SENSITIVITY TO HYDROCARBONS AND CYTOCHROME P4501A ENZYME ACTIVITY IN ARCTIC MARINE BIRDS AND WATERFOWL

By

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Abstract

The Arctic is host to a taxonomically diverse group of birds, including species of conservation and subsistence importance that spend many months of their annual cycle in the region. With prospects for oil and gas resource development and increases in vessel traffic in the Beaufort and Chukchi Seas, arctic birds could be valuable bioindicators to monitor contaminants and specifically hydrocarbons from crude oil. Using liver cytochrome P4501A (CYP1A) activity, I measured levels of hydrocarbon exposure in three bird species of subsistence importance: king eiders (*Somateria spectabilis)*, common eiders (*Somateria mollissima),* and greater white-fronted geese (*Anser albifrons*). Over the course of three years, I collected liver samples during spring and fall hunts near Utqiaġvik (formally Barrow) and validated methods for both direct-take and opportunistic liver sampling. Enzyme activity results show significant differences in CYP1A activity levels among species, seasons, and years. Except birds collected during fall 2014, when significantly high enzyme activity was observed in all sampled species, all other collections resulted in median activity levels similar to those reported in other sea duck species in Alaska from un-oiled or non-industrialized habitats. I also used species-specific hepatocyte culture in a broader selection of arctic marine birds and waterfowl candidate bioindicators to assess and compare species CYP1A activity responses as a measure of sensitivity to hydrocarbons. Cytochrome P4501A results from hepatocyte cultures dosed with positive control reference reagents and Alaska North Slope crude oil showed differences in species responses. Based on sensitivity results, I recommend the common eider and common murre (*Uria aalge*) as bioindicators for use in CYP1A monitoring due to their consistent and measureable responses in our experiments. However, additional species are promising candidates (e.g., tufted puffin; *Fratercula cirrhata*) but further testing is needed. This is the first study of reference hydrocarbon exposure and comparative laboratory assessment of CYP1A inducing compounds for arctic marine birds and waterfowl and these results form the basis for hydrocarbon monitoring programs and risk assessments.

Dedication

To my husband, children and parents
for their unwavering support and love.

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Introduction

The Arctic is host to a range of species including a diverse group of marine-associated waterfowl, alcids, loons, gulls, and shorebirds which rely on the region for important stages in their life cycle including mating, nesting, brood rearing, molting, and migration staging. These birds represent a variety of foraging strategies (surface, sub-surface, benthic, terrestrial) that consume marine prey including small fishes, euphausiids, zooplankton, mussels, and coastal and tundra plants and insects. Among these species are birds of conservation concern due to population declines. Additionally, some arctic birds (e.g., sea ducks) are an important subsistence food resource for local coastal communities. As prospects grow for arctic industrial development, such as drilling for oil and gas resources and vessel traffic, (BOEM 2015, Smith and Stephenson 2013), these activities increase the risk for potential contaminant exposures for arctic marine birds and waterfowl. Due to diversity in life histories, habitat use, and foraging strategies, birds may serve as valuable bioindicators for environmental contaminants in the Arctic. This thesis focuses on assessing sensitivity to and measuring levels of exposure of arctic marine birds and waterfowl to polycyclic aromatic hydrocarbons (PAHs) found in oil using enzyme responses. Based on those results, I make recommendations for species to use as bioindicators for hydrocarbon monitoring programs.

Large numbers of migratory birds use the nearshore area and marine waters of the Beaufort and Chukchi seas as migration corridors and staging grounds to accumulate body reserves for breeding and migration. For instance, satellite transmitter data showed that migrating king eiders (*Somateria spectabilis)* staged in the eastern Chukchi Sea for 21 ± 10 (standard deviation; SD) days in mid April-early June and 13 ± 13 (SD) days between late June and early November (Oppel et al. 2009). King eiders also used the Beaufort Sea in late summer as a post-breeding migratory staging area (Phillips et al. 2007). Common eiders (*Somateria mollissima*) that nested along the Beaufort Sea coast used both the Beaufort and Chukchi seas as migration staging areas (Petersen 2009). Additionally, spectacled eiders (*Somateria fischeri)* used the Beaufort and Chukchi seas as offshore migration corridors after breeding on the North Slope of Alaska (AK; Petersen et al. 1999). To get to their coastal nesting grounds, black brant (*Branta bernicla*) migrate along the southeastern Beaufort Sea coast (Reed et al. 1998). Shorebirds stage

for southern migration at Beaufort Sea coastal river deltas in the Arctic National Wildlife Refuge (Perkins et al. 2016). Many birds spend several months in the region using the coastal tundra, lakes, and nearshore islands for nesting and brood or chick rearing. Islands and coastal areas with cliffs and rocky slopes in the southern Chukchi Sea and northern Bering Sea support millions of breeding marine birds including auklets (*Aethia sp.*), murres (*Uria sp.*) and puffins (*Fratercula sp.*; Stephenson and Irons 2003). Several waterfowl species, including king eiders, common eiders, spectacled eiders, Steller's eiders (*Polysticta stelleri)*, greater white-fronted geese (*Anser albifrons)*, black brant, and tundra swans (*Cygnus columbianus*) nest on coastal tundra in the North Slope (Monda et al. 1994, Ely and Takekawa 1996, Reed 1998, Petersen 1999, Quakenbush et al. 2004, Phillips and Powell 2006, Petersen 2009). Several species of shorebirds including American golden-plover (*Pluvialis dominica*), sandpipers (*Calidris sp.*) and phalarope (*Phalaropus sp.*) nest near Utqiaġvik (formally Barrow) in northern Alaska (Perkins et al. 2016). The yellow-billed loon (*Gavia adamsii*) uses large tundra lakes in northern Alaska for breeding (Earnst et al. 2005a)

Several bird species of conservation concern use the Beaufort and Chukchi seas region. All four eider species nesting in the Arctic have experienced population declines. Spectacled eider and the Alaska-breeding Steller's eider are both federally listed as threatened under the United States Endangered Species Act (Federal Register 1993, 1997) and king and common eider populations have decreased by 50% compared to historical levels (Suydam et al. 2000). Populations of long-tailed duck (*Clangula hyemalis*), another arctic sea duck, have declined since the 1970s (Hollmén et al. 2003 and references therein). Reasons for population declines in all these species are not well understood and many factors may be responsible. The yellow-billed loon is also a species of conservation concern due, in part, to primarily breeding in areas proposed for oil and gas developments (Earnst et al. 2005b).

Arctic coastal communities rely on marine resources for subsistence and include harvest of meat and eggs of several species of sea ducks and geese. Of sea ducks, common eiders, long-tailed ducks and king eiders are harvested with king eiders, the most abundant sea duck, favored by hunters. Geese, such as greater white-fronted goose, black brant, and Canada goose (*Branta canadensis)* are hunted during spring arrival onto the North Slope with greater white-fronted geese as the main resource (Robert Sarren, personal communication).

Arctic oil and gas resource exploration (BOEM 2015), development of coastal infrastructure for drilling activities, and increased shipping traffic through ice-free arctic waters (Smith and Stephenson 2013) elevate the risk for accidental release of oil into the environment. Many arctic marine birds tend to aggregate in large flocks while at sea making large proportions of the population susceptible to disturbances such as oil spills. Alexander et al. (1997) reported over 25% of the Beaufort Sea population of common eiders congregated in open water off Cape Bathurst in western Canada during spring migration. Large aggregations of auklets, murres and puffins were record in the Chukchi Sea from ship-based surveys during summer and fall (Kuletz et al. 2015). During wing molt, when birds are flightless for several weeks, they are also vulnerable to environmental disturbances. Spectacled eiders, common eiders, and king eiders all molt along coastal areas of the western Chukchi Sea with some king eiders using the Beaufort Sea as well (Petersen et al. 1999, Petersen and Flint 2002, Phillips et al. 2007).

PAHs are environmental contaminants, often found in complex mixtures, that can originate from natural sources (e.g., forest fires), but major sources are crude oil, coal, and oil shale (Douben 2003). PAHs, including those found in crude oil, can induce a wide range of toxic effects in birds and have been frequently studied in laboratory projects involving chickens (*Gallus domesticus*), mallards (*Anas platyrhynchos*), and turkeys (*Meleagris gallopavo*; Albers 2006 and references therein). When injected into fertile eggs, applied to surface of eggshells, or given orally to birds, developmental abnormalities, reduced weight gain, decreased survival, and endocrine and immune system effects were noted (Butler et al. 1979, Hoffman and Gay 1981, Peakall et al. 1982, Brunstrom et al. 1991, Brunstrom 1991, Trust et al. 1994). In the wild, birds can be exposed to PAHs from oil through ingestion of water and diet items, preening of oil contaminated feathers, dermal absorption, and inhalation (Albers 2006).

Several studies have used a biochemical approach to measuring and evaluating the effects of PAHs in birds by using induction of the liver enzyme cytochrome P4501A (CYP1A; Albers 2006 and references therein). CYP1A, found in high levels in vertebrate liver, aids in the detoxification process of xenobiotic compounds (Livingstone 1996). After exposure to PAHs and persistent organic pollutants (POPs), the aryl hydrocarbon receptor (AhR), a nuclear transcription factor, is activated and induces CYP1A and liver enzyme 7-ethoxyresorufin-*O*-deethylase (EROD) activity (Mohammadi-Bardbori 2014, Head et al. 2015). The catalytic

activity of CYP1A is commonly measured as EROD activity using a fluorescent assay and is a standard, and widely used, measurement for assessing exposure to hydrocarbons (Kennedy et al. 1996, Short et al. 2008, Esler et al. 2010). Due to post-ingestion metabolism of PAHs, which prevents direct measurement of oil constituents in tissues, and the limited number of compounds that cause strong induction (PAHs, polychlorinated biphenyls (PCB), and dioxins; Payne et al. 1987, Rattner et al. 1994, Goksøyr 1995), CYP1A activity is often used as a biomarker of oil exposure in marine waterfowl (Trust et al. 2000; Miles et al. 2007, Esler 2008, Esler et al. 2010). Previous field studies have validated elevated CYP1A activity as resulting from oil exposure, and not from another inducing compound, by measuring plasma biomarkers (Trust et al. 2000, Miles et al. 2007) or analyzing the bioavailable contaminants present in water (Short et al. 2008). It is important to note that CYP1A induction does not necessarily indicate harmful effects (Lee and Anderson 2005) and indicates only that there has been exposure to inducing compounds and, as a result, a potential for toxic impacts.

