMS analysis (see "Instrumental Analysis" section below).
14. After the 40 minute sampling procedure, the option exists to set the bird free or to kill it and remove the liver for subsequent analysis (such as for EROD activity or chemical concentrations). The following section describes that procedure:
15. Kill the bird via cervical dislocation or decapitation. Open the body cavity and remove the liver quickly, taking care to separate the gall bladder without puncture. (Gall bladder contents can damage the enzymes which will be assayed.)
16. Weigh the liver.
17. For subsequent EROD activity analysis, place liver segments in several liquid nitrogen-safe 2 ml cryovials such that each cryovial is approximately 2/3 filled. Label the outside with a sharpie if it has a writing surface; otherwise label it with cryotape (Baxter). Insert a piece of paper inside the cryovial with the liver which has also been labelled with a pencil. Place the liver samples in liquid N_2 as soon as possible (preferably within 5 to 10 min after death). If the liquid N_2 is not immediately available, immerse deeply into dry ice immediately and keep it there until transfer to the liquid N_2 (storage in dry ice for up to 2 weeks prior to transfer to liquid N_2 is acceptable).
18. For subsequent chemical analysis, place the liver in a chemically-cleaned jar (3 times acetone, 3 times hexane) with a Teflon-lined lid. Store in a -20°C freezer.

INSTRUMENTAL ANALYSIS OF CBT SAMPLES:

The ratio of $^{13}CO_2$ to $^{12}CO_2$ in breath samples is analyzed by use of isotope ratio mass spectroscopy (IRMS). In my experiments, a Europa Scientific ANCA-SL Stable Isotope Analysis System with an autosampler was utilized. Breath samples were flushed by a helium carrier, passed through a reduction furnace to remove oxygen, through a gas drying stage, and then through a gas chromatograph column to resolve CO_2 from N_2 and any trace hydrocarbons, before passing into the mass spectrometer ion source for measurement of ^{13}C enrichment. Enrichment of ^{13}C was analyzed with reference to a 5% CO_2 in N_2 working standard (1.06558 atom percent ^{13}C) (Europa Scientific Ltd., 1988), and expressed relative to the ^{13}C content of the historical calcium carbonate standard known as PDB (Boutton, 1991a).

CALCULATIONS:

In order to calculate the recovery of ^{13}C from labelled caffeine in the breath, both the breath isotopic composition and the overall rate of CO_2 production must be known. Total CO_2 production in humans is often estimated from body weight or body surface area (Boutton, 1991b). The relationship of body weight to CO_2 production in birds was calculated with equation 1:

$$CO_2 \text{ excretion} = V_g \times P_E CO_2 / RT \qquad (1)$$

where: V_g = volume of gas
$P_E CO_2$ = partial pressure of end expiratory CO_2
RT = gas constant times the absolute temperature

At the body temperature of chickens, 1/RT is approximately 0.051 mmol/(liter torr) (Burger, 1980).

The partial pressure of end expiratory CO_2 in the chicken was averaged from literature values to be approximately 33 torr (Piiper et al., 1970; McLelland and Molony, 1983; Fedde, 1986). Substitution of these values into equation 1 and simplification yields equation 2:

$$CO_2 \text{ excretion} = V_g \text{ (liter/min)} \times 33 \text{ torr} \times 0.051 \text{ mmol/liter torr)}$$
$$= 1.683 \text{ (MV) mmol/min} \qquad (2)$$

where: MV = minute-volume liters/min

The value of MV may be estimated with units of ml/min by equation 3:

$$\log MV = 2.4533 + 0.77(\log BW) \quad \text{(Lasiewski and Calder, 1971)} \qquad (3)$$

where: BW = body weight (kg)

In the following sample calculation, CO_2 excretion was calculated for a 1.6 kg chicken using equations 2 and 3.

log MV	= 2.4533 + 0.77(log 1.6)	= 2.6105 ml/min
MV	= Antilog (2.6105)	= 407.85 ml/min
MV (L/min)	= 407.85 ml/min / 1000ml/L	= 0.40785 L/min
CO_2 excretion	= 1.683 (0.40785) mmol/min	= 0.68641 mmol/min

Once this value was calculated, a series of calculations were performed to convert data from the mass spectrometer into a cumulative amount of caffeine metabolized during the CBT (Boutton, 1991a, b). Raw mass spectrometry data was expressed as a $\delta^{13}C_{PDB}$ value (percent ^{13}C in comparison to the PDB standard). The first step was to convert the $\delta^{13}C_{PDB}$ value to an R value (the absolute ratio of the sample) by use of equation 4.

$$R_{sample} = {}^{13}C/{}^{12}C = [\delta^{13}C/1000 + 1] \times R_{PDB} \qquad (4)$$

where: R_{PDB} = 0.0112372

Next, the fractional abundance (F) of ^{13}C was calculated by use of equation 5:

$$F = \frac{R_{sample}}{R_{sample} + 1} \quad (5)$$

From this value, the atom percent excess (APE) of ^{13}C between CBT samples and background breath samples was calculated by use of equation 6:

$$APE = (F_{postdose} - F_{baseline}) \times 100 \quad (6)$$

The mmol excess ^{13}C produced per minute at each timepoint was then calculated by use of equation 7:

$$\frac{mmol\ excess\ ^{13}C}{min} = \frac{APE\ breath}{100} \times \frac{mmol\ total\ CO_2}{min} \quad (7)$$

note: "APE breath" was calculated from equation 6 and "mmol total CO_2/min" was calculated from equation 2.

The mmol excess ^{13}C in the caffeine dose was calculated by use of equation 8:

$$excess\ ^{13}C\ in\ dose = \frac{DOSE\ (mg)}{MW} \times (\%label) \times (\#atoms) \quad (8)$$

where:				
	DOSE	= caffeine dose (mg)	=	variable
	MW	= molecular weight of 3-methyl-^{13}C-caffeine	=	195.19
	%label	= % of all molecules labelled	=	99%
	#atom	= number of labelled atoms per molecule	=	1

The percent caffeine dose metabolized per min (PCD) could then be calculated for each time point by use of equation 9:

$$PCD = \frac{mmol\ excess\ ^{13}C\ /min}{mmol\ excess\ ^{13}C\ in\ dose} \times 100 \quad (9)$$

Note:The numerator was calculated by use of equation 7 and the denominator was calculated by use of equation 8.

