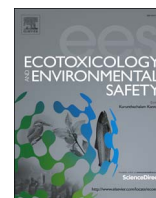




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Reprint of: CYP1A protein expression and catalytic activity in double-crested cormorants experimentally exposed to Deepwater Horizon Mississippi Canyon 252 oil



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ABSTRACT

Double-crested cormorants (*Phalacrocorax auritus*, DCCO) were orally exposed to Deepwater Horizon Mississippi Canyon 252 (DWH) oil to investigate oil-induced toxicological impacts. Livers were collected for multiple analyses including cytochrome P4501A (CYP1A) enzymatic activity and protein expression. CYP1A enzymatic activity was measured by alkoxyresorufin O-dealkylase (AROD) assays. Activities specific to the O-dealkylation of four resorufin ethers are reported: benzyloxyresorufin O-debenzylase (BROD), ethoxyresorufin O-deethylase (EROD), methoxyresorufin O-demethylase (MROD), and pentoxyresorufin O-depentylase (PROD). CYP1A protein expression was measured by western blot analysis with a CYP1A1 mouse monoclonal antibody. In study 1, hepatic BROD, EROD, and PROD activities were significantly induced in DCCO orally exposed to 20 ml/kg body weight (bw) oil as a single dose or daily for 5 days. Western blot analysis revealed hepatic CYP1A protein induction in both treatment groups. In study 2 (5 ml/kg bw oil or 10 ml/kg bw oil, 21 day exposure), all four hepatic ARODs were significantly induced. Western blots showed an increase in hepatic CYP1A expression in both treatment groups with a significant induction in birds exposed to 10 ml/kg oil. Significant correlations were detected among all 4 AROD activities in both studies and between CYP1A protein expression and both MROD and PROD activities in study 2. EROD activity was highest for both treatment groups in both studies while BROD activity had the greatest fold-induction. While PROD activity values were consistently low, the fold-induction was high, usually 2nd highest to BROD activity. The observed induced AROD profiles detected in the present studies suggest both CYP1A4/1A5 DCCO isoforms are being induced after MC252 oil ingestion. A review of the literature on avian CYP1A AROD activity levels and protein expression after exposure to CYP1A inducers highlights the need for species-specific studies to accurately evaluate avian exposure to oil.

1. Introduction

The Deepwater Horizon (DWH) oil spill in the northern Gulf of Mexico began April 20th, 2010, with the explosion and sinking, two

days later, of the Deepwater Horizon drilling rig and the deaths of 11 rig workers (BP, 2010). Before it was capped, the uncontrolled well in the Macondo Prospect of Mississippi Canyon block 252 (MC252) released more oil than any other oil spill in the history of the United

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States (Camilli et al., 2010). Many birds died from the overwhelming effects of direct oiling, while many more were observed alive with varying amounts of oil on skin and plumage, exposures whose consequences are not well established (Haney et al., 2014).

Birds frequently come in contact with oil after a spill due to their feeding and behavioral habits (Haney et al., 2014). Contaminated food sources can cause increased oil ingestion while oiled feathers can lead to increased ingestion of oil through preening, reduced buoyancy and thermoregulation, and disruption of flight. Two different routes of exposure (oral ingestion and oiled feathers) were recently explored in a series of studies to investigate the health, behavioral, and mechanical effects of MC252 oil in birds at acute, sub-lethal levels (Bursian et al., 2017, this volume). Double-crested cormorants (*Phalacrocorax auritus* DCCO) are migratory birds, travelling from the Great Lakes region to the Gulf of Mexico during winter. This particular piscivorous species of water bird is commonly chosen as a model bird in toxicology because of its widespread population and habitats (Dorr et al., 2012; Lavoie et al., 2015; Ofukany et al., 2012). DCCO was one of the many avian species affected by the DWH oil spill, resulting in fledglings lost due to death of reproductively mature birds (USFWS, 2015). MC252 oil was fed to birds to explore the toxicity of ingested spilled oil, a common route of exposure for birds inhabiting the contaminated area. Feather oiling, a second route of exposure, was studied to evaluate the resulting metabolic and thermoregulatory effects and lab- and field-based flight effects. The profile of health effects endpoints in these studies included analysis of cytochrome P450 and its induction in liver tissues.