Field and laboratory studies have linked increased EROD activity to oil exposure in harlequin ducks (*Histrionicus histrionicus*), Steller's eider, and Barrow's goldeneye (*Bucephala islandica*; Trust et al. 2000, Miles et al. 2007, Esler 2008, Esler et al. 2010). These studies used non-lethal surgery or direct-take to collect liver biopsies from wild and captive birds and their methodology included freezing of liver samples with-in 10 minutes of collection. Residual oil from the *Exxon Valdez* oil spill in Prince William Sound, AK is likely thought to be the cause of elevated EROD activity in livers from harlequin ducks and Barrow's goldeneye sampled 8-20 years post-spill (Trust et al. 2000, Esler 2008, Esler et al. 2010). Miles et al. (2007) found increases in EROD activity in harlequin ducks and Steller's eider near industrialized seaports in the eastern Aleutian Islands, AK when compared to non-industrialized habitats. To further understand EROD activity, Miles et al. (2007) also dosed captive Steller's eiders with a CYP1A inducing reagent. While the captive birds induced CYP1A, enzyme activity was higher in wild Steller's eiders potentially due to repeated exposure and exposure to higher concentrations and mixtures of PAHs and other inducing compounds. It is of interest to determine reference levels of EROD activity in arctic birds of the Beaufort and Chukchi seas prior to further industrial development, but these have not been reported. Additionally, the rate of CYP1A degradation in the liver after sampling has not been investigated. Defining the degradation rate could potentially

expand the collection window beyond the established 10 minute protocol and increase feasibility of field sampling.

As an alternative to field or animal dosing studies, EROD activity in primary hepatocyte cultures provides a diagnostic tool to define species-specific sensitivity and responses to CYP1A inducing toxins. Cell culture techniques allow for testing and comparing species responses to individual and compound hydrocarbons by assessing the magnitude of EROD activity. In several studies, chickens were determined to be most sensitive to PAHs and halogenated aromatic hydrocarbons (HAHs) based on their EROD responses when compared to a variety of species including ring-necked pheasants (*Phasianus colchicus),* turkeys, mallards, herring gulls (*Larus argentatus*), greater scaup (*Aythya marila*), bald eagles (*Haliaeetus leucocephalus*) and common terns (*Sterna hirundo*) (Kennedy et al. 1996, Lorenzen et al. 1997, Kennedy et al. 2003, Head et al. 2015). Understanding a species response to hydrocarbon exposure, and how responses among species compare to each other, aids in identification of candidate species for monitoring programs. Species and individuals will likely respond differently to the same contaminant depending on their sensitivities to different chemicals (Head et al. 2015), and knowledge about variability in CYP1A responses among species and individuals would help prioritize sampling efforts. To date, assessment of sensitivity, using CYP1A activity responses, of arctic marine birds and waterfowl of the Beaufort and Chukchi seas to exposure from PAHs found in oil has not been investigated.

A good bioindicator species acts as an ecosystem sentinel showing the first signs of exposure or physiological impacts, i.e., a "canary in the coalmine" (Bonisoli-Alquati 2014). Birds in general are good candidate bioindicators because of their ecological niche diversity, presence across different environments, abundance, visibility, and typically, unexpected changes in population sizes, health, or reproductive success provides an "alarm" to potential ecosystem changes or contaminants (Furness and Camphuysen 1997, Bonisoli-Alquati 2014). For instance, bird species were an early indicator of dichlorodiphenyltrichloroethane (DTT) bioaccumulation, which led to reproductive failures and population declines (Hellou et al. 2012). Early detection of pollutants through bioindicator species allows for remedial or preventative actions to be taken (Livingstone 1996). Using both field and CYP1A response laboratory data, this thesis provides

information for the selection of candidate bird species from the Beaufort and Chukchi seas region for monitoring hydrocarbon exposure.

Herein, I report field methods and CYP1A activity observed in liver samples collected from 2014 to 2016 from three arctic bird species; king eider, common eider and greater white-fronted goose. Additionally, I determine species responses to hydrocarbon exposure by using species-specific liver cell culture as a tool to measure CYP1A activity in a broader selection of arctic birds: Steller's eider, spectacled eider, common eider, king eider, long-tailed duck, greater white-fronted goose, black brant, common murre (*Uria aalge*), tufted puffin (*Fratercula cirrhata*) and horned puffin (*Fratercula corniculata*). Throughout the remainder of this thesis, I use the term "arctic birds" to encompass study species that use marine, near shore, or terrestrial habitats. Species were selected for the study based on their conservation status and subsistence importance. The selected species represent hosts for a variety of potential hydrocarbon exposure pathways due to diversity in habitat use and foraging strategies. Additionally, species life history diversity increases the chances an exposure event or changes in the ecosystem will be captured. Lastly, I make recommendations for using arctic birds in hydrocarbon monitoring programs based on field sampling and laboratory CYP1A results.

My specific objectives were to:

1. Measure levels of CYP1A activity in three arctic bird species.
2. Validate field liver collection methods and determine the feasibility of using liver samples collected from hunter-killed arctic birds as a CYP1A monitoring tool for the Beaufort and Chukchi seas.
3. Measure CYP1A enzyme degradation in liver samples postmortem to confirm a collection window.
4. Use species-specific CYP1A activity in hepatocyte culture in ten arctic bird species to identify which species are comparatively more sensitive to hydrocarbon exposure.
5. Using both field and sensitivity CYP1A results; identify potential bioindicator species and make recommendations for using arctic birds in hydrocarbon monitoring programs.

The laboratory and field results for the first three objectives I discuss in Chapter 1. In Chapter 2, I address my fourth objective by measuring CYP1A activity in cell cultures dosed with a variety of concentrations of testing reagents and Alaska North Slope crude oil. The fifth objective I discuss in part in both chapters with final biomonitoring recommendations in the general conclusion.

Chapter 1 Cytochrome P4501A Enzyme Activity in Arctic Marine Birds and Waterfowl: Opportunistic Field Collection Methods Validation and Enzyme Levels 2014-2016[1]

1.1 Abstract

The Arctic is host to taxonomically diverse birds that rely on the habitat for many stages in their annual cycle. With potential increases in oil and gas resource development in the Beaufort and Chukchi Seas reference levels of exposure to polycyclic aromatic hydrocarbons have not been reported in arctic birds. Using liver cytochrome P4501A (CYP1A) activity we evaluated hydrocarbon exposure in king eiders (*Somateria spectabilis*), common eiders (*Somateria mollissima),* and greater white-fronted geese (*Anser albifrons*) by validating field methods and collecting livers during seasonal hunts over three years. Results show differences in CYP1A activity among species and years, with most activity levels similar to those reported in other sea duck species from un-oiled habitats, except significantly high enzyme activity in fall 2014 birds. Our results provide a first assessment of CYP1A activity in these species and form a basis for development of programs monitoring exposure levels in arctic birds.

[1] Riddle, A. E., T. E. Hollmén, R. Suydam, R. Sarren, and R. Stimmelmayr. Cytochrome P4501A Enzyme Activity in Arctic Marine Birds and Waterfowl: Opportunistic Field Collection Methods Validation and Enzyme Levels 2014-2016. Prepared for submission to Marine Pollution Bulletin.

1.2 Introduction

Arctic birds may be exposed to a variety of harmful contaminants in the Arctic and during life stages spent away from the region (e.g., wintering grounds). For example, waterfowl are at risk for lead poisoning by consuming lead shot left on hunting grounds (Flint et al. 1997, Wilson et al. 2004). Elevated, and potentially detrimental, levels of mercury exposure have been recorded in arctic-breeding shorebirds (Perkins et al. 2016). High levels of lead in spectacled eiders (*Somateria fischeri*), common eiders (*Somateria mollissima*), and juvenile long-tailed ducks (*Clangula hyemalis*) sampled near Prudhoe Bay, Alaska (AK), an active oil field, may

indicate local exposure (Wilson et al. 2004, Miller et al. 2016). Also near Prudhoe Bay, Franson et al. (2004) reported low concentrations of analyzed trace elements and persistent organic pollutants except for selenium in long-tailed ducks and common eiders, and strontium in common eider eggs. Miller et al. (2016) sampled birds near Prudhoe Bay and Utqiaġvik (formally Barrow), AK during pre-breeding through post-breeding, and found selenium, copper, and cadmium in spectacled eiders, common eiders, Steller's eiders (*Polysticta stelleri*), king eiders (*Somateria spectabilis*) and long-tailed ducks but suggested the elements were acquired on non-breeding habitats. Spectacled eiders wintering off of St. Lawrence Island, AK in the Bering Sea accumulated elevated levels of copper, cadmium, and selenium, which may decrease fecundity or survival of young but currently no direct health impacts have been noted on adults (Trust et al. 2000b, Lovvorn et al. 2013). Toxicity levels of many trace elements have not been determined for arctic birds.