Finally, the cumulative percent of the caffeine dose recovered (CUMPCD) was calculated for each CBT time point by use of equation 10:

$$CUMPCD = \left[\frac{(PCD_t + PCD_{t-1}) \times \Delta t\ (min)}{2}\right] + CUMPCD_{t-1} \quad (10)$$

The cumulative percent of the caffeine dose recovered over the entire 40-min CBT was used in subsequent analyses.

AQUATIC TOXICOLOGY LABORATORY

DEPARTMENT OF FISHERIES AND WILDLIFE
PESTICIDE RESEARCH CENTER
INSTITUTE FOR ENVIRONMENTAL TOXICOLOGY
MICHIGAN STATE UNIVERSITY

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Prepared by Lori A. Feyk
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Method Standard Operating Procedure Check List:

TITLE: **THE AVIAN CAFFEINE BREATH TEST (CBT) WITH RADIOACTIVELY (^{14}C) LABELLED SUBSTRATE**

SCOPE: This protocol describes the procedure used to perform the caffeine breath test in birds using radiolabelled caffeine. This method allows for an *in vivo* assessment of the subject's cytochrome P-450-1A (CYP1A) activity.

REFERENCED DOCUMENTS:

A description of the methods for the avian CBT using caffeine labelled with the stable isotope ^{13}C can be found in the accompanying SOP entitled "The caffeine breath test (CBT) in birds using ^{13}C-labelled caffeine".

SUMMARY OF METHOD:

A bird is weighed, a mask is fitted onto its head, and the mask is connected to a breath collection apparatus. Before caffeine is injected, a ten-minute breath sample is obtained for a background measurement. Then, a caffeine solution is injected into the bird's brachial vein which consists of 5 μCi of ^{14}C activity with approximately 1 mg caffeine/kg body weight in 1 ml physiological saline. Upon injection, the CBT pump and timer are started. The trapping solution in the sample impinger is changed before each interval of CO_2 collection, at 10, 20, 30 and 40 min post-injection. A 4 ml

aliquant of the used trapping solution is placed in a 20 ml scintillation vial along with 16 ml of Safety-Solve® High Flash Point Cocktail, and the ^{14}C activity (from respired $^{14}CO_2$) is determined by liquid scintillation. The total mass of caffeine metabolized during the first 40 min of the CBT is used for comparisons between individuals; this value was calculated by summing the activities (μCi) of $^{14}CO_2$ expired during 40 min post-injection and then converting the total activity to mass of caffeine (pmol) using the specific activity of the caffeine.

SIGNIFICANCE AND USE:

The induction of CYP1A activity is an important biomarker of exposure of birds to polyhalogenated aromatic hydrocarbons (PHDHs) such as some PCB and dibenzodioxin congeners. These are environmental contaminants of concern in some areas of the world, including the Great Lakes of North America. CYP1A activity is traditionally measured *ex vivo* in liver tissue, and test subjects are usually killed to obtain the liver sample. The CBT method is a less invasive *in vivo* method to measure CYP1A activity which obviates the need to kill the test bird. Since the method is non-destructive, it can be used to measure changes in CYP1A activity over time in response to changing PHDH exposure, to changing environmental factors such as temperature, or to internal changes such as fluctuating levels of sex hormones. In addition, the less invasive nature of the breath test may enable the monitoring of the CYP1A induction status of threatened or endangered bird species in PHDH-contaminated areas.

Relationship to similar assay; comparative advantages of each

This assay is similar to the CBT for birds using ^{13}C-labelled caffeine. Both assays measure *in vivo* CYP1A activity. The assay described in this document involves radiolabelled caffeine. Its relative advantage is that the samples which are obtained are easy and cheap to analyze, and involve the use of commonly available laboratory equipment (a scintillation counter). Also, the data which are obtained are more straightforward to interpret. Since the natural abundance of ^{14}C is very low, all measurable ^{14}C activity in the sample can be attributed to caffeine metabolism. It is a simple matter to calculate the amount of caffeine metabolized based on the scintillation counts, without a need to take metabolic rates into account. In contrast, approximately 1.1% of carbon in the environment is in the form of the stable isotope ^{13}C. The exact ratio of abundance can be different in various environmental

compartments, including the levels of the food chain. This makes data interpretation more difficult, as background metabolic processes need to be taken into account. Also, data analysis is more difficult for the stable isotope CBT, because it requires the use of a complex, expensive, and relatively uncommon instrument: an isotope ratio mass spectrometer. Data are expressed as ratios of ^{13}C to ^{12}C, and it is not straightforward to relate this information back to the amount of caffeine metabolized. However, radioactive caffeine is inherently more hazardous to work with than the corresponding compound labelled with the stable isotope ^{13}C. The CBT with radioactive caffeine is not suitable for field work, since permits are too difficult to obtain. Therefore, it is recommended that the radioactive ^{14}C-CBT be used when possible during laboratory studies, but that the stable-isotope ^{13}C-CBT be used during field studies.

INTERFERENCES:

It has been found that sunlight can react with scintillation cocktail in the field. The trapping solution/scintillation cocktail complex becomes photoactivated, and this interferes with scintillation counting for ^{14}C activity. Therefore, it is recommended that if the CBT is performed in the field, the scintillation cocktail should not be added until the samples are in the laboratory.

APPARATUS:

The apparatus for collecting CO_2 from exhaled air during the CBT consists of several components. A 16"x11"x8" cage is used to hold each bird during laboratory tests with chickens. (This cage is not essential and is generally not used in the field with cormorants or gulls; instead, a hand can be placed on the bird for light restraint when needed). A lightweight leather or Spandex® mask is attached to the bird with several Velcro® straps. The mask is not airtight, so a sufficient air supply is present. The beak fits into a plastic cone, which is connected to flexible latex tubing that allows bird movement. The latex tubing is attached to Teflon tubing anchored to the cage. The Teflon tubing is connected to a 30 ml bubbler impinger (SKC Inc., Eighty Four PA), then to a glass cold trap, and finally to a sample pump (SKC Inc., Eighty Four PA) with an adjustable low-flow controller (SKC Inc., Eighty Four PA).