The cytochrome P450 superfamily of phase I metabolism enzymes catalyzes the oxidative biotransformation of a vast array of exogenous and endogenous compounds (Kubota et al., 2009; Nebert et al., 2006). The cytochrome P4501A (CYP1A) subfamily is of particular interest because it biotransforms polycyclic aromatic hydrocarbons (PAHs) present in oil, leading to both activation and detoxication of these compounds (Denison and Whitlock, 1995; Sarasquete and Segner, 2000). Additionally, the metabolism of xenobiotics by CYP1A can result in the production of harmful reactive oxygen species (ROS) that have the ability to alter an organism's immune, erythroid, reproductive, and endocrine systems (Jennifer et al., 2006; Stegeman et al., 1992). The aryl hydrocarbon receptor (AhR), a multiprotein transcription factor, regulates the expression of CYP1A (Walker et al., 2000; Whitlock, 1999). AhR agonists include PAHs and planar halogenated aromatic hydrocarbons (PHAH) such as planar polychlorinated biphenyls, dioxins and furans (Safe, 1986, 1994). Exposure to these agonists results in CYP1A induction and this highly sensitive process is the basis for the wide use of CYP1A expression as a biomarker of exposure to these compounds (Bucheli and Fent, 1995; Stegeman and Lech, 1991; Stegeman et al., 1992; van der Oost et al., 2003; Whyte et al., 2000).

CYP1A induction is a recognized and widely used measure of exposure to, and molecular effects of, oil and PAHs in humans, laboratory animals and wildlife species (Godard et al., 2004, 2006; Stegeman et al., 1992; Webb et al., 2014) including birds (Brausch et al., 2010; Brunström et al., 1991; Custer et al., 2000; Lee et al., 1985; Lee et al., 1986; Peakall et al., 1989). Avian species express two forms of CYP1A: CYP1A4 and CYP1A5 (Gilday et al., 1996). Evolutionary studies indicate the avian CYP1As, CYP1A4 and CYP1A5, are orthologous to the mammalian CYP1As, CYP1A1 and CYP1A2, respectively; the pairs diverging after a single duplication event of CYP1A (Goldstone and Stegeman, 2006; Kubota et al., 2006a). Constitutive expression of CYP1A is low, yet highly inducible in the presence of certain xenobiotics, strengthening the utility of this enzyme subfamily as a biomarker (Whitlock, 1999).

Cytochrome P4501A analysis is performed by measuring the rate of the enzyme's metabolic degradation of synthetic substrates (its catalytic activity) or by directly quantifying the amount of the specific CYP1A protein. Activity measurement of the CYP1A subfamily of enzymes is based on the rates of O-dealkylation of four substituted resorufin ethers by what are generally known as alkoxyresorufin O-dealkylase (AROD)

reactions. Activities of the CYP1A-catalyzed O-dealkylation of the methoxy-, ethoxy-, pentoxy-, and benzoxy-substituted resorufin ether substrates are known, respectively, as methoxyresorufin O-demethylase (MROD), ethoxyresorufin O-deethylase (EROD), pentoxyresorufin O-depentylase (PROD) and benzyloxyresorufin O-debenzylase (BROD). AROD assays are a widely recognized method for studying CYP1A activity and its induction in response to chemical exposure (Bucheli and Fent, 1995; Burke et al., 1985; Kubota et al., 2009). On a species-to-species basis, CYP1A varies in its forms and tissue distributions. There is a resultant variability in the measured activities and induction responsiveness of the four AROD assays (Brunstrom and Halldin, 1998; Fossi et al., 1995; Giorgi et al., 2000; Helgason et al., 2010; Herve et al., 2010; Kubota et al., 2009; Walker, 1998). Therefore, all four AROD activities were explored to assess which of the dealkylase activities are relevant in each exposure scenario and in the particular DCCO species. Hepatic expression of CYP1A was also studied by directly measuring the amount of protein present using semi-quantitative western blotting. Differences in band intensity allow direct quantification of changes in protein concentration following MC252 oil-exposures and the resulting induction of protein synthesis.

Though a considerable amount of research has been done on CYP1As in mammals, less is known regarding other vertebrates such as birds (Heubeck et al., 2003; Kubota et al., 2009; Walker and Ronis, 1989). The acute and sub-chronic sub-lethal responses to oil exposure in the double-crested cormorant were examined in two separate studies in order to help close this gap in the specific case of the DWH spill. We report here on both CYP1A catalytic activity and protein expression in DCCO exposed orally to DWH oil in various exposure scenarios.