With prospects for increasing development of oil and gas resources and vessel traffic in the Arctic, specifically the Beaufort and Chukchi seas (BOEM 2015, Smith and Stephenson 2013), current measurements of exposure in arctic birds to polycyclic aromatic hydrocarbons (PAH) found in crude oil are needed as a baseline, but have not been reported. As industrialization increases, understanding baseline exposure levels to PAHs is essential to assist with monitoring and protection of arctic bird populations. The Beaufort and Chukchi region supports a wide array of taxonomically diverse birds (e.g., waterfowl, alcids, loons, gulls, shorebirds) that rely on the region for key stages in their annual cycle. Many birds depend on the nearshore, marine waters, coastal tundra, and near-by barrier islands for migration, migration staging, breeding, and brood/chick rearing. Additionally, many arctic birds and their eggs are important subsistence resources for coastal communities. King eiders are abundant and commonly hunted sea duck, with common eiders and long-tailed ducks harvested as well. Among geese, greater white-fronted geese (*Anser albifrons)*, black brant (*Branta bernicla)*, and Canada geese (*Branta canadensis)* are also hunted in the region.

Due to post-ingestion metabolism, direct measurement of oil constituents in bird tissues cannot be used to assess exposure to PAHs (Lee et al. 1985, Näf et al. 1992). Instead, cytochrome P4501A (CYP1A) enzyme induction has been used as a biomarker of oil exposure in wildlife, including marine waterfowl (Trust et al. 2000a, Miles et al. 2007, Esler 2008, Esler et

al. 2010). CYP1A is activated with exposure to polychlorinated biphenyls (PCB), dioxins, and PAH constituents found in crude oil (Payne et al. 1987, Rattner et al. 1994, Goksøyr 1995). Activity of another liver enzyme, 7-ethoxyresorufin-*O*-deethylase (EROD) is used as an indicator of CYP1A induction. EROD activity measures the catalytic function of CYP1A and is a standard and widely used measurement for assessing exposure to hydrocarbons from oil (Kennedy et al. 1996, Short et al. 2008, Esler et al. 2010). Previous sea duck field studies have linked increased EROD activity to exposure from residual oil from the *Exxon Valdez* oil spill (Prince William Sound, AK) in harlequin ducks (*Histrionicus histrionicus*) and Barrow's goldeneyes (*Bucephala islandica*) 8-20 years after the spill (Trust et al. 2000a, Esler 2008, Esler et al. 2010). Miles et al. (2007) also found increases in EROD activity in harlequin ducks and Steller's eiders near industrialized seaports and PAH dosed captive Steller's eiders.

In this study, our primary objectives were to establish levels of CYP1A activity in three arctic avian species and assess the feasibility of using liver samples from hunter-killed birds as a PAH exposure monitoring tool. We selected the common eider, king eider, and greater white-fronted goose as study species based on their population status, subsistence importance, and role as a potential bioindicator in the Beaufort and Chukchi areas. Additionally, sampling feasibility for use in long-term monitoring, including the potential for opportunistic sample collections from subsistence hunters, aided in the selection of these three species. Due to their relative abundance in easily accessible hunting areas, greater white-fronted geese can also be sampled to explore seasonal differences in CYP1A activity between arrival onto the breeding grounds (spring) and again at the end of breeding (fall). The Chukchi and Beaufort seas and adjacent terrestrial and nearshore areas provide important habitat for all of these species (Suydam et al. 2000, Dickson and Gilchrist 2002, Oppel et al. 2009) and population declines have been noted in king eider and common eider populations in the Beaufort Sea (Suydam et al. 2000). Also, due to differences in habitat use and foraging strategies, eiders have the potential to serve as bioindicators of the at-sea benthic environment and geese more terrestrial and near shore environments. The ability to monitor different habitats through these species expands our potential to capture various hydrocarbon exposure pathways.

1.3 Methods

1.3.1 Field Collections

Liver samples were collected during seasonal hunts near Utqiaġvik, AK (Figure 1.1) between August 2014 and May 2016 following State and Federal hunting regulations and University of Alaska Fairbanks Institutional Animal Care and Use Committee approval (project 593132-7; Appendix 1.A). King and common eider samples were collected during fall (post-breeding; August-October) and greater white-fronted goose samples were collected during spring (pre-breeding; May) and fall hunts. Eiders were not collected during spring as they migrate along the ice edge making them more difficult to sample. In previous CYP1A analysis studies, liver biopsies were frozen within 10 minutes of death/biopsy removal (Trust et al. 2000a), and information about potential postmortem enzyme degradation beyond this time frame has not been reported. To maintain a 10 minute postmortem sampling window, we targeted birds with a verifiable time of death by using direct-take (shotgun) or observing the death when collecting opportunistic samples from subsistence hunters. Postmortem, livers were quickly removed through one or two incisions near the spine along the bird's back. Livers were cut into small pieces, samples placed in cryovials (~1 cm^3 tissue minimum), and immediately frozen in a cryogenic vapor phase liquid nitrogen dewar (-150ºC) in the field. When possible, samples were also frozen at less than 10 minutes postmortem in addition to the standard collection after 10 minutes. To explore the postmortem degradation of CYP1A activity and determine if the sampling window can be expanded, additional liver vials were frozen at 20 and 30 minutes and 1, 2, 4, 6, and 24 hours postmortem. Cryovials were kept at outdoor ambient temperatures until their assigned freeze time. Extra vials were frozen at 10 minutes, and 1 and 2 hours to use for laboratory QA/QC and determination of CYP1A activity degradation during long-term freezer storage. All birds underwent a necropsy and additional tissues were collected for archival and health assessment. Liver samples were transported in the vapor phase liquid nitrogen dewar to the Alaska SeaLife Center (ASLC) in Seward, AK, for laboratory analysis. Once at the ASLC, liver samples were transferred to -80ºC for storage until analysis.

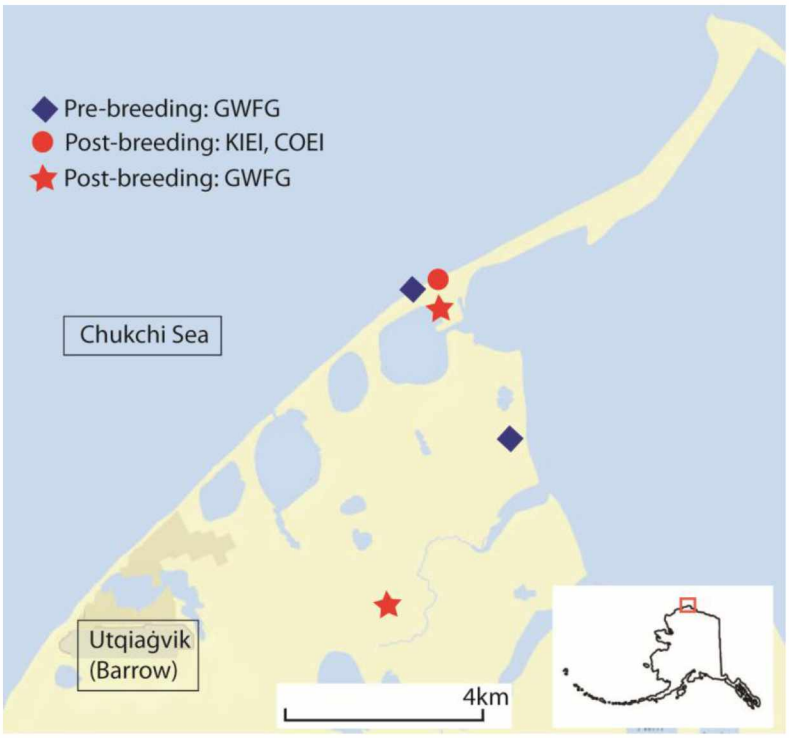

Figure 1.1: Spring and fall liver sample collection areas. Spring (pre-breeding) greater white-fronted goose (GWFG) locations were hunted from snow blinds (blue diamonds). Fall (post-breeding) GWFG locations were hunted inland on the tundra and at a pond near Pigniq (local hunting station; red stars). Fall (post-breeding) king eider (KIEI) and common eider (COEI) were taken also near Pigniq (red circle) as birds flew toward the Chukchi Sea.

1.3.2 Laboratory Analyses

We used microsome EROD activity to measure CYP1A induction in liver samples using methods adapted from Trust et al. (2000a) and Miles et al. (2007) and standard laboratory QA/QC procedures (Appendix 1.B). Microsomes were extracted within six months of collection (Daniel Esler personal communication) from 50-100 mg of liver tissue homogenized with 500 µL cold homogenizing buffer (0.05 M Tris, 0.15 M KCl, pH 7.4). Each homogenate was then centrifuged for 20 minutes at 10,000 g at 2ºC. The resulting supernatant was transferred to a new cold vial and spun for 60 minutes at 20,800 g at 2ºC. This supernatant was removed and the microsome pellet was resuspended in 100 µL cold resuspension solution (50 mM Tris, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 20% (v/v) glycerol, pH 7.4). To

determine EROD activity, microsomes were plated in triplicate in a black walled 96-well plate and read using a Gemini EM Dual-Scanning microplate spectrofluorometer fluorescent plate reader (Molecular Devices, Sunnyvale, California). Each well contained 200 µL consisting of 10 µL microsomes (or resorufin standard), 150 µL 2.5 µM 7-ethoxyresorufin (7-ER) in 50 mM Tris buffer (pH 8.0), and 40 µL 1.34 µM catalyst nicotinamide adenine dinucleotide phosphate (NADPH) in 50 mM Tris buffer (pH 8.0). Fluorescence was measured once every minute for 6 minutes at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. EROD activity was averaged over the triplicates with mean calculated and then divided by the slope of the resorufin product curve (0, 5, 10, 20, 60 pmol) to yield pmol/min. Protein levels (mg/mL) were determined by diluting 1 µL microsomes with 4 µL distilled water (dH_2O) and using the Bradford reagent (Sigma, St. Louis, Missouri) following manufacture protocols. Final EROD activity was expressed as pmol/min/mg protein and calculated using the formula:

EROD activity = (EROD pmol/min) / EROD sample volume (0.01 mL) / (mg/mL protein)

To validate the extraction process we tested EROD activity in the post-mitochondrial fraction before the 20,800 g 60 minute spin, the supernatant resulting from that spin, and finally the microsome pellet. Each fraction showed EROD activity but was on average 3-10 times lower than microsome fraction activity, confirming that we were using the fraction with the highest EROD activity.