MATERIALS:

1. **Instruments**
    1. Scintillation counter
    2. Scales to weigh bird and liver (laboratory or portable for field)

2. **Work supplies**
    1. test procedures, laboratory or field notebook
    2. pump (charged)
    3. low flow controller
    4. dry ice in cooler
    5. mask
    6. tubing
    7. spare battery pack for pump; charged
    8. Dewar flask (Thermos-sized, for cold trap)
    9. bubbler impinger
    10. cold trap glassware (Kontes)
    11. squirt bottle of methanol
    12. CO_2 trapping solution
    13. box large (20 ml) scintillation vials and caps
    14. Safety-Solve® High Flash Point Cocktail (Research Products International Corporation, Mount Prospect IL)
    15. sharpies (fine black), pen & pencil
    16. lab tape
    17. radioactive labelling tape
    18. (2) ring stands, one fitted with bubbler impinger holder (SKC Inc., Eighty Four PA)
    19. Tuberculin syringes (1-ml)
    20. alcohol swabs
    21. needles (25-gauge)
    22. injection solution
    23. tools to make dry ice slurry (hammer and stirrer)
    24. 10-ml pipettes, pipette bulb

3. **Supplies for removing the liver (if desired)**

    1. liquid nitrogen-safe 2 ml vials with caps
    2. paper
    3. filet knife
    4. small sharp scissors
    5. garden shears
    6. liquid nitrogen

HAZARDS AND PRECAUTIONS:

This CBT involves the use of trace quantities of ^{14}C radioactivity. This is a low energy radioisotope which cannot penetrate skin. However, every effort should be made to avoid exposure to ^{14}C, particularly via ingestion. Any trace quantities of untrapped $^{14}CO_2$ from the subject's exhaled breath should not pose a hazard to workers, since the majority of inhaled CO_2 is rapidly exhaled and not retained by the body. Workers should wear protective clothing, including a lab coat and gloves, while performing the CBT. No eating, drinking or smoking should be allowed during the CBT. Syringes should be placed in a labelled sharps container immediately after use, and all waste should be disposed of appropriately.

PREPARATION OF APPARATUS:

1. **Preparation of CO_2 trapping solution**

    The CO_2 trapping solution is a 10% ethanolamine/90% methanol solution. It is not necessary to use HPLC-grade methanol. In order to make 3 liters of trapping solution, combine 300 ml of ethanolamine with 2700 ml of methanol and shake to mix.

2. **Preparation of caffeine injection solution**

    The target solution will consist of 5 μCi of ^{14}C activity with approximately 1 mg caffeine/kg body weight in 1 ml physiological saline (0.9%). The two types of labelled caffeine which have produced satisfactory results to date are tri-labelled caffeine (1,3,7-^{14}C-trimethylxanthine synthesized in our laboratory) and 3-methyl-^{14}C-caffeine (Moravek Biochemicals, Brea CA). The solution is prepared in the following manner:

    1. Determine the average weight of the birds to be sampled, and the number of doses to be prepared in a given tube. Always make an extra half dose (500

μl) per tube.

2. Calculate the amount of labelled caffeine solution to add to an empty, sterile Vacutainer, such that the delivered dose will contain exactly 5 μCi of ^{14}C activity. Add this labelled caffeine solution to the Vacutainer, and blow off the carrier solvent to dryness using a gentle stream of nitrogen.
3. Add cold caffeine to the Vacutainer, such that a bird of the average weight would receive 1 mg caffeine/kg body weight.
4. Add 1 ml of sterile saline (0.9%) to the Vacutainer for each dose. Vortex to mix.

3. On the day prior to a breath test, all equipment should be gathered and solutions prepared if needed. Determine a pump setting using the low flow controller such that air will be drawn through the bubbler impinger at a rate which is sufficient to capture the volume of exhaled breath and produce sufficient bubbling, without being so vigorous as to cause excessive volatilization and sample loss. (I determined such a setting at less than 500 ml/min, and then left the setting intact for the duration of all future studies). On the morning of a breath test, prepare a dry ice slurry with crushed dry ice and methanol. Place the cold trap in a Dewar flask, and pour the dry ice slurry around it. Place 12 ml of trapping solution in the bubbler impinger. Assemble all of the components of the CBT apparatus.

PROCEDURE:

1. Weigh the bird.
2. Place the mask on the bird, and connect the bird to the CBT apparatus. Turn on the pump and obtain a 10 min "background" breath sample.
3. Exchange the bubbler impinger with another which contains 12 ml of fresh trapping solution. Pipette 4 ml of the used trapping solution into a 20 ml scintillation vial, along with 16 ml of scintillation cocktail. (In field situations, do not add the scintillation cocktail until you have returned to the lab.) Label the vial with the bird ID, the date, and the sampling period (-10 min).
4. Remove the bird from the apparatus, while leaving the mask intact. Inject 1 ml of a solution which contains 5 μCi of ^{14}C activity with approximately 1 mg caffeine/kg body weight in physiological saline (0.9%) into the bird's brachial vein.
5. Connect the mask to the CBT apparatus, and re-start the pump and timer.

6. Sample the breath continuously for 40 min, changing the trapping solution every 10 min. As in step 3, take a 4 ml sub-sample of the used trapping solution at each time point and store in a labelled scintillation vial. To clean the bubbler impinger between samples, perform a methanol rinse of the outer sleeve 3 times before filling it with 12 ml of fresh trapping solution.
7. The CBT is now concluded. Before performing a CBT on another bird, place fresh trapping solution in the bubbler impinger, turn on the pump, and run it for 5 min without a bird attached. This will thoroughly clean the bubbler impinger so that the background reading for the next bird is not affected.
8. Count each sample for 5 min in a scintillation counter.
9. After the 40 minute sampling procedure, the option exists to set the bird free or to kill it and remove the liver for subsequent analysis (such as for EROD activity or chemical concentrations). The following section describes that procedure:
10. Kill the bird via cervical dislocation or decapitation. Open the body cavity and remove the liver quickly, taking care to separate the gall bladder without puncture. (Gall bladder contents can damage the enzymes which will be assayed.)
11. Weigh the liver.
12. For subsequent EROD activity analysis, place liver segments in several liquid nitrogen-safe 2 ml cryovials such that each cryovial is approximately 2/3 filled. Label the outside with a sharpie if it has a writing surface; otherwise label it with cryotape (Baxter). Insert a piece of paper inside the cryovial with the liver which has also been labelled with a pencil. Place the liver samples in liquid N_2 as soon as possible (preferably within 5 to 10 min after death). If the liquid N_2 is not immediately available, immerse deeply into dry ice immediately and keep it there until transfer to the liquid N_2 (samples can be stored for up to 2 weeks in dry ice prior to their transfer to liquid N_2).
13. For subsequent chemical analysis, place the liver in a chemically-cleaned jar (3 times acetone, 3 times hexane) with a Teflon-lined lid. Store in a -20°C freezer.