2. Materials and methods

2.1. Dosing studies

CYP1A enzymes and induction were investigated in two studies of captive double-crested cormorants. Wild caught double-crested cormorants were young of the year or sub-adults captured and maintained on a clean diet of captive-reared fingerling channel catfish (*Ictalurus punctatus*) from 14 to 21 days prior to dosing. Study 1 DCCO (13 males, 7 females) were collected November 6, 2012 from Little Mossy Lake, Mississippi. Study 2 DCCO (19 males, 1 female) were collected March 12, 2013 from McIntyre Scatters, Leflore County, Mississippi. In study 1, artificially aged MC252 oil, originating from the DWH oil spill (DWH7937, batch # B030112; Forth et al., 2016), was mixed with ground catfish filets (1:1) and the resulting slurry was gavaged directly to the birds' stomach twice each dosing day. Control birds received a gavage dose of only ground catfish fillets at the same times as the dosed birds. To optimize dose absorption and retention in study 2, cormorants were fed catfish that were lightly anesthetized (tricaine methanesulfonate) and injected with artificially aged MC252 oil (same source as Study 1). Control catfish received similar anesthesia but no oil. All feed fish were allowed to recover from anesthesia before being fed to cormorants. A detailed description of all animal care and experimental procedures can be found elsewhere (Cunningham et al., 2017, this issue). In study 1, the birds received 20 ml/kg bw/day of artificially weathered MC252 oil on a single day, day 1, of the study or for 5 days, on days 1 through 5. All birds were euthanized on day 6 and liver samples (approximately 1 g) were placed in cryovials, flash frozen in liquid nitrogen and maintained at -80C until analyzed. Final number of birds for the AROD and Western blot analyses are reported in Table 1. Mean body weights of Study 1 birds were 1977.3, 1792.5, and 1854.7 before exposure and 1957.1, 1762.7, and 1921.9 after exposure for control, 1-day, and 5 day treatments, respectively. Additional information on DCCO organ weights can be found in Harr et al. (2017) (this issue).

In study 2, the cormorants received a nominal dose of either 5 or 10 ml/kg bw/day of the artificially weathered MC252 oil, daily, for 21

Table 1

CYP1A protein expression and activities in livers of double-crested cormorants dosed with artificially aged MC252 petroleum associated with the Deepwater Horizon oil spill. Values are means (\pm standard deviation) of CYP1A protein or activity. Lower case letters indicate significant differences among treatment groups within each enzyme measure. Induction over control values are fold-increases of enzyme measured in petroleum-dosed versus control cormorants.

Treatment	Duration	N	CYP1A Protein Intensity	N	Activity (pmol/min/mg protein)			
					BROD	EROD	MROD	PROD
<i>Study 1</i>								
Control		3	0.03a \pm 0.0001	3	1.77a \pm 1.57	11.80a \pm 19.07	2.41 \pm 1.48	0.47a \pm 0.44
20 ml/kg	1 day	5	1.93b \pm 0.0018	5	26.00b \pm 14.72	56.40b \pm 39.79	10.20 \pm 6.53	1.09b \pm 0.46
20 ml/kg	5 days	7	1.88b \pm 0.0022	7	25.30b \pm 11.58	39.30a,b \pm 18.48	12.80 \pm 6.94	2.69b \pm 1.09
Induction over control								
20 ml/kg	1 day		71.5		14.7	4.8	4.2	2.3
20 ml/kg	5 days		69.6		14.3	3.3	5.3	5.7
<i>Study 2</i>								
Control		4	0.07a \pm 6.61E-05	6	4.01a \pm 3.97	15.20a \pm 18.98	5.68a \pm 7.44	0.77a \pm 0.49
5 ml/kg	21 days	5	2.07a,b \pm 0.0030	5	37.29b \pm 14.02	39.69a,b \pm 12.73	16.89b \pm 5.74	3.25b \pm 0.85
10 ml/kg	12–15 days	4	1.68b \pm 0.0019	6	21.29a,b \pm 21.70	56.89b \pm 27.98	25.69b \pm 11.39	1.92a,b \pm 1.19
Induction over control								
5 ml/kg	21 days		30.4		9.3	2.6	3.3	4.3
10 ml/kg	12–15 days		24.7		5.3	3.7	4.5	2.5

days in the form of oil-injected catfish. Doses based on actual fish consumption data were 5.2 ± 0.2 and 8.5 ± 0.4 ml/kg bw/day (mean \pm SE). Because of deteriorating physical condition, high dose birds were euthanized and tissues collected on days 12 through 15. Signs of deteriorating physical condition include lack of alertness, tucking of head under wings, and a cloacal temperature of 39.4°C or less. Control and low dose birds were euthanized on day 21 and tissues collected and processed as in Study 1. Final numbers of birds for the AROD and Western blot analyses are reported in Table 1. Mean body weights of Study 2 birds were 2001, 1880, and 1968 g before exposure and 1839, 1673, and 1545 g after exposure for control, 5 ml/kg bw/day, and 10 ml/kg bw/day treatments, respectively. Additional information on DCCO organ weights can be found in Harr et al. (2017) (this special issue).

Both studies received IACUC approval from USDA/APHIS/WS/NWRC.

2.2. Sample preparation

2.2.1. Microsome or S9 preparation

Microsomes and S9 fractions were prepared as described previously (Trust et al., 2000). Samples were homogenized and then centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant was centrifuged at $20,000 \times g$ for 20 min at 4°C . The resulting supernatant, the S9 fraction, was saved. Microsomes were prepared from larger S9 fractions by centrifuging at $105,000 \times g$ for 1 h and 10 min at 4°C in an ultracentrifuge and resuspending the pellet (Beckman, Optima XL-100K ultracentrifuge).