Liver microsomes from embryonated mallard (*Anas platyrhynchos)* eggs, injected with a dose of 4 mg β-naphthoflavone (BNF; dissolved in peanut oil) at day 11 of incubation, acted as a positive EROD assay control. Dosed mallard microsome pools from three different extraction rounds resulted in mean enzyme activity of 13.51 ± 0.83 pmol/min, CV = 6.1%, 16.5 ± 2.6 pmol/min, CV = 16%, and 49.7 ± 9.9 pmol/min, CV = 19.9%. Resorufin 10 pmol was used for an inter- and intra-assay control and greater white-fronted goose 10 minute postmortem samples (birds #9 and #14) were used for inter-assay controls. EROD 10 pmol intra-assay results were 13.4 pmol/min ± 1.6 CV = 11.8%. EROD inter-assay control results were as follows: 10 pmol mean 8.9 ± 1.2 pmol/min, CV = 13%, greater white-fronted goose #9 mean 2.4 ± 3.9 pmol/min, CV = 16%, great white-fronted goose #14 mean 3.4 ± 0.56 pmol/min, CV = 16%. Bradford assay

controls included 2 mg/mL protein standard (BSA) as an intra- and inter-assay control and greater white-fronted goose birds #9 and #14 as inter-assay controls. Bradford 2 mg/mL intra-assay results were mean 1.95 ± 0.08 mg/mL, CV = 4% and inter-assay results were mean 2.04 ± 0.09 mg/mL, CV = 4.3%. Greater white-fronted goose Bradford inter-assay results were mean 3.1 ± 0.31 mg/mL, CV = 10% and mean 3.0 ± mg/mL, CV 12% for birds #9 and #14, respectively.

1.3.3 Statistical Analyses

Ten minute post mortem liver samples were used for all EROD (pmol/min/mg protein) result comparisons. EROD activity data was not normally distributed. For analysis, 10 minute postmortem median EROD results by year, season, and species were tested using the Kruskal-Wallis test and a post-hoc analysis performed using the Dunn test (p-values adjusted with the Holm method). EROD results for freezer storage and duplicate vial QA/QC were also analyzed using this method. All tests were calculated using *R* (*R* Core Team 2014) and an alpha level of 0.05 was used to determine statistical significances.

1.4 Results

1.4.1 Liver Collections

Table 1.1 presents the sample size for birds collected during spring and fall. All birds were collected using direct-take, except one king eider in fall 2015 was sampled opportunistically within 10 minutes of death. Complete time series samples were collected from all birds, and 81 of 92 birds had an additional liver sample collected and frozen at <10 minutes postmortem. In 2014, time series samples included 10, 20, 30 minutes and 1 and 2 hours. In 2015, the time series was adjusted to include vials frozen at 4, 6, and 24 hours in response to learning that hunters often do not butcher their birds until hours or days later.

Table 1.1: Numbers of birds collected during spring and fall hunts 2014-2016.

Species	Spring 2015 (n=)	Spring 2016 (n=)	Fall 2014 (n=)	Fall 2015 (n=)
Greater white-fronted goose	20	18	9	13
King eider	---	---	6	15
Common eider	---	---	9	2

1.4.2 EROD Activity

Median EROD activity results (pmol/min/mg protein) from microsome liver samples frozen at 10 minutes postmortem are shown in Figure 1.2. Due to small sample sizes in some sampling seasons, we combined all ages and sexes for each species for analysis. Individual EROD activity results ranged from 0-284 pmol/min/mg protein in king eiders, 27-367 pmol/min/mg protein in common eiders, and 0-915 pmol/min/mg protein in greater white-fronted geese. The highest EROD activity responses were found in greater white-fronted geese in fall 2014. Among all species fall 2014 was significantly different from fall 2015 ($p = 0.031$) and in geese also significantly different than spring 2015 and 2016 ($p = 0.025$ and 0.022 respectively). Enzyme activities in greater white-fronted geese were significantly different in fall 2015 than in spring 2015 ($p = 0.044$) but not spring 2016 ($p = 0.072$). There was no significant difference between spring 2015 and 2016 in the EROD activity in greater white-fronted geese ($p = 0.88$).

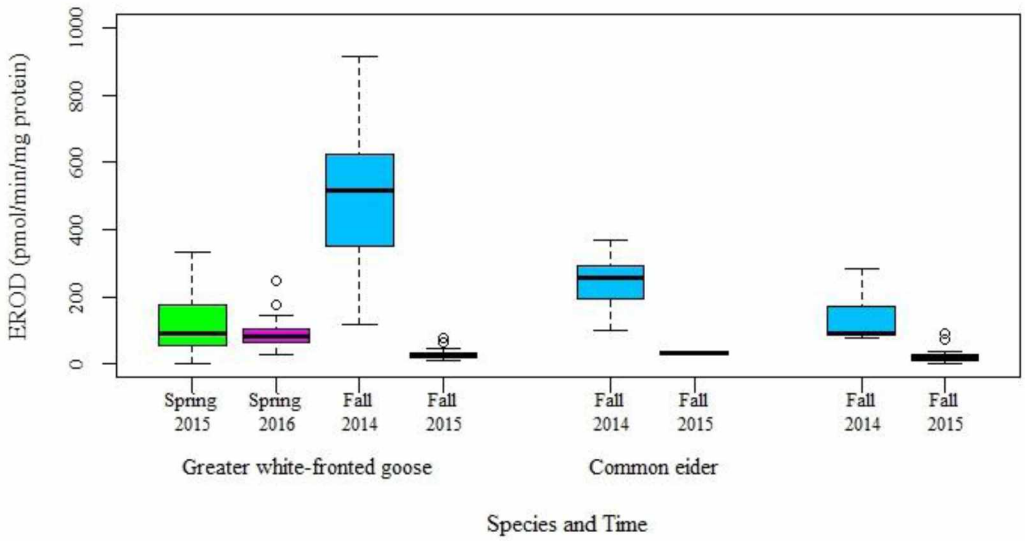

Figure 1.2: Median EROD activity in liver samples frozen at 10 minutes postmortem in all birds from all sampling periods.

EROD activity was detected throughout the postmortem time series samples, including liver samples frozen 24 hours after death. Enzyme activity after 10 minutes postmortem had a high degree of individual variability within and among species (Figure 1.3). Vials frozen at <10 minutes (2-9 minutes, average 5.5 minutes) showed similar activity to vials frozen at 10 minutes postmortem in all species and seasons (p = 1.0).

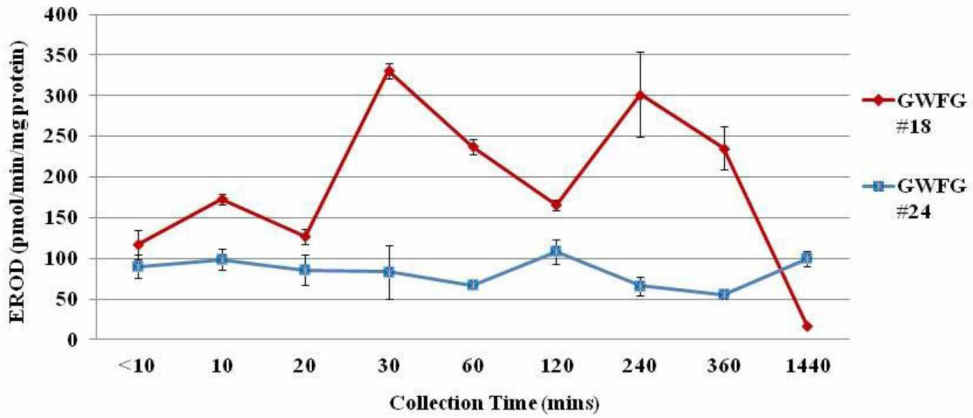

Figure 1.3: Mean EROD activity for triplicate sample wells (with standard error) for each time series point postmortem in two greater white-fronted geese sampled spring 2015.

Figure 1.3 cont: Greater white-fronted goose (GWFG) #24 represents a sample with little variability over the time series while goose #18 is an example of variable enzyme activity.

Results for QA/QC duplicate microsome extractions (2015 and 2016 samples, no duplicate vial was collected in 2014) for 10 minute postmortem samples are in Table 1.2 and show a similar response between the two extracts ($p = 1.0$). Fall 2015 common eider samples were not included in analysis due to small sample size (n=2). Replicates from vials frozen at 1 hour ($p = 1.0$) and 2 hours ($p = 1.0$) also showed no significant difference in EROD activity (data not shown). The first replicate vial from 10 minutes postmortem was used in all data analysis for consistency. EROD activity results for the freezer storage experiment with the 10 minute postmortem samples are in Table 1.3. The original samples from fall collections in 2014 were extracted in February 2015 (four to six months after collection). The same samples, from a vial that had not been previously thawed, were extracted in December 2015 after 16 months in storage at -80°C. The EROD results show a decrease in enzyme activity during long-term freezer storage with significant differences in common eiders ($p = 0.001$) and greater white-fronted geese ($p = 0.01$). King eider liver samples also decreased in EROD activity but not significantly ($p = 0.2$).