CALCULATIONS:

Sample calculation for preparation of an injection solution:

Assume the following:

a. The radiolabelled stock caffeine solution to be used is 3-methyl-^{14}C-caffeine, which is 0.05 mCi in 1 ml ethanol.

b. Five doses of injection solution are desired, plus an additional half dose which will not be used.

c. Average bird weight is 1200 g.

1. Activity desired: 5.5 doses x 5 μCi = 27.5 μCi total activity

2. Volume of stock solution to use:

$$\frac{50\ \mu Ci}{1\ ml} = \frac{27.5\ \mu Ci}{x\ ml}\ ;\ x = 0.55\ ml$$

3. Amount of cold caffeine to use (1 mg caffeine/kg bird weight): 5.5 x 1.20 mg = 6.6 mg caffeine

4. Amount of saline to use (1 ml/dose): 5.5 x 1 ml = 5.5 ml saline

Sample calculation of CBT data:

Assume the following:

a. The above caffeine solution was used with a 1200 g chicken.

b. Disintegrations per minute (DPM) of a background breath sample was 100 DPM.

c. A 10 min sample following caffeine injection was 1300 DPM.

A. Calculate the specific activity (S.A.) of the caffeine.

1. Used 1.20 mg caffeine; caffeine molecular weight is 194.2

2. 1.20 mg/194.2 mg/mmol = 6.18 μmol caffeine

3. S.A. = 5 μCi/6.18 μmol = 0.81 μCi/μmol caffeine

B. Calculate amount of caffeine metabolized over 10 min period.

1. Correct for background. Sample DPM = 1300 - 100 = 1200 DPM

2. Multiply by three (only counted 4 of 12 ml trapping solution): 3600 DPM

3. Convert sample DPM to μCi: 2.22×10^6 DPM/μCi
   3600 DPM/2.22×10^6 DPM/μCi = 1.62×10^{-3} μCi

4. Convert to nmol caffeine by use of specific activity
   1.62×10^{-3} μCi/0.81 μCi/μmol caffeine = 2.0×10^{-3} μmol caffeine
   = 2.0 nmol caffeine

C. Can convert to % dose metabolized:
   2.0×10^{-3} μmol caffeine met./6.18 μmol caffeine admin. = 3.24×10^{-4} = 0.03%

D. Cumulative 40 min caffeine metabolism is calculated by summing the 10, 20, 30 and 40 min time points as calculated above.

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MICHIGAN STATE UNIVERSITY

Release 2.0

Prepared by Lori A. Feyk
January 31, 1996

Method Standard Operating Procedure Check List:

Title: **PROTOCOL FOR AVIAN LIVER MICROSOME PREPARATION**

Scope: This protocol describes the procedure used to prepare avian liver microsomes so that they can used in subsequent enzyme activity assays.

Referenced Documents:

This protocol is adapted from the following reference:

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

Terminology:

microsomes are closed vesicles formed by the self-sealing of fragments of the endoplasmic reticulum membrane when a cell is homogenized

precipitate refers to the solid pellet formed during centrifugation

supernatant refers to the liquid layer following centrifugation

Summary of Method:

An avian liver sample is removed from liquid nitrogen, where it has been stored since its collection from the bird. All subsequent steps are performed at 4°C. A 0.75 gram subsample of the liver is homogenized with Tris buffer, and the homogenate is centrifuged at 10,000 x g for 20 minutes. The precipitate is discarded and the supernatant is centrifuged at 100,000 x g for 60 minutes. The supernatant is discarded and the microsomal pellet is resuspended in EDTA buffer. This suspension is centrifuged at 100,000 x g for 60 minutes. The supernatant is discarded and the microsomal pellet is resuspended in a microsomal stabilizing buffer. The suspension is placed in several eppendorf tubes in 100 μl aliquants and stored in a -80°C freezer.

Significance and Use:

Cytochrome P-450 and its associated mixed-function oxygenase enzymes are located in the smooth endoplasmic reticulum of cells, particularly in the liver. It is necessary to homogenize the cells and isolate the microsomes from tissue samples in order to perform assays to determine cytochrome P-450-dependent enzyme activity. It is desirable to perform such enzyme activity assays because several of the cytochrome P-450 isozymes are specifically induced by certain chemicals. In some cases, measurements of enzyme activity can be used as biomarkers of exposure to specific classes of chemical compounds. For example, the isozyme cytochrome P-450-IA1 is induced by planar diaromatic compounds such as some PCB and dibenzodioxin congeners. These are environmental contaminants of concern in some areas of the world, including the Great Lakes of North America.

Interferences:

1. When collecting the liver from the bird, care must be taken to remove the gall bladder from the liver without puncture. Gall bladder contents can damage liver proteins.

2. Throughout the microsomal preparation procedure the sample temperature should not be allowed to rise above 4° C. The cold temperatures keep the microsomes and associated enzymes from degrading.

3. Blenders, homogenizers and utensils must be rinsed carefully between samples with distilled water to avoid cross-contamination. It is also recommended that control livers be processed prior to treated samples, for the same reason.

Materials:

1. Clinical centrifuge with refrigeration unit, capable of attaining 10,000 x g at 4° C
2. High-speed centrifuge with refrigeration unit, capable of attaining 100,000 x g at 4° C
3. "Tri-R Stir-R" homogenizer with teflon stir stick
4. -80° C freezer
5. liver samples in liquid nitrogen
6. small pair of forceps
7. small pair of scissors
8. vortexer
9. two coolers full of ice
10. 25 ml beaker
11. (2) thick plastic centrifuge tubes per sample - labelled
12. plastic-coated glass homogenizer tube for Teflon stir stick
13. (2) high-speed ultra-centrifuge tubes with caps and gaskets per sample - labelled
14. 10 ml pipette and bulb
15. Parafilm®
16. 100 μl pipette and tips
17. disposable pipettes
18. (2) waste beakers
19. (6) small eppendorf tubes, (1) large (1 ml) eppendorf tube and (4) liquid nitrogen vials (2 ml) per sample - labelled
20. Tris buffer
21. EDTA buffer
22. Microsomal stabilizing buffer
23. DTT aliquant (500 μl)

Hazards and Precautions:

1. Caution should always be exercised when using a centrifuge. Samples must be perfectly balanced in a centrifuge to avoid the risk of a serious accident. This is especially critical for the high-speed centrifuge, where the samples in their tubes must share the same weight to the nearest hundredth of a gram.