2.2.2. Protein analysis

Protein concentration was determined using the Bradford assay (Bradford, 1976) on $10 \mu\text{l}$ of liver microsomes. Bovine serum albumin (BSA) was used to create the standard curve. All samples were measured in triplicate. Protein assay dye (Bio-Rad) was added to the sample and standard wells. BioTek Synergy 4 plate reader read the absorbance of all wells at 595 nm.

2.3. Sample analysis

2.3.1. Alkoxyresorufin-O-dealkylation analysis

Catalytic activity of cytochrome P4501A was assessed as previously described in birds (Brausch et al., 2010) but with four AROD activities (BROD, EROD, MROD, and PROD) measured using a fluorescence plate reader (BioTek Synergy 4) that took 16 readings over 15 min using

530 nm excitation and 590 nm emission wavelengths. Resorufin standard and all resorufin substrates were purchased from Sigma. The standard curve was created using 8 resorufin concentrations (0, 0.02, 0.05, 0.2, 1, 2, 20, and 40 pmol). Rat liver S9 induced with Aroclor 1254 (Celsis) was the positive control. Each well had final assay concentrations of 0.01 mM dicumarol, 1.42 mM NADPH, $40 \mu\text{g}$ of sample protein, and $2 \mu\text{M}$ of either 7-benzyloxy, ethoxy, methoxy, or pentoxyresorufin substrate in a final well volume of $200 \mu\text{l}$. NADPH was added to the wells last to initiate the reaction. Limit of detection for the plate reader was $0.0005 \text{ pmol resorufin}/\mu\text{g}$ of protein (Pezdek, 2014). An R^2 value greater than 0.47 indicated positive activity. A 10% coefficient of variance (CV) was acceptable for the positive control and a CV of 50% was acceptable for samples. If the above mentioned criteria were not met, values were discarded before final calculations for the overall AROD activity. Final AROD activity was expressed as pmol/min/mg protein and the following equation was used:

$$\text{Final AROD activity} = \frac{((\text{Sample slope} - \text{Blank slope}) / \text{RR standard slope})}{\text{Protein mg}}$$

2.3.2. Western Blotting technique

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was used to separate either microsomal or S9 samples. A 4% SDS-PAGE stacking gel and a 12% SDS-PAGE resolving gel were used. Microsomal samples in amounts of $16.6 \mu\text{g}$ protein for high treatment groups (to avoid saturation of the total protein stain) and $33.3 \mu\text{g}$ protein for medium and control treatment groups were run in the SDS-PAGE. A semi-dry transfer instrument (Bio-Rad) transferred the protein from the polyacrylamide gel to a PVDF membrane. Total protein staining was carried out with SYPRO Ruby total protein stain according to the manufacturer's (Thermo Fisher Scientific) manual. The membrane was exposed to a UV-CCD camera (Alpha Innotech, FluorChem SP) to reveal the total protein bands that were quantified using Quantity One software (Bio-Rad). The PVDF membrane was then blocked in 5% TBS-Blotto. Mouse monoclonal CYP1A1 antibody (Santa Cruz Biotechnology, sc-393979) was used as primary antibody (1:500 dilution) and followed by a goat anti-mouse secondary antibody (Santa Cruz Biotechnology, sc-2005, 1:2000 dilution). ECL A and ECL B (ThermoFisher) photo developers were applied to the membrane to reveal the CYP1A bands. These were revealed by exposing the membrane to a CCD camera (Alpha Innotech, FluorChem SP) for 20 min. Intensities of the bands were quantified in arbitrary units using Quantity One software 4.6.9 (Bio-Rad) after normalization using SYPRO Ruby total protein stain.

2.4. Statistical analysis and boxplot design

Differences among treatment groups were assessed with the Kruskal-Wallis test. If statistically significant differences (defined as $p < 0.05$) were detected, pairwise comparisons among treatment groups were assessed with Dunn's test using the Bonferroni correction for multiple comparisons. Correlations among CYP1A AROD activities and band intensity were assessed using Spearman's rank correlation (ρ). Statistical calculations were performed with R software version 3.2.3 (R Core Team, 2015).

For boxplots: letter codes indicate statistically significant pairwise comparisons (Dunn's test; $p < 0.05$, $\alpha = 0.05$). The lower and upper limits of the boxes represent the 25th and 75th percentiles, respectively. The horizontal black line within the boxes represents the median. The lower and upper whiskers represent the data range and dots depict the individual measured values.