Table 1.2: Median EROD results (pmol/min/mg protein) with sample size by species and season, for vials frozen 10 minutes after death for duplicate microsome extraction QA/QC (KIEI=king eider, GWFG=greater white-fronted goose).

Sample	**EROD activity (pmol/min/mg protein)**	
	First Replicate	**Second Replicate**
GWFG Spring 2015 (n=16)	99.09	90.91
GWFG Fall 2015 (n=13)	19.99	28.01
GWFG Spring 2016 (n=18)	88.83	73.36
KIEI Fall 2015 (n=15)	19.62	25.86

Table 1.3: Median EROD results (pmol/min/mg protein) for 10 minute postmortem samples (collected 2014 fall) from the freezer storage QA/QC experiment. Enzyme activity results for original microsome extraction (within 6 months of collection) in February 2015 and the same samples re-extracted in 2015 after 16 months in storage at -80°C (KIEI=king eider, GWFG=greater white-fronted goose). *p-value <0.05

Species	EROD activity (pmol/min/mg protein)	
	Frozen for 6 months	**Frozen for 16 months**
GWFG (n=9)	518.57	68.49*
KIEI (n=5)	94.64	36.62
COEI (n=9)	254.51	30.23*

1.5 Discussion

1.5.1 Methods Validation

In this project samples were primarily collected by direct-take to ensure liver samples were frozen within the recommended 10 minute time frame postmortem. This approach made collecting and sampling all three species within 10 minutes of death feasible. We found that opportunistic sampling within 10 minutes of death can be challenging, but is possible with a close relationship with subsistence hunters. Due to the nature of goose and eider hunting around Utqiaġvik, king and common eider opportunistic samples will be most feasible to obtain in the fall. During fall, eider hunters gather outside of town in a small area (Pigniq; Figure 1.1) and the close proximity between hunters gives more opportunities for interactions between hunters and researchers. Other opportunistic events may include spring eider collections from birds hunted and butchered at whaling camps set along the ice edge. Also, during spring goose hunts collaborating hunters could be provided a small, portable, liquid nitrogen dewar to take to remote field camps for sample collections. Opportunistic samples from greater white-fronted geese in the fall are unlikely because very few hunters target geese due to the preference for eiders during the fall season.

Part of our methods validation was also to understand postmortem CYP1A enzyme degradation in the field and during freezer storage of liver samples. Our results indicate some EROD activity in liver samples through the time series samples, including those frozen 24 hours after death. However, EROD results for individual birds showed a high degree of variability in enzyme activity over the course of postmortem samples (<10 minutes–24 hours). It is unlikely this represents a sampling artifact since enzyme activity variability occurred in all collection periods and species, and our QA/QC replicates from single time-points showed similar EROD activity. The finding could be due to uneven degradation of proteins postmortem but whether protein degradation or some other biochemical processes are causing the uneven activity across the avian liver is unknown. We recommend maintaining the current protocol to freeze liver samples within 10 minutes of death for use in CYP1A analysis. Also, EROD activity significantly declines in liver samples during in long-term storage. We found samples kept at -80°C for over one year lost enzyme activity when compared to the same samples extracted within 6 months of collection. Based on these findings, we also recommend processing of samples within the currently used time frames.

1.5.2 EROD Activity

While our absolute enzyme activities are not necessarily directly comparable to other studies due to slight differences in laboratory protocols and different species, most of our median EROD results are similar to those reported for sea duck species in Alaska sampled in areas considered unexposed to oil. Trust et al. (2000a) reported average activity of 49.5 and 70.7 pmol/min/mg protein in Barrow's goldeneye and harlequin ducks in areas untouched by the *Exxon-Valdez* oil spill in Prince William Sound and 94.3 and 204.6 pmol/min/mg protein in spill areas. Measuring EROD activity in harlequin ducks in Prince William Sound up to 20 years after the *Exxon-Valdez* oil spill, Esler et al. (2010) reported activity of 15-25 pmol/min/mg protein in un-oiled and 75-100 pmol/min/mg protein in oiled areas. Mean EROD activity in Steller's eiders and harlequin ducks around Unalaska, AK at a clean reference site were 10-15 and 50 pmol/min/mg protein, respectively and enzyme activity around industrial areas were 20-50 and 100-275 pmol/min/mg protein, respectively (Miles et al. 2007). Our median EROD activity for king eider were 92.65 and 19.97 pmol/min/mg protein, common eider 54.51 and 32.97 pmol/min/mg

protein and greater white-fronted goose 518.57 and 25.04 pmol/min/mg protein (fall 2014 and fall 2015 respectably). EROD activity was significantly higher in greater white-fronted geese collected in fall 2014 than all other goose sampling periods. Enzyme activity in common eiders and king eiders from fall 2014 were also significantly higher than fall 2015 results in each species. Our EROD results from fall 2014 are also similar to enzyme activities from oiled areas. Greater white-fronted geese median EROD activity results for spring samples in 2015 and 2016 were 89.90 and 82.78 pmol/min/mg protein respectably. These results, which are discussed in more detail below, may also indicate PAH exposure in some individuals.

There has been little research into the half-life of CYP1A enzyme in avian species but CYP1A half-life was estimated at ~19 hours in rats injected with a CYP1A inducer (BNF; Chen et al. 2010). Additionally, CYP1A activity continued in fish livers 4 days after removal from a PAH contaminated environment (Fragoso et al. 1998) and 10+ days after a BNF injection (Kloepper-Sams 1989). It is likely the fall 2014 birds were exposed while on the North Slope, instead of experiencing exposure in wintering areas, since the birds had been on the breeding ground for several months at the time of sampling.

We detected a seasonal difference in EROD activity between spring and fall in greater white-fronted geese; spring 2015 and 2016 were statistically similar, both significantly different than fall 2014, and spring 2015 significantly different from fall 2015. Additionally, while median greater white-fronted goose spring activities are similar to those measured in unexposed sea ducks, our results indicate exposure in some individual geese. This may be residual enzyme activity if exposure occurred before arrival onto the breeding grounds. If there was exposure, genetic polymorphisms in CYP1A coding regions could also contribute to variation in responses to CYP1A induction between individuals within a species (Courtenay et al. 1994). Previous studies have shown age, sex, and season can affect CYP1A induction in some fish and birds (Kammann et al. 2005, Lee and Anderson et al. 2005 and references therein). However, Esler et al. (2010) found age, sex, and body mass of harlequin ducks sampled in early and late winter had no influence on EROD activity between oiled and un-oiled habitats. In this study, sex, and age was not considered due to sample sizes but our samples do include juveniles and adults of both sexes in spring and fall collections. Future Beaufort and Chukchi seas PAH monitoring programs

should include sampling adults and juveniles of both sexes during spring and fall hunts to further investigate how these parameters may impact CYP1A activity.

It is unknown at this time the source and identity of hydrocarbon, or other CYP1A inducing compound, which caused the elevated enzyme activity in 2014 in some spring geese. Also, with the current data we are unable to identify if the exposure was dietary or environmental (e.g., preening contaminated plumage) and acute or chronic. Areas close to Utqiaġvik, where birds nest and forage, such as greater white-fronted geese, include historical military sites. Several of these sites are designated "contaminated" by the Alaska Department of Environmental Conservation (ADEC) due to fuel spills and possible PCB contamination in the 1940-1970s (ADEC 2014). The role these contaminated sites might play in contaminant exposure and the elevated EROD activity in some sampled birds is uncertain. Previous studies in Prince William Sound, AK and St. Lawrence Island, AK have measured various PCBs in sea duck tissue and while some PCBs were detected, either potent CYP1A inducers were not, or levels were at trace concentrations (Trust et al. 2000a, Trust et al. 2000b, Esler et al. 2010). PAHs have been found in invertebrate sea duck diet items (e.g., blue mussels; *Mytilus trossilus*) and were tied to elevated sea duck EROD activity in the Eastern Aleutian Islands, AK (Miles et al. 2007) but direct measurement PAH load in arctic bird diet items haven't been completed to our knowledge. Lastly, high natural concentrations of PAHs, from biogenic and terrestrial sources, have been found in the Beaufort Sea nearshore and shelf surface sediments (Yunker and MacDonald 1995, Vankatesan et al. 2013). However, it is unlikely the natural concentration of PAH are at levels to induce toxic effects but their presence may make the area more sensitive to releases of additional PAHs from human activities (Yunker and MacDonald 1995). Archived liver and kidney samples could be used for direct measurement of potential contaminants to aid in identification of the potential source of exposure and ruling out other toxins (e.g., PCBs) that can also activate CYP1A.

High activity levels of CYP1A only indicate exposure has occurred and there is a potential for detrimental effects. On postmortem examination the majority of all sampled birds appeared to be healthy and in good body condition. One king eider and one white-fronted goose were found in poor body condition (little to no subcutaneous fat) in fall 2014, but their EROD activity fell near the median results of each species. Additional health evaluations and

biomarkers are necessary to fully determine the impact of elevated enzyme activity. Future research should involve supplementary tests, such as histopathology analysis, to further determine the health of all sampled birds and physiological factors potentially affecting seasonal variation in enzyme activity.

1.6 Conclusion

In summary, we present the first CYP1A reference data set for three arctic bird species that heavily use the Arctic during their annual life cycle: common eider, king eider, and greater white-fronted goose. We validated field methods for sample collections showing both direct-take and opportunistic sampling can be done within the current, and confirmed in this study, 10 minute postmortem time frame. Our enzyme activity results show exposure during fall 2014 that likely occurred while the birds were in the Arctic, but the exact source is unknown. We also noted high EROD activity in individual greater white-fronted geese collected during spring 2015 and 2016. Due to differences in EROD activity, diversity in diet and habitat use, and potential for opportunistic sampling, these three species are valuable candidates as bioindicators for hydrocarbon exposure monitoring.