Sampling and Sample Preparation:

The liver must be removed from the bird immediately upon death. The gall bladder should be removed with the liver, and then carefully excised from the liver without

puncture. The liver should be weighed. Then, small liver sections should be stuffed inside of multiple pre-labelled 2 ml liquid nitrogen vials (Sarstedt 72.694.100, Newton NC) until the vials are 2/3 full, using forceps. The vials should then be immersed immediately in liquid nitrogen. Be sure to put a paper label, written in pencil, inside the cap of each vial. The liver sample should be stored in liquid nitrogen until the microsomes can be prepared as described in this document.

Preparation of Apparatus:

1. **Tris buffer preparation**

   This buffer is 0.05 M Tris and 1.15% KCl in nanopure water, pH 7.5. To prepare the buffer, the majority of the water is combined with the Tris and KCl in a large Erlenmeyer flask. After dissolving the KCl using a stir plate, the solution must be cooled to 4° C in a refrigerator before adjusting the pH because Tris is pH-dependent on temperature. After adjusting the pH, the solution is transferred to a volumetric flask, brought to volume, and then transferred to a storage bottle and stored in a 4° C refrigerator.

   Detailed Instructions:

   1. Put around 850 ml of nanopure water into a 1000 ml erlenmeyer flask.
   2. Add 6.055 g Tris.
   3. Add 11.5 g KCl
   4. Dissolve solids, etc. as described above.

   Reference: Lab notebook #2 pg 87-88.

2. **EDTA buffer preparation**

   This buffer is 10 mM EDTA and 1.15% KCl in nanopure water, pH 7.4. It is prepared as described in section 12.1, except that the pH can be adjusted at room temperature because EDTA is not temperature-dependent for pH. This buffer should also be stored in a 4° C refrigerator.

   Detailed Instructions:

   1. Add around 850 ml of nanopure water into a 1 liter erlenmeyer flask.
   2. Add 3.7224 g EDTA.
   3. Add 11.5 g KCl.
   4. Dissolve and adjust pH as described above.

3. **Microsomal stabilizing buffer preparation**

This buffer consists of 20% glycerol, 0.1 M KH_2PO_4, 1 mM EDTA and 1 mM dithiothreitol (DTT) at pH 7.25. Most of this buffer is stable. However, the dithiothreitol is a reducing agent and it must be replenished in the buffer each time that the buffer is used. Therefore aliquants of the dithiothreitol should be stored in a -20° C freezer, and one should be always be added to the buffer right before it is used.

Detailed Instructions for preparing the DTT aliquants:

1. Add 0.9258 g of DTT to a 15 ml centrifuge tube.
2. Add 6 ml of nanopure water; mix thoroughly.
3. Transfer 500 μl to each of 12 small eppendorf tubes.
4. Store in conventional freezer.
5. Each time the buffer is used, add 1 μl of DTT solution to the buffer per ml of buffer, after approximating the volume of buffer remaining. Discard any of the DTT aliquant which remains.

Reference: Lab notebook 5, pg 66.

Detailed Instructions for preparing the microsomal stabilizing buffer:

1. Add 100 ml glycerol to a 500 ml erlenmeyer using a glass cylinder. It is very thick. Flush the cylinder several times with distilled water, putting rinsings in the flask. Bring total volume in erlenmeyer up to around 425 ml.
2. Add 0.18612 g of EDTA.
3. Add 6.80 g of KH_2PO_4. Stir on stir-plate.
4. Adjust pH, bring to volume in a volumetric, and transfer to a 500 ml Nalgene plastic storage container.

References: Lab notebook #4 pg 29, and #5 pg 67.

4. On the day prior to the microsomal preparation, allequipment should be gathered and tubes should be properly labelled. On the morning of the microsomal preparation, tubes and beakers which will hold the sample should be chilled on ice, and the refrigeration units of the centrifuges should be turned on.

Calibration and Standardization:

In lieu of calibrating the centrifuges used, the investigator will evaluate the suitability of the pellets which are formed.

Procedure:

1. Place 5 ml of chilled Tris buffer in the chilled 25 ml beaker. Remove liver sample from the liquid nitrogen. Weigh out a liver portion of approximately 0.75 - 1.2 grams using forceps, record the weight, and place the portion in the beaker with the buffer.
2. Use a small pair of scissors to mince the liver in the buffer. Let the minced liver settle for a minute or so. Pour the excess buffer off into the waste beaker.
3. Place 2 ml of Tris buffer into the beaker and chill to 4°C. Swirl, then rapidly transfer all beaker contents into the homogenizer tube.
4. Place the homogenizer tube inside a tall, thin beaker filled with ice to serve as a cooling jacket. Homogenize the sample using the Tri-R Stir-R by moving the sample carefully up and down 5 times with the speed setting at approximately 5.0.
5. Place 8 ml of Tris buffer in a thick plastic (low speed) centrifuge tube. Add the contents of the homogenizer tube to the buffer in the centrifuge tube. Mix the contents by vortexing, cover the tube with Parafilm, and keep on ice.
6. Wash the 25 ml beaker, scissors, forceps, homogenizer, and homogenizer tube. Begin to process the next liver using steps 1 to 6. Work from controls to low dosed to high dosed samples if possible. Repeat until 8 liver samples are at this stage. Start them in the centrifuge (step 7 below), and then return to prepare the last 4 samples.
7. Place all samples in the Sorvall High Speed centrifuge in Dr. Hart's lab, first floor PRC. The centrifuge should be pre-cooled to 4° C, with the blue adapters inserted into the rotor so that our centrifuge tubes fit. Set the speed control notch to16,000 R.P.M. Put the timer at 20 minutes, and push the start switch. This is 20 minutes at approximately 10,000 x g.
8. Prepare for the next round of centrifugation by placing a set of ultra-centrifuge tubes on ice. Turn on the refrigeration unit of Dr. Haug's lab centrifuge (first floor PRC). To do this, set the temperature limits at 3° to 8° C. Turn on the main power switch, and turn on the vacuum. The refrigeration knob should be in the "on" position, and the light should be illuminated.
9. The precipitate from the first centrifugation is discarded at this time. A lipid layer on the top also needs to be avoided. Use a disposable pipette to suck as much lipid off

as possible without losing too much supernatant or contaminating it. Place a small beaker on a scale and tare. Place an ultra-speed centrifuge tube in the beaker, and place the gasket and cap on the scale also. Then, use a disposable pipette to transfer the purest supernatant possible into the tube on the scale. Record the final weight of the centrifuge tube and its contents. Cap and place on ice. Repeat with each sample, making sure that the final weight of each tube is the same to the nearest hundredth of a gram. If a sample does not have sufficient supernatant to meet the weight, Tris buffer can be used to bring the weight up to the mark.