3. Results and discussion

Crude oil is known to differ among sources in its hydrocarbon content and composition. A series of studies was specifically conducted to understand possible physiological, enzymatic, phenotypic and metabolic changes in birds after exposure to MC252 oil through multiple routes (this special issue). Here, we report exclusively on CYP1A catalytic activity and protein expression in captive double-crested cormorants exposed orally to MC252 oil. Crude oil studies have been performed on a variety of species, especially fish and seaducks, to explore potential association between exposure and CYP1A catalytic activity or protein induction (Holth et al., 2014; Miles et al., 2007; Stagg et al., 2000; Esler et al., 2010; Trust et al., 2000). To our knowledge, this is the first study reporting CYP1A catalytic activity and protein induction in double-crested cormorant after exposure to crude oil.

3.1. CYP1A catalytic activity

CYP1A AROD activity in birds is a widely used and accepted biomarker of exposure to toxicants that are AhR agonists. Significant positive correlations between such toxicant concentration/toxic equivalents (TEQs) and AROD activities have been found previously in double-crested cormorants and common cormorants exposed to CYP1A inducers (Kubota et al., 2005; Sanderson et al., 1994; van den Berg et al., 1994; Guruge and Tanabe, 1997) as well as in many other avian species exposed to PAHs or crude oil such as lesser scaup (*Aythya affinis*, Custer et al., 2000), and herring gull (*Larus-Argentatus*, Peakall et al., 1989). In addition, EROD activity in Steller's eiders (*Polysticta stelleri*) was positively correlated with PAH concentration in blue mussels (*Mytilus edulis*), a common prey item (Miles et al., 2007).

Significant induction in AROD activity was detected in the livers of double-crested cormorants in both studies (Table 1, Figs. 1 and 2). In study 1, oil exposure led to significant BROD, EROD, and PROD induction and a trend of increased MROD activity. EROD was found to have the highest basal and induced activities of all four ARODs in both single and multiple Study 1 treatment groups. BROD had the highest fold increase (over 14-fold) seen in both treatment groups when compared to the control. Study 1 EROD, MROD and PROD inductions ranged from 3- to 6-fold. In study 2, all four ARODs were significantly induced in liver microsomes upon oil exposure. EROD was found to have the highest activity in both the 5 ml/kg and 10 ml/kg study 2 treatment groups. One bird in the 10 ml/kg study 2 treatment group had a low EROD activity but that outlying datapoint could not be excluded after review of raw data or pathology findings. BROD had the highest fold-induction (over 9-fold and 5-fold) in both the 5 ml/kg and 10 ml/kg study 2 treatment groups when compared to the control group. Study 2 EROD MROD and PROD inductions ranged from 2.5-fold to 4.5-fold. The AROD activity profile of control birds was EROD >

MROD > BROD > PROD in both studies. This profile changed to EROD > BROD > MROD > PROD in study 1 treated birds and in study 2 birds treated with 5 ml/kg MC252 oil daily. Hepatic AROD profiles were similar in both dosing studies: EROD and PROD activities were the highest and the lowest, respectively, in both control and treated birds, and BROD activity had the highest fold increase.

AROD activity levels and profiles from untreated animals have been reported previously in double-crested cormorants as well as a variety of avian species and appear to vary widely, likely reflecting the different age groups and provenance of the birds (Custer et al., 2001; Davis et al., 1997; Esler et al., 2010; Hofius, 1992; Sanderson et al., 1994; Verbrugge et al., 2001). Basal AROD activities and CYP1A protein expression level were 1.3–2.3 fold higher in study 2 than in study 1 but low in both studies (Table 1). Study 2 had a greater proportion of sub-adults and birds of the year than study 1, which may explain the observed difference in basal CYP1A expression. Basal hepatic EROD activities in the present study were within a similar range to findings in wild DCCO embryos at pipping from reference colonies in South Dakota and Minnesota (16–18 pmol/min/mg microsomal protein, Custer et al., 2001) but lower than wild embryos at pipping from a reference site in Oregon (54 pmol/min/mg protein, Davis et al., 1997), wild 10-day old chicks from a control site (31–56 pmol/min/mg microsomal protein, Custer et al., 2001), untreated nestlings from Gull Island, Lake Huron (around 80 pmol/min/mg microsomal protein according to publication figure, Verbrugge et al., 2001), and day-old hatchlings from a Canadian reference site (283 pmol/min/mg microsomal protein Sanderson et al., 1994). Some of the above basal EROD values are comparable or higher than EROD activities found after treatment in the present study. Verbrugge and coauthors (2001) measured additional hepatic ARODs in untreated nestlings from Gull Island and reported higher BROD and MROD levels than in our studies (around 70 and 20 pmol/min/mg microsomal protein, respectively, according to publication figure) but did not detect PROD. EROD and PROD activities (the only two studied) stayed at similar activities from hatching to fledgling (Hofius, 1992). Similar results were found in wild birds collected from reference sites (Esler et al., 2010).