1.7 Acknowledgments

We thank the Coastal Marine Institute and the Bureau of Ocean Energy Management (BOEM) for funding this project (AK-13-03-02). We thank Ruth Post and Rick Raymond for support and assistance during the course of this project. For field assistance we thank Dave Ramey, Ryan Klimstra, Nathan Wilen, and Callie Nelson from the North Slope Borough Department of Wildlife Management, and Sarah Tanedo and Jane Belovarac from the Alaska SeaLife Center. We also thank Alaska SeaLife Center Eider Research staff for support, especially Sadie Ulman. For statistical advice we thank Charles Frost U.S. Fish and Wildlife Service.

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1.9 Appendices

Appendix 1.A

University of Alaska Fairbanks Institutional Animal Care and Use Committee Approval Letter

Institutional Animal Care and Use Committee

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

April 29, 2016

To:	Tuula Hollmen Principal Investigator
From:	University of Alaska Fairbanks IACUC
Re:	[593132-7] Baselines of Hydrocarbon Exposure in Marine Birds of the Beaufort and Chukchi Seas

The IACUC has reviewed the Progress Report by Designated Member Review and the Protocol has been approved for an additional year.

Received:	April 18, 2016
Initial Approval Date:	May 29, 2014
Effective Date:	April 27, 2016
Expiration Date:	May 29, 2017

This action is included on the May 12, 2016 IACUC Agenda.

PI responsibilities:

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*

- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates).*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures detailed in the form 005 "Reporting Concerns".*

Appendix 1.B

Laboratory Protocols for Microsome Extraction, EROD, and Egg Dosing

Liver Tissue Microsome Extraction

1. Process on ice as much as possible and keep buffers ice cold to prevent any enzyme activity in the liver sample
    a. Put buffers and tubes in fridge/freezer
2. Weigh 50-100 mg (wet weight) liver
3. Homogenize with pestle in Tris+KCl buffer pH 7.4 on ice
    a. Add 500 µL buffer to each sample
    b. Use Teflon pestle, cleaning with ethanol (ETOH) after each sample
4. Centrifuge for 20 minutes at 10,000 g at 2°C
5. Transfer post-mitochondrial supernatant (PMS) to new cooled microcentrifuge tube
6. Centrifuge PMS highest possible speed (20,800 g) for 60 minutes at 2°C
7. Remove supernatant and toss
8. Resuspend microsome pellet in 100 µL 50mM Tris Resuspention Solution pH 7.4
9. Freeze microsomes at -80°C until ready to assay
    a. If running the same day keep in fridge short term before EROD
    b. If running Bradford assay later save 10 µL in separate tube

Bradford Protein Assay

1. Follow kit protocol
    a. Sigma catalog number B6916
    b. 96 well assay protocol
    c. Use SpectraMax Plus 384 absorbance microplate plate reader [Molecular Devices, Sunnyvale, California (CA)].
2. Gently mixed the Bradford reagent in the bottle and bring to room temperature
3. Prepare protein standards in buffer ranging from 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0mg/mL using a BSA standard or an equivalent protein standard
    a. Use 2 mg/mL protein BSA standard (Sigma catalog number P0834)
    b. 2 mg/mg: straight standard
    c. Example: 1 mg/ mL= 30 µL 2 mg + 30 µL distilled water (dH_2O)
    d. Negative control (0) is dH_2O
4. Add 5 µL of the protein standards to separate wells in the 96 well plate. To the blank wells add 5 µL of dH_2O.
5. Prepare the unknown samples(s) with an approximate concentration between 0.1-1.4 mg/mL.
    a. Dilute all unknown samples: 1 µL sample to 4 µL dH_2O
6. To each well being used, add 250 µL of the Bradford reagent and mix in the plate reader for ~30 seconds (hold shake button down).
7. Let the samples incubate at room temperature for 10 minutes.
    a. Assay can be incubated from 5-45 minutes, results don't change with shorter/longer incubation, but be consistent with 10 minutes.
8. Measure the absorbance at 595 nm.
    a. The absorbance of the samples must be recorded before 60 minutes (protein dye is stable for 60 minutes) and within 10 minutes of each other
9. Plot the absorbance vs. the protein concentration of each standard
10. Determine the protein concentration of the unknown sample(s) by comparing the net A595 values against the standard curve
    a. Multiple concentration by dilution amount to get final concentration

Microsome EROD

1. Run everything in triplicate on 96 well black plate
2. Use the Gemini EM Dual-Scanning Microplate Spectrofluorometer fluorescent plate reader (Molecular Devices, Sunnyvale, CA).
3. Add controls to plate
    a. Resorufin standards for curve
    b. Negative control (buffers only, no sample)
    c. Positive biological control (dosed mallard liver)
    d. Inter-assay biological control (microsome sample to run on every plate)
4. Resorufin standard dilution series
    a. Add 10 µL of each control to well
    b. Control 0, 5, 10, 20, 40 and 60 pmol (note stock used on each plate; use concentrations 100, 140 and 200 pmol if needed):
        i. negative control: 10 µL dH_2O
5. Total well volume 200 µL
6. Add to each sample well:
    a. 10 µL microsome
    b. 150 µL 7-ER buffer [2.5 µM 7-ethoxyresorufin (7-ER) in 50 mM Tris buffer at 8.0 pH (final concentration)]
        i. Color should be peach, if it is pink remake
        ii. For a full plate make: 538 µL 7-ER + 15.5 mL 50 mM Tris
    c. Add 40 µL of 1.34 mM of nicotinamide adenine dinucleotide phosphate (NADPH) in buffer to initiate activity (final concentration)
7. Measure fluorescence excitation wavelength of 530 nm and emission wavelength of 590 nm at 1 minute intervals for 6 minutes at room temperature
8. Use *Excel* to calculate mean wavelengths for each sample and control.
9. Graph resorufin control pmol activity (x-axis) against fluorescence (y-axis). This should be a tight linear relationship.
        i. Use a scatter plot graph

ii. Standard line R-squared value should be close to 1 (i.e., 0.9973).
iii. Show the equation of the line to find slope of standard curve

10. The sample slope (fluorescence/min) is divided by the slope of the resorufin product standard curve (fluorescence/pmol) to yield pmol/min
11. Use Bradford protein concentration (mg/mL protein) for each sample for final activity calculation.
    a. Activity is expressed as pmol/min/mg protein
12. Formula:

    EROD activity = (EROD pmol/min) / EROD sample volume (0.01 mL) / (mg/mL protein)

Egg Injections

1. Embryonated mallard eggs
    a. Artificial incubate in a Grumbach incubator (Grumbach, Germany)
    b. Monitor eggs every 3-5 days by candling
        i. Remove infertile or eggs that stop developing from the incubator
    c. Incubator settings:
        i. 37.5°C with 50-60% humidity
        ii. Eggs gently rolling on automatic rollers over 24 hours
        iii. Two 30 minute breaks with no heat per 24 hour period (10 and 14 hours)
2. Dissolve BNF into peanut oil to prepare dose
    a. 4 mg BNF/50 µL peanut oil
3. Inject into air cell on day 11
    a. Clean area with beta iodine
    b. Helpful to stick sterile needle into a black rubber stopper. This will leave just a small section of needle poking out and help prevent the needle from going too far into the egg.
    c. Make two holes in egg to allow for air to escape while injecting dose
    d. Use small grade needle as the doses are chucky and can clog the needle
    e. Once finished use liquid *Band-Aid* to cover holes

4. Doses:
    a. BNF/peanut oil dose inject ~50-100 µL
    b. Dose with ~50-100 µL just peanut oil (control)
    c. Dose with ~50-100 µL sterile 1X PBS (control)
5. Place eggs blunt end upward for 10-60 minutes following injection
6. Return to horizontal position in incubator
7. Harvest livers after 24 hours on day 12
    a. Use sterile techniques (follow cell extraction protocol for liver removal; Chapter 2)
    b. Place liver samples in cryovial
        i. Can pool multiple eggs with the same dose
    c. Freeze in liquid N_2 with-in 10 minutes of death

Extraction Reagents:

Homogenizing Buffer (pH 7.4)

500 mL total volume

0.05 M Tris: Add 25 mL 1 M Tris

0.15 M KCl: Add 75 mL 1 M KCl

400 mL dH_20

50 mM Tris Resuspention Solution (pH 7.4)

250 mL total volume

250 ul 1 M EDTA (1 mM EDTA)

250 ul 1 M DTT (1 mM DTT)

50 mL glycerol (20% glycerol v/v)

12.5 mL 1 M Tris (50 mM Tris)

Bring volume to 250 mL with dH_20 (187 mL)

1 M KCl Buffer

250 mL total volume

18.64 g KCl

Bring volume to 250 mL with dH_20

1 M Tris Buffer

250 mL total volume

30.285 g Tris

Bring volume to 250 mL

1 M DTT

1 g/vial

6.5 mL total volume

1 g DTT to 6.5 mL dH_20

1 M EDTA

250 mL total volume

93.05 g EDTA

Bring volume to 250 mL

Need NaOH to go into solution

May need HCl to add more acidity to reach correct pH

EROD Reagents:

7-ER buffer

200 µL assay well volume

2.5 µM final concentration 7-ER

2.5 µM concentration in total well volume of 200 µL. Stock needs to be 3.33 µM to have the addition of 150 µL = 2.5 µM.