10. Place the high-speed centrifuge tubes in the Ultracentrifuge in Dr. Haug's lab. To do this, get the holder out of the refrigerator. Turn the vacuum "off" so that you can open the centrifuge door (turn the door knob to the right to open it). Place the holder inside, with the lid and screw-top intact. Place the holder in the bottom slot of the centrifuge. Turn the vacuum on after closing the door. Also turn on the diffusion, brake, and water (see red knob at top right). Set speed at 45,000 R.P.M., and time at 60 minutes. In order to run, oil light must be on. Push the start button; hold a few seconds. This is 60 minutes at approximately 100,000 x g. It is wise to return after 10 minutes or so to make sure it is running OK.
11. The solid microsomal pellet is what gets saved after the spin in section 10. First, fill a 1 ml eppendorf tube about 2/3 full of supernatant (this is a cytosolic fraction Bob Crawford is interested in). Then, discard the rest of the supernatant into the waste beaker.
12. Place 5 ml of the EDTA buffer into the homogenizer tube; chill. For each sample, use a disposable pipette to take some of the buffer; transfer it into the other tube with the pellet. Work to resuspend the pellet into the buffer. Transfer everything into the homogenizer tube with the rest of its buffer. Thoroughly resuspend the sample by moving up and down 5 times with the Tri-R Stir-R, using the cooling jacket as before.
13. Prepare to Ultracentrifuge again. Tare beaker, weigh clean ultra-speed centrifuge tube with gasket and lid (you can use the gasket and lid from step 9 if you want). Transfer all of the sample from the homogenizer tube into the centrifuge tube using a transfer pipette. Then add enough EDTA buffer to make the tube more than 3/4 full and up to an even weight to match. Place on ice.
14. Wash the Tri-R Stir-R and homogenizer tube. Repeat steps 11, 12 and 13 for each sample, making sure all samples weigh the same.
15. Ultracentrifuge the samples for 60 minutes at approximately 100,000 x g, by using the same settings as outlined in step 10. Log the run, and put the rotor back in the refrigerator.

16. Discard the supernatant from the centrifuge spin in step 15. Add 1 ml of microsomal stabilizing buffer to the centrifuge tube per gram of liver used originally. Use a disposable pipette to resuspend the pellet, and then transfer all of the contents to the homogenizer tube. Use the Tri-R Stir-R to homogenize the sample, using a cooling jacket. Transfer the contents to an appropriately labelled low-speed centrifuge tube using a transfer pipette. Cover with Parafilm and place on ice. Repeat for each sample, making sure to wash the Tri-R Stir-R and homogenizer tube between samples and to work from "clean" to "dirty" samples.
17. Vortex, and then transfer 100 μl of the homogenate to each of six small labelled eppendorf tubes, and 4 liquid nitrogen vials. Place on ice. Repeat for each sample.
18. Store the eppendorf samples in a -80° C freezer, and the liquid nitrogen vials on liquid nitrogen, until enzyme activity assays can be performed. Samples should not be held for more than a few months in the -80° C freezer, because activity decreases 1-2% per month of storage. Activity seems well preserved even after several years on liquid nitrogen.

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DEPARTMENT OF FISHERIES AND WILDLIFE
PESTICIDE RESEARCH CENTER
INSTITUTE FOR ENVIRONMENTAL TOXICOLOGY
MICHIGAN STATE UNIVERSITY

Version 2.1

Prepared by Lori A. Feyk
February 16, 1996

LABORATORY PROTOCOL FOR HEPATIC MICROSOMAL PROTEIN MEASUREMENT AND EROD ASSAY IN THE SAME 96-WELL PLATE

References:

Protocol by Suzanne Trudeau obtained at SETAC, Nov. 1994, entitled "Adaptation of the Simultaneous Measurement of Cytochrome P4501A Catalytic Activity and Total Protein concentration with a fluorescence Plate reader to Wildlife Samples". Her address and phone are: National Wildlife Research Centre, Canadian Wildlife Service, 100 Gamelin Blvd., Hull Quebec K1A OH3. (819) 953-2635

See also, my Lab Notebook #5 pgs 68-77.

Part 1: Advance preparation of stock solutions

A. Fluorescamine

This solution is 60 mg fluorescamine/100 ml acetonitrile.

B. HEPES buffer, 0.05 M, pH 7.8

To make 500 ml of buffer:

1. Add about 450 ml of nanopure water to a 500 ml erlenmeyer flask.
2. Add 5.96 g HEPES.

3. Bring to 37°C in incubator.
4. Adjust pH, bring to volume in a volumetric, and transfer to a bottle for storage.

C. 7-Ethoxyresorufin stock (876 μM in methanol)

To make 5 ml:

1. Weigh out 0.00106 g of 7-ER. Add to a 5 ml volumetric flask.
2. Bring to the mark with methanol.
3. Add micro-stir bar. Seal with a ground glass stopper and parafilm, and cover with aluminum foil. Leave on a stir plate for several hours until all 7-ER is dissolved. Store in freezer.

D. Resorufin stock (150 μM in methanol)

To make 100 ml:

1. Weigh out 0.00352 g resorufin. Add to a 100 ml volumetric flask.
2. Bring to the mark with methanol. Transfer to a storage bottle. Cover with parafilm and aluminum foil; leave on a stir-plate for several hours until all resorufin is dissolved. Store in freezer.

E. Bovine Serum Albumin (BSA)

BSA at a concentration of 2 mg/ml HEPES buffer should be prepared and stored as many 1.0 ml aliquants in the freezer.