Many studies have reported CYP1A induction after crude oil or PAH exposure in duck species and other bird species. Herring gull chicks orally exposed to either Prudhoe Bay or Hibernia crude oil had elevated hepatic microsomal EROD activities of approximately 19-fold over the control birds (Lee et al., 1985). Rates of EROD activity in herring gull chicks exposed to Prudhoe Bay crude oil revealed greater induction by the aromatic fraction than the aliphatic fraction when compared to control birds (6.6-fold versus 2-fold, Peakall et al., 1989). Studies performed 10 years after the Exxon-Valdez oil spill revealed EROD activity was still significantly higher than in birds from unoiled sites: pigeon guillemots (*Cepphus columba*, 1.6-fold), harlequin duck (*Histrionicus histrionicus*, 2.9-fold), and Barrow's goldeneyes (*Bucephala islandica*, 1.9-fold) (Golet et al., 2002; Trust et al., 2000). Twenty years after the spill, harlequin ducks from oiled sites had EROD activity levels 3.7-fold greater than unoiled sites (Esler et al., 2010). Lesser scaup collected from areas with widespread petroleum contamination had higher BROD, EROD, and MROD levels (11.8-, 10.3-, 3.5-fold) than those from reference sites (Custer et al., 2000). Both Harlequin ducks and Steller's eiders from a petroleum-contaminated site exhibited induced EROD levels when compared to birds from a control site (Miles et al., 2007).

Induced AROD profiles of cormorants and other avian species exposed to a variety of CYP1A inducers highlight species differences. Double-crested cormorant chicks exposed twice to β -Naphthoflavone (BNF) and sampled 48 h after the last intraperitoneal injection had an AROD profile (EROD > BROD \geq MROD > PROD) similar to that in our two studies (Verbrugge et al., 2001). Contrasting with our study, an AROD profile of EROD > MROD > BROD > PROD was found in male and female, juvenile and adult, common cormorants (*Phalacrocorax carbo*) environmentally exposed to PHAHs (Kubota et al., 2005). A

Study 1 CYP1A Activity and Protein Expression

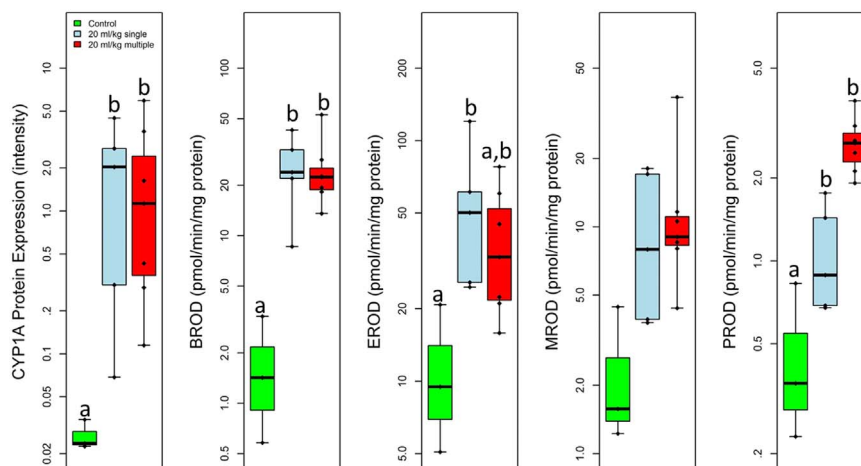


Fig. 1. CYP1A western blot band intensity and AROD activity in study 1 liver microsomes after oral exposure to 20 ml/kg MC252 oil per day for one day (single) or 5 days (multiple). The superscript lowercase indicates significant differences within each boxplot among treatment groups. See Table 1 for sample sizes and mean values.

profile of EROD > PROD > BROD > MROD was induced in black-eared kites (*Milvus migrans lineatus*) contaminated with dioxin-like compound exposure (Kubota et al., 2006b). CYP1A4 and CYP1A5 have been shown to preferentially bind to different resorufins and have uneven catalytic potential, likely contributing to the array of AROD profiles found in different studies reporting CYP1A activity in birds (Kubota et al., 2009). Common cormorant CYP1A4 and CYP1A5 expressed individually in yeast microsomes and exposed to dioxin-like compounds, revealed EROD activity values were highest for both enzymes followed by BROD for CYP1A4 and MROD for CYP1A5 and that CYP1A4 showed a higher binding affinity and catalytic potential for BROD than CYP1A5 for MROD (Kubota et al., 2009). In the present study, all 4 ARODs were significantly induced after oil ingestion suggesting induction of both CYP1A isoforms in DCCO.