Stock 3.33 µM: 20 mL (make fresh)

7-ER stock=100 µM stored at -80°C (suspended in MeOH)

For assay combine: 538 mL 100 µM 7-ER and 15.5 mL 50 mM Tris buffer

50 mM Tris Buffer (pH8.0)

500 mL total volume

25 mL 1 M Tris buffer stock

475 mL dH_20

NADPH

10 mg/vial

Need starting stock of 6.7 mM to get 1.34 mM in 200 µL assay well total volume (40 µL NADPH added to assay well)

6.7 mM stock

Add 1.8 mL 50 mM Tris buffer (to keep buffers the same as in 7-ER) to 10 mg vial

Egg Dosing Reagents:

BNF

1 g/vial

4 mg/50 µL = 0.04 g BNF + 500 µL peanut oil

Control Reagents:

Resorufin

1 g/vial

Add 79 mL dH_20 to 100 mg for 5 mM concentration

Dilute:

1 mM= 200 µL 5 mM stock + 800 µL dH_20

20 µM= 20 µL 1 mM stock + 980 µL dH_20

1 µM = 10 µL 20 uM stock + 190 µL dH_20

All buffer and media reagents were purchased from Invitrogen (Grand Island, New York), Sigma-Aldrich (St. Louis, Missouri), Fisher Scientific (Pittsburg, Pennsylvania), or VWR (Radnor, Pennsylvania)

Chapter 2 Assessing Arctic Marine Bird and Waterfowl Sensitivity to Hydrocarbon Exposure Using P4501A Enzyme Response in Liver Cell Cultures[1]

2.1 Abstract

As industrial activities increase in the Beaufort and Chukchi seas, determining the sensitivity of arctic wildlife to hydrocarbon exposure can provide reference data and information for monitoring programs and management of species potentially impacted by an oil spill. Using species-specific hepatocyte culture we assessed polycyclic aromatic hydrocarbon sensitivity by measuring liver enzyme cytochrome P4501A (CYP1A) activity in selected species of arctic birds. Our CYP1A results showed differences in species responses to dosing reagents chrysene, phenanthrene, β-naphthoflavone, and Alaska North Slope crude oil. Based on these results, we recommend the common eider (*Somateria mollissima*) and common murre (*Uria aalge*) as bioindicators for use in CYP1A monitoring due to their consistent and measureable CYP1A responses in our experiments. However, additional species are promising candidates (e.g., tufted puffin; *Fratercula cirrhata*) but further testing is required. This study is the first comparative laboratory assessment for arctic marine birds and waterfowl to CYP1A inducing compounds.

[1] Riddle, A. E., T. E. Hollmén, R. Suydam, R. Sarren, C. Frost, K. Counihan, C. Latty, R. Stimmelmayr. Assessing Arctic Marine Bird and Waterfowl Sensitivity to Hydrocarbon Exposure Using P4501A Enzyme Response in Liver Cell Cultures. Prepared for submission to Marine Pollution Bulletin.

2.2 Introduction

In the Arctic, the Beaufort and Chukchi sea region and nearby coastal habitat supports a rich variety of waterfowl and marine birds that use the area for breeding and migration (Reed et. al. 1998, Stephenson and Irons 2003, Phillips et al. 2007, Oppel et al. 2009, Petersen 2009, Perkins et al. 2016). The Beaufort and Chukchi seas are also areas of interest in oil and gas development (BOEM 2015), increasing commercial vessel traffic through ice-free arctic waters (Smith and Stephenson 2013), and is home to many hundreds of current and formally used defense sites with a high degree of contamination (von Hippel et al. 2016). This industrial

activity adds a potential source of polycyclic aromatic hydrocarbon (PAH) exposure for arctic birds through accidental releases of oil. PAHs, including those found in oil, and can induce a wide range of toxic effects in birds (Albers 2006 and references therein) and are poorly studied in many arctic marine birds and waterfowl species.

Several previous studies have researched the toxic effect of PAHs on eggs, embryos, and birds primarily using chicken (*Gallus domesticus*), mallard (*Anas platyrhynchos*), or turkey (*Meleagris gallopavo*) and less frequently, the common eider (*Somateria mollissima*) as test species (Albers 2006 and references therein). Single or mixtures of PAHs injected into fertile eggs, or applied to the eggshell, resulted in embryo cellular damage, decreased survival, developmental abnormalities, and reduced body weight (Hoffman and Gay 1981, Brunstrom et al. 1991, Brunstrom 1991). Exposure to PAHs during an egg injection study (Brunstrom et al. 1990) identified domestic ducks as the most sensitive and chickens the least sensitive, when compared with turkeys and common eiders. Studies dosing juvenile or adult birds in controlled laboratory exposure experiments showed endocrine disruption, immune system effects, and reduced weight gain (Butler et al. 1979, Peakall et al. 1982, Trust et al. 1994).

Hydrocarbon studies often use induction of the liver enzyme cytochrome P4501A (CYP1A) to assess the relative potency of or exposure to PAHs in wildlife including birds (Trust et al. 2000, Miles et al. 2007, Esler et al. 2010, Head et al. 2015). Cytochrome P4501A (CYP1A) is activated by exposure to environmental contaminants such as polychlorinated biphenyls (PCB) and dioxins but also to PAHs including the constituents of crude oil (Payne et al. 1987, Rattner et al. 1994, Goksøyr 1995). Activity of the liver enzyme, 7-ethoxyresorufin-*O*-deethylase (EROD), is a standard and widely used measurement of the catalytic activity of CYP1A induction (Kennedy et al. 1996, Short et al. 2008, Esler et al. 2010).

Measuring EROD responses in hepatocyte cultures can provide diagnostic tools to characterize species-specific sensitivity and responses to CYP1A inducing toxins. Cell culture techniques allow testing of unique species-specific responses and comparisons among species by using a suite of reference compounds under controlled laboratory conditions. Additionally, the magnitude and duration of CYP1A induction to single or compound toxin mixtures can be assessed in individual species. Kennedy et al. (1996) used CYP1A induction in hepatocyte cultures, measured by EROD activity, to predict the sensitivity of chickens, ring-necked

pheasants (*Phasianus colchicus),* turkeys, mallards, and herring gulls (*Larus argentatus*) to several halogenated aromatic hydrocarbons (HAHs). They ranked chicken as the most sensitive and herring gull the least sensitive to their testing reagents. Also using primary hepatocyte cultures, Head et al. (2015) ranked chickens more sensitive than Pekin duck (*Anas platyrhynchos domesticus*) and greater scaup (*Aythya marila*) to 18 PAHs. Bald eagle (*Haliaeetus leucocephalus*) and common tern (*Sterna hirundo*) hepatocyte cultures had low EROD activity responses to dioxins and HAHs when compared to chickens (Lorenzen et al. 1997, Kennedy et al. 2003). Species will likely respond differently to the same mixture of PAHs and HAHs depending on their individual sensitivities to different chemicals (Head et al. 2015).

CYP1A responses in arctic birds of the Beaufort and Chukchi seas from exposure to PAHs found in crude oil have not been investigated in controlled laboratory experiments. We selected ten arctic bird species to assess their sensitivity to PAHs and CYP1A inducing reagents: Steller's eider (*Polysticta stelleri*), spectacled eider (*Somateria fischeri)*, common eider, king eider (*Somateria spectabilis*), long-tailed duck (*Clangula hyemali*), greater white-fronted goose (*Anser albifrons*), black brant (*Branta bernicla)*, common murre (*Uria aalge*), tufted puffin (*Fratercula cirrhata*), and horned puffin (*Fratercula corniculata*). We chose species based on their conservation status, subsistence importance, and potential to act as a bioindicator of different exposure pathways due to diversities in habitat use and foraging strategies. A good bioindicator species can also serve as an ecosystem sentinel and birds have previously been used as early indicators to environmental toxin bioaccumulation (Hellou et al. 2012).

Due to significant population declines and range contraction, spectacled eiders and Alaska-breeding Steller's eiders, respectively, are listed as threatened under the United States Endangered Species Act (Federal Register 1993, 1997). King and common eider population numbers have decreased by 50% compared to historical levels (Suydam et al. 2000), and long-tailed duck populations have declined as well (Hollmén et al. 2003 and references therein). Currently, reasons for population declines in all these species are not well understood and many factors may be responsible. Arctic coastal communities rely on several avian species including king eider, common eider, greater white-fronted goose, long-tailed ducks, and black brant for subsistence use. Greater white-fronted geese and eggs from geese and ducks are important

resources during spring with migrating king eiders and common eiders as the primary targets hunted during fall (Robert Sarren, personal communication).

Birds may come in contact with hydrocarbons via their diet (e.g., ingestion of contaminated prey) or habitat (e.g., preening oil from feathers; Albers 2006). To represent different hydrocarbon exposure pathways in the food web, we chose both piscivorous (alcids) and benthic foraging (sea ducks) arctic bird species for the experiments. Two goose species were selected, 1) greater white-fronted goose represent a near shore and terrestrial foraging species and 2) black brant represent a goose species with a strong marine association (Ward et al. 2005). We also chose species based on their use of different habitats while in the Beaufort and Chukchi sea region. Many birds spend several months of their annual cycle in the Arctic for nesting and rearing young. For example, millions of murres (*Uria sp.*), puffins (*Fratercula sp.*), and auklets (*Aethia sp.*) use islands and coastal areas with cliffs and rocky slopes for nesting and chick rearing (Stephenson and Irons 2003), while all four eider species and black brant use the coastal tundra (Reed 1998, Petersen et al. 1999, Quakenbush et al. 2004, Phillips et al. 2007, Petersen 2009). Life history diversity in a monitoring program increases the chances an exposure event or changes in the ecosystem will be captured by one of the indicator species.

This study aims to understand arctic bird comparative sensitivity to PAHs from oil using EROD activity responses in species-specific hepatocyte culture. Specific goals were to:

1) Establish species-specific EROD reference curves from hepatocyte cultures dosed with known CYP1A inducers chrysene, phenanthrene, and β-naphthoflavone (BNF).
2) Establish species-specific EROD reference curves from hepatocyte cultures dosed with Alaska North Slope crude oil.
3) Compare tested species and identify sensitive responders using EROD activity and cellular cytopathic effects.
4) Make recommendations for bioindicator species to use for PAH monitoring in the arctic marine environment.