Part 2: Working solutions prepared on the day of the assay

A. NADPH in buffer

Make a 2 mM solution such that the final well concentration is 0.3 mM NADPH.

The proper ratio is 5.0 mg NADPH/3 ml buffer. Calculate the volume of NADPH which will be needed for the day (approximately 2.1 ml per plate plus necessary excess for pipetting). Make only as much as needed; this stuff is expensive.

B. Working resorufin solution: 7.5 μM

Add 50 μl of stock resorufin (150 μM) to 950 μl of HEPES buffer.

C. Working 7-ER solution

Place 76 μl of stock 7-ER solution (876 μM) to 4.924 ml HEPES buffer (5 ml total volume per plate).

D. Fluorescamine in acetonitrile

Pour 5 ml per plate (plus excess) into centrifuge tube.

Part 3: Addition of reagents to the 96-well plate

Add reagents in the following order, according to the volumes specified in table 1

1. Add 200 μl of HEPES buffer to all unused wells.
2. Add specified quantity of HEPES buffer to all other wells.
3. Add BSA to standard wells.
4. Add resorufin to standard wells.
5. Add microsomes to sample wells (4 wells each).
6. Add 7-ethoxyresorufin to all wells (except unused wells).
7. Pre-incubate 10 min at 37°C
8. Start the reaction by adding NADPH to sample wells (except blanks, columns 5 and 9), and all standard wells.
9. Incubate for 10 min at 37°C
10. Stop the reaction by adding fluorescamine in acetonitrile to all wells (except unused wells).
11. Cover plate to exclude light. Wait 15 min, read plate.

Step 4: Read the plate and analyze the data

Use the following settings, and read them simultaneously:

filter #	reads	EX	filter	EM	filter	Sensitivity
1	EROD	C	530/25	C	590/35	3
2	protein	E	400/30	A	460/40	3

Save the data on disk as a .csv file. Analyze it in Excel using the macro.

Table 7. Chart for 96-well plate: Simultaneous hepatic microsomal protein measurement and EROD assay

Well #	Sample Info	Buffer (μl)	2 mg/ml BSA (μl)	Final BSA (mg)	7.5 μM resorufin (μl)	Final resorufin (pmol)	13.4 μM 7-ER (μl)	Final [7-ER] (μM)	2 mM NADPH (μl)	Final [NADPH] (mM)	Fluoresc. in acetonit. (μl)	Total well volume (μl)
1 A-H	Unused wells	200										200
A& H 1-4												200
others												200
2 B-D	standards	81	0	0	0	0	30	2.85	30	0.425	60	201
2 E-G	BSA and resorufin	77	3	.006	1	7.5	30	2.85	30	0.425	60	201
3 B-D		73	6	.012	2	15	30	2.85	30	0.425	60	201
3 E-G		61	12	.024	8	60	30	2.85	30	0.425	60	201
4 B-D		47	18	.036	16	120	30	2.85	30	0.425	60	201
4 E-G		33	24	.048	24	180	30	2.85	30	0.425	60	201
			micro-somes(μl)									
A5	Blank a	105	6	XXX	XXXXX	XXXXX	30	2.85	0	0	60	201
A6-8	Sample a	75	6	XXX	XXXXX	XXXXX	30	2.85	30	0.425	60	201
......	etc		6	XXX	XXXXX	XXXXX						
H5	Blank stnd ck 34	105	6	XXX	XXXXX	XXXXX	30	2.85	0	0	60	201
H6-8	stnd ck 34	75	6	XXX	XXXXX	XXXXX	30	2.85	30	0.425	60	201
A9	Blank x	105	6	XXX	XXXXX	XXXXX	30	2.85	0	0	60	201
A10-12	sample x	75	6	XXX	XXXXX	XXXXX	30	2.85	30	0.425	60	201
....	etc		6	XXX	XXXXX	XXXXX						
H9	Blank z	105	6	XXX	XXXXX	XXXXX	30	2.85	0	0	60	201
H10-12	sample z	75	6	XXX	XXXXX	XXXXX	30	2.85	30	0.425	60	201

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MICHIGAN STATE UNIVERSITY

Version 2.2: Effect of inhibitors

Prepared by Lori A. Feyk

June 3, 1996

LABORATORY PROTOCOL FOR HEPATIC MICROSOMAL ALKOXYRESORUFIN-O-DEALKYLASE ASSAY IN A 96-WELL PLATE: EFFECT OF INHIBITORS

References:

Protocol by Suzanne Trudeau obtained at SETAC, Nov. 1994, entitled "Adaptation of the Simultaneous Measurement of Cytochrome P4501A Catalytic Activity and Total Protein concentration with a fluorescence Plate reader to Wildlife Samples". Her address and phone are: National Wildlife Research Centre, Canadian Wildlife Service, 100 Gamelin Blvd., Hull Quebec K1A OH3. (819) 953-2635

See also, my Lab Notebook #5 pgs 68-77.

Part 1: Advance preparation of stock solutions

A. Fluorescamine

This solution is 60 mg fluorescamine/100 ml acetonitrile.

B. HEPES buffer, 0.05 M, pH 7.8

To make 500 ml of buffer:

1. Add about 450 ml of nanopure water to a 500 ml erlenmeyer flask.
2. Add 5.96 g HEPES.
3. Bring to 37°C in incubator.
4. Adjust pH, bring to volume in a glass cylinder, and transfer to a bottle for storage.

C. 7-Ethoxyresorufin stock (876 μM in methanol)

To make 5 ml:

1. Weigh out 0.00106 g of 7-ER. Add to a 5 ml volumetric flask.
2. Bring to the mark with methanol.
3. Add micro-stir bar. Seal with a ground glass stopper and parafilm, and cover with aluminum foil. Leave on a stir plate for several hours until all 7-ER is dissolved. Store in freezer.

D. Resorufin stock (150 μM in methanol)

To make 100 ml:

1. Weigh out 0.00352 g resorufin. Add to a 100 ml volumetric flask.
2. Bring to the mark with methanol. Transfer to a storage bottle. Cover with parafilm and aluminum foil; leave on a stir-plate for several hours until all resorufin is dissolved. Store in freezer.

E. Bovine Serum Albumin (BSA)

BSA at a concentration of 2 mg/ml HEPES buffer should be prepared and stored as many 1.0 ml aliquants in the freezer.