EROD activity was highest for both dosing scenarios in the present study. EROD activity (599 pmol/min/mg microsomal protein) was also highest out of all four ARODs in double-crested cormorant chicks intraperitoneally exposed to BNF (Verbrugge et al., 2001). Studies on male, female, juvenile, and adult wild common cormorants exposed to PHAHs all reported EROD activity as highest (110–470 pmol/min/mg) (Kubota et al., 2005). Studies that focused on EROD activity alone in double-crested cormorant chicks reported median values at 193 pmol/

min/mg (embryos environmentally exposed to dioxin-like compounds, Davis et al., 1997) and a range of 21.2–68.3 pmol/min/mg microsomal protein (environmentally exposed to p,p'-DDE and PCBs, Custer et al., 2001).

BROD has been cited as a better indicator of CYP1A activity in birds than in mammals (Elliott et al., 1996; Verbrugge et al., 2001). BROD activity, in both studies 1 and 2, was found to be induced to the greatest degree compared to controls. Comparable results were found in lesser scaup exposed to PAHs (Custer et al., 2000). In contrast, MROD activity was the most highly induced activity in double-crested cormorant chicks exposed to BNF (19.5-fold, Verbrugge et al., 2001). Wild black-footed albatrosses (*Phoebastria nigripes*) and jungle crows (*Corvus macrorhynchos*) exposed to dioxin-like compounds, were reported to have low BROD activity and high MROD activity (Kubota et al., 2010; Watanabe et al., 2005). PROD activities were the lowest AROD activities in both studies reported here, which was consistent with studies in other species of cormorant (Kubota et al., 2005; Verbrugge et al., 2001; Guruge and Tanabe, 1997; van den Berg et al., 1994). This is not consistent across all bird species, as studies of black-eared kites and black-footed albatrosses with environmental exposures to dioxin-like compounds demonstrated PROD activities higher than MROD and/or BROD activity (Kubota et al., 2006, Kubota et al., 2010). Despite the

Study 2 CYP1A Activity and Protein Expression

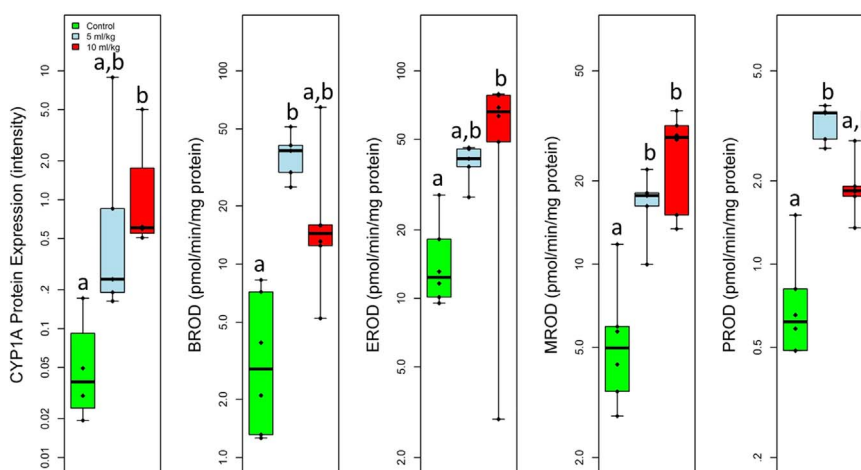


Fig. 2. CYP1A western blot band intensity and AROD activity in study 2 liver microsomes after oral exposure to 5 or 10 ml/kg MC252 oil daily for up to 21 days. The superscript lowercase indicates significant differences within each boxplot among treatment groups. See Table 1 for sample sizes and mean values.

Table 2

Correlative relationships among hepatic microsomal AROD activities in double-crested cormorants orally dosed with artificially aged MC252 oil. Rho values from Spearman's rank correlation analysis. Italics represent statistically significant values ($p < 0.05$). For the EROD-PROD correlation in Study 2, $p = 0.05$.

EROD	MROD	PROD	
Study 1			
0.79	<i>0.51</i>	<i>0.60</i>	BROD
	<i>0.84</i>	<i>0.52</i>	EROD
		<i>0.76</i>	MROD
Study 2			
0.72	<i>0.65</i>	<i>0.87</i>	BROD
	<i>0.67</i>	<i>0.48</i>	EROD
		<i>0.69</i>	MROD

low PROD activities found in the present study, fold increase was high, usually the second highest, and therefore, cannot be dismissed as biologically irrelevant.

AROD induction pattern and levels vary among bird species and between inducers in the same species. Thus AROD monitoring for CYP1A induction in wild birds should include all four of the resorufin substrates to ensure capturing these species-, tissue-, and inducer-specific variations. Induction profiles of CYP1A catalytic activity in the present study occurred in response to oral exposures of artificially aged oil from the Deepwater Horizon oil spill. Relative to crude oil from other spills, MC252 DWH oil was found to be high in alkanes and low in PAH composition (Faksness et al., 2015; Turner et al., 2014). As petroleum oils are complex mixtures of CYP inducers and inhibitors, variability in induction responses should be anticipated in other DWH-associated species. Exposures to spills of other, non-DWH petroleum oils might lead to different AROD response patterns, even in double-crested cormorants, the species studied here.

3.2. CYP1A protein expression

CYP1A protein expression has been used as a biomarker of PAH exposure in many organisms including seabirds, fish, and sea turtles (Verbrugge et al., 2001; Nakayama et al., 2008; Giannetti et al., 2012). CYP1A1 mouse monoclonal antibody was used to probe CYP1A4/1A5 in the double-crested cormorants. While the antibody of choice was monoclonal and a single band was detected for each liver microsome sample, we choose to refer to the protein detected as CYP1A rather than CYP1A4. A single band may result because the two bird isoforms (1A4/1A5) exhibit high molecular similarities and have similar weights differing by less than 1 kDa (Kubota et al., 2009), thus both isoforms could possibly be contributing to band intensity.

Western blot analysis revealed significant CYP1A protein induction in livers of DCCO exposed to oil in both of our studies. A 71-fold and 69-fold induction was noted in study 1 DCCO from the single and multiple dose treatment groups, respectively (Table 1). Study 2 showed a significant 30-fold and 24-fold increase in CYP1A band intensity in the liver for the 5 and 10 ml/kg MC252 oil treatment groups, respectively, when compared to the control group (Table 1).

CYP1A protein levels have been shown to increase in birds after exposure to various AhR agonists. Induction of CYP1A protein expression varies among species and contaminant exposure. For example, CYP1A band intensity increased 2–3-fold in double-crested cormorant chicks exposed to halogenated aromatic hydrocarbon contamination when compared to reference sites (Sanderson et al., 1994). In the same study, TEQs and CYP1A protein concentration were correlated at 5 different sites across Canada. Double-crested cormorant chicks exposed to BNF resulted in induced CYP1A-like protein (Verbrugge et al., 2001). Bald eagle chicks exhibited 6-fold higher CYP1A protein expression at

PCB contaminated sites when compared to reference sites. The fold-increase found in the present study is greater than those found in the previously mentioned studies.

3.3. Correlation analyses among ARODs and between AROD and CYP1A protein induction

Significant positive correlations were found among all four hepatic AROD activities in both studies (Table 2). Similar findings have been reported in black-eared kites, common cormorants, and lesser scaups exposed to PAHs (Kubota et al., 2006; Kubota et al., 2005; Custer et al., 2000). In study 2 DCCO livers, a significant positive correlation was detected between CYP1A band intensity and MROD ($\rho = 0.65$, $p = 0.018$) and PROD activity ($\rho = 0.62$, $p = 0.027$). A previous study conducted on wild common cormorants environmentally exposed to AhR agonists reported significant positive correlations between hepatic AROD activity and CYP1A but not CYP2B, CYP2C, nor CYP3A protein (Kubota et al., 2005). No significant correlation between DCCO liver CYP1A band intensity and EROD or BROD in study 2 nor any of the AROD activities in study 1 were detected, possibly due to the small sample size of the studies or the catalytic specificities of the DCCO CYP1As. Further studies are needed to elucidate in greater details correlation patterns between AROD and CYP1A protein in birds exposed to oil.

Further analyses will investigate potential correlations among these detected CYP1A changes and other endpoints of oxidative stress examined in the birds. Oxidative stress endpoints include measurements of antioxidants, antioxidant enzymes, and % Heinz bodies.

4. Conclusion

Our data show that acute and subchronic ingestion of MC252 oil induces a hepatic CYP1A response in double-crested cormorants. Significant induction was found through both CYP1A catalytic activity (AROD) and protein expression (western blot). Based on the AROD profiles observed in treated birds and the known catalytic specificity of CYP1A isoforms in common cormorant, both CYP1A4 and CYP1A5 were likely induced in DCCO in the present studies. Exposure to MC252 oil resulted in significant induction of all four ARODs. EROD had the highest activity and BROD the highest fold induction in both studies and MROD and PROD correlated with CYP1A protein expression in study 2. We suggest the measurements of all four AROD activities as well as CYP1A protein expression to ensure a thorough evaluation of oil exposure in birds. AROD activities and induction profiles are known to vary between species indicating species-specific studies are needed to accurately evaluate avian exposure to oil. The biomarkers of exposure to oil used in this study should be included as part of a weight-of-evidence approach to assessing oil-induced injury to birds.

Declaration of interest

There were no financial, professional, or personal conflicts of interest with any author listed on this manuscript.

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