F. Ellipticine (11.6 mM)

Purchase 5 mg vial from Sigma. This substance is very difficult to transfer or weigh, so it is best to assume a real weight of 5 mg and add DMSO directly to the vial. (Sigma rep. says the maximum overweight for this product is 0.2 mg).

1. Add 1.75 ml DMSO to the original Sigma vial.
2. Vortex. Transfer all contents to an autosampler vial for storage.

G. Furafylline (20.4 mM)

1. Tare empty autosampler vial.
2. Add entire contents of a 5 mg batch of furafylline to the vial, weigh.
3. Add sufficient DMSO to the vial; vortex (MW 260.25). (In first experiment, 1 ml

DMSO was added to 5.31 mg furafylline).

Part 2: Working solutions prepared on the day of the assay

A. NADPH in buffer

Make a 2 mM solution such that the final well concentration is 0.3 mM NADPH.

The proper ratio is 5.0 mg NADPH/3 ml buffer. Calculate the volume of NADPH which will be needed for the day (approximately 2.1 ml per plate plus necessary excess for pipetting). Make only as much as needed; this stuff is expensive.

B. Working resorufin solution: 7.5 μM

Add 50 μl of stock resorufin (150 μM) to 950 μl of HEPES buffer.

C. Working substrate solution

Make 2.1 ml per plate plus 2 ml excess per day.

i. EROD - final well concentration of ethoxyresorufin: 15 μM
Working solution is 70.5 μM

Add 322 μl of working stock (876 μM) 7-ER to 3.678 ml buffer for 4 ml.
Add 241 μl of working stock to 2.759 ml buffer for 3 ml.

ii. MROD - final well concentration of methoxyresorufin:
15 μM for "low activity" samples; 5 μM for "high activity" samples
Working solution is 70.5 μM

Add 322 μl of working stock (876 μM) to 3.678 ml buffer for 4 ml.
For "high activity" samples, dilute this solution 1:3.

iii. PROD and BROD: final well concentration of alkoxyresorufin: 15 μM
Working solution is 70.5 μM
Also need final well concentration of 0.044% BSA.

Working solution needs 2 μg/μl BSA. For 4 ml of working solution:

1. Add 322 μl of working stock (876 μM) to a conical bottom glass test tube with screw-top

lid.

2. Weigh out and add 0.008 g BSA.
3. Add 3.678 ml buffer; vortex.

D. Fluorescamine in acetonitrile

Pour 5 ml per plate (plus excess) into centrifuge tube.

E. Inhibitor solutions (ref: notebook #6 pg 17-19)

i. Ellipticine

Need 10 μM in 12 wells/plate. For two plates:
Add 3.16 μl stock (11.6 mM) to 256.8 μl buffer.

ii. Furafylline

Need 200 μM in 12 wells/plate. For two plates:
Add 36 μl stock (20.4 mM) to 224 μl buffer.

Part 3: Addition of reagents to the 96-well plate

Add reagents in the following order, according to the volumes specified in table 1

1. Add 200 μl of HEPES buffer to all unused wells.
2. Add specified quantity of HEPES buffer to all other wells.
3. Add BSA to standard wells.
4. Add resorufin to standard wells.
5. Add microsomes to sample wells, including their blanks (column 5).
6. Add inhibitor to applicable sample wells.
7. Add alkoxyresorufin substrate(s) to all wells (except unused wells).
8. Pre-incubate 10 min at 37°C
9. Start the reaction by adding NADPH to sample wells (except blanks, column 5), and all standard wells.
10. Incubate for 10 min at 37°C
11. Stop the reaction by adding fluorescamine in acetonitrile to all wells (except unused

wells).

12. Cover plate to exclude light. Wait 15 min, read plate.

Step 4: Read the plate and analyze the data

Use the following settings, and read them simultaneously:

filter #	reads	EX	filter	EM	filter	Sensitivity
1	EROD	C	530/25	C	590/35	3
2	protein	E	400/30	A	460/40	3

Save the data on disk as a .csv file. Analyze it in Excel using the macro.

Table 8. Chart for 96-well plate: Effect of inhibitors on alk-ROD activity

Well #	Sample Info	Buffer (μl)	2 mg/ml BSA (μl)	Final BSA (mg)	7.5 μM resorufin (μl)	Final resorufin (pmol)	Alk-OR (μl)	2 mM NADPH (μl)	Final [NADPH] (mM)	Fluoresc. in acetonit. (μl)	Total well volume (μl)
1 A-H	Unused wells	200									200
A&H 1-4											200
others											200
2 B-D	standards	81	0	0	0	0	30	30	0.425	60	201
2 E-G	BSA and resorufin	77	3	.006	1	7.5	30	30	0.425	60	201
3 B-D		73	6	.012	2	15	30	30	0.425	60	201
3 E-G		61	12	.024	8	60	30	30	0.425	60	201
4 B-D		47	18	.036	16	120	30	30	0.425	60	201
4 E-G		33	24	.048	24	180	30	30	0.425	60	201
			micro-somes(μl)	Ellipt (μl)	Furafyl. (μl)						
5B	SamplABlank,OR1	105	6	XXX	XXXXX	XXXXXX	30-OR1	0	0	60	201
5C	SamplABlank,OR2	105	6	XXX	XXXXX	XXXXXX	30-OR2	0	0	60	201
5D-G	Blanks,SamplB&C	105	6	XXX	XXXXX	XXXXXX	30	0	0	60	201
B6-7	SampleA,OR1	75	6	XXX	XXXXX	XXXXXX	30-OR1	30	0.425	60	201
C6-7	SamA,OR1,Ellipt	65	6	10	XXXXX	XXXXXX	30-OR1	30	0.425		
D6-7	SamA,OR1,Furaf	65	6	XXX	10	XXXXXX	30-OR1	30	0.425	60	201
E6-7	SampleA,OR2	75	6	XXX	XXXXX	XXXXXX	30-OR2	30	0.425	60	201
F6-7	SamA,OR2,Ellipt	65	6	10	XXXXX	XXXXXX	30-OR2	30	0.425	60	201
G6-7	SamA,OR2,Furaf	65	6	XXX	10	XXXXXX	30-OR2	30	0.425	60	201
B-G8-9	SampleB as above					XXXXXX					
CG10-11	SampleC as above					XXXXXX					

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