2.3 Methods

2.3.1 Source Material

Eggs from several species were acquired from captive birds from private or commercial breeders: mallard eggs from Murray McMurray Hatcheries (Webster City, Iowa), efowl.com (Denver, Colorado), and Metzer Farms (Gonzales, California); chicken eggs from Murray McMurray Hatcheries, efowl.com, and Stromberg's Chick and Game Birds Unlimited (Pine River, Minnesota); common eider and king eider eggs from Dry Creek Waterfowl (Port Angeles, Washington); and black brant eggs from private breeders (Washington). Additionally, the Alaska SeaLife Center [(ASLC) Seward, Alaska (AK)], a public aquarium and marine research facility, provided eggs from Steller's eider, spectacled eider, tufted puffin, horned puffin, long-tailed duck, and common murre. Greater white-fronted goose and common eider eggs were collected on the North Slope of Alaska [Utqiaġvik (formally Barrow) area and Kaktovik, respectively] in 2015 and 2016.

2.3.2 Egg Development

After arrival at the laboratory, eggs were cleaned by dipping them in a 1X chlorhexidine solution. They were then allowed to air dry and given an individual identification number. Eggs were either placed directly into an incubator or kept at room temperature at a 45° angle for 1-48 hours to stagger incubation start times. Eggs kept at room temperature longer than 12 hours were turned 180° every 12 hours. Artificial incubation occurred in a Grumbach incubator (Grumbach, Germany) at 37.5°C with 50-60% humidity and two 30 minute breaks with no heat per 24 hour period (10 and 14 hours). Eggs were placed on shelves in the incubator on top of slowly moving rollers which gently moved the egg over 24 hours. During incubation, eggs were monitored every 3-5 days by candling and eggs that were infertile, or stopped developing, were removed from the incubator.

2.3.3 Hepatocyte Extraction

We used established primary cell culture protocols (Brendler-Schwaab et al. 1994; Hollmén et al. 2013,) and followed standard cell culture QA/QC (Freshney 2000) to harvest

embryonic liver tissue at approximately 11-18 days of incubation for all species (Appendix 2.A). To be able to directly compare species responses, incubation times were adjusted for each species so embryos represented a similar stage of development at the time of cell line establishment. Eggshells were sterilized with povidone iodine and rinsed with distilled water (dH_2O) prior to opening. Using scissors to cut the shell, the top of the shell around the air cell was removed, the embryo quickly located and decapitated using scissors, and the torso placed in a Petri dish. The liver, without the gallbladder, was removed from the body and placed in cold 1X phosphate-buffered saline (PBS). Livers were pooled in groups of 1-4 and rinsed with pre-perfusion buffer II [Hanks Balanced Salt Solution 1X (HBSS) without $CaCl_2$ and $MgCl_2$, 10 mM HEPES, and 0.5 mM ethylene glycol tetraacetic acid (EGTA, pH 7.4)] after removal of the 1X PBS. The buffer was removed and livers were minced gently using sterile scalpels. Next, perfusion medium II [minimum essential medium (MEM) without L-glutamine, collagenase IV (100 U) and 0.125% trypsin] was added to the minced liver and the mixture added to a trypsinizing flask. For 1-2 livers 32 mL of perfusion medium II were added and for 3-4 embryos 48 mL. Livers were digested for 30 minutes at 37°C with gentle stirring and digestion was stopped with the addition of 20% heat inactivated fetal bovine serum (HI FBS). The resulting mixture was filtered through nylon mesh (100 μm) into a sterile 50 mL tube containing 10 mL pre-perfusion buffer. The hepatocyte suspension was then centrifuged at 63 g and 4°C for 5 minutes. The supernatant was removed and the remaining cell pellet was gently resuspended in 10 mL pre-perfusion buffer. At this point, additional liver suspensions were combined, if necessary, to bring the total number of livers used per assay culture to 4-12. Cell suspensions were spun at 110 g and 4°C for 5 minutes, the supernatant removed, and the cell pellet gently resuspended in 10 mL low glucose media [high glucose Dulbecco's Modified Eagle Medium (DMEM), MEM, antibiotic mixture (nystatin, penicillin-streptomycin, and gentamycin) and L-glutamine] and 20% HI FBS. A cell count was then performed using a hemocytometer and cells diluted with low glucose media with 20% HI FBS as needed. Finally, hepatocytes were seeded in a black-walled 96-well plate at 30,000 cells/well and a total well volume of 200 μL. Hepatocyte lines from all species were incubated at 37°C in 5% CO_2 atmosphere for 24-48 hours before dosing. Cultures were given fresh media with 20% HI FBS and checked by microscopy for confluency level, health, and morphology every 24 hours (Hollmén et al. 2002). All buffer and

media reagents were purchased from Invitrogen (Grand Island, New York), Sigma-Aldrich (St. Louis, Missouri), Fisher Scientific (Pittsburg, Pennsylvania), or VWR (Radnor, Pennsylvania).

2.3.4 Cell Culture Assays

Before dosing, media was removed from the wells, cells gently washed 1-2 times with 1X PBS to remove any cellular debris, and replaced with fresh media (100 µL with no FBS). Cultures were dosed, in triplicate, with carrier solvent control dimethyl sulfoxide (DMSO) or ethanol, negative controls (media only and cells with no dose), and different concentrations or amounts of single hydrocarbons or a reference laboratory chemical (chrysene, phenanthrene, or BNF) or compound mixture Alaska North Slope crude oil (ANS oil; Marathon Alaska Beaver Creek Crude Oil-Sweet). BNF, a standard laboratory chemical, is a known inducer of CYP1A activity and has been used in previous studies to measure avian EROD responses (Miles et al. 2007, Hollmén et al. 2013). Phenanthrene and chrysene represent PAHs of different molecular weights (3 and 4 rings respectively) that are found in crude oil and induce CYP1A activity (Incardona et al. 2005, Short et al. 2008). Doses were prepared with low glucose media, when necessary, and stored in amber vials at 4°C. Cultures were returned to the incubator following dosing. After 24 hours of dose exposure, cells were first evaluated using microscopy for general cell morphology and cytopathic effects (CPE). Types of CPE observed were characterized and described for each cell line and dose used. Cells were scored on a semi-quantitative response scale from 0-4 (0=none to 4=100 % effect) for a suite of CPE responses including: vacuolarization, cytoplasmic swelling, granularization, non-viability, cellular debris, and other abnormality (Hollmén et al. 2013).

We used EROD methods adapted from both Hodson et al. (1996) and Hahn et al. (1996). First, a solution of 6 µM 7-ethoxyresorufin (7-ER) was freshly prepared from 100 µM 7-ER stock and $NaPO_4$ buffer and 25 µL added to each cell well. Next, catalyst nicotinamide adenine dinucleotide phosphate (NADPH; 10 mg) was resuspended in 1.5 mL $NaPO_4$ buffer and 10 µL added to each well. Immediately, fluorescence was measured at excitation wavelength of 530 nm and an emission wavelength of 590 nm using a Gemini EM Dual-Scanning microplate spectrofluorometer fluorescent plate reader (Molecular Devices, Sunnyvale, California). To assess the cellular dose-response over time, and capture the threshold activity level, EROD

activity was measured every 15 minutes for 3 hours. Protein levels were not quantified because cell counts per well were uniform (Hahn et al. 1996). Mallard cultures were used to validate protocols and test reagents.

2.3.5 Analysis

The number of eggs we were able to acquire for each species varied and we were unable to complete the full testing regimen on all species. Common murre cultures occasionally had a high EROD response to control doses of carrier reagents DMSO and ethanol. To account for this background activity, EROD responses from DMSO and ethanol control wells were subtracted when these carrier reagents were used in doses. In all species, due to high background EROD activity in controls of media with neat ANS oil (no cells), the fluorescent responses from these controls were removed from the cellular responses before analysis. Cell culture results were expressed as pmol/min/metabolic activity.

After visualizing trends in the EROD responses, we found it more appropriate to use a linear mixed modeling approach than a traditional logistic response. The linear mixed model had fixed effects of dose and EROD assay read time (0-180 minutes in 15 minute increments) and a random effect of individual cell line to create estimated EROD response curves with 95% confidence intervals. Data from each individual cell culture well was used in the model. Chrysene 1.0 μM, BNF 1.0 μM and ANS oil 5.0 μL dose estimated EROD response curves for each species were visually compared to each other to assess relative sensitivity between species. Using those curves, species were categorized as having a high, moderate, or low EROD response to the testing reagent based on the highest activity reached during the 3 hour assay. Chrysene 1.0 μM EROD responses of ≥125 pmol/min/metabolic activity were considered high, 124-51 pmol/min/metabolic activity moderate, and ≤50 pmol/min/metabolic activity low. EROD responses of ≥150, 149-51, and ≤50 pmol/min/metabolic activity were ranked high, moderate, and low, respectably, for BNF 1.0 μM. ANS oil 5.0 μL estimated EROD response curves ≥350 pmol/min/metabolic activity were ranked high, 349-101 pmol/min/metabolic activity moderate, and ≤100 pmol/min/metabolic activity low responders. Categories were chosen by comparing the range of responses from tested species at each reagent. Cytopathic effects were used to evaluate the dose response and subsequent EROD activity.

2.4 Results

Table 2.1 summarizes test doses per species for this project. In most species, for the single reagent testing compounds, the highest EROD activity was recorded at the end of the assay (3 hours post-addition of catalyst). We rarely observed a decrease in EROD activity over the course of a single assay. Cellular responses to the compound mixture ANS oil were less consistent than the single compound testing reagents during the course of a single assay: EROD activity increased, decreased, or remained stable. Curve fitting results are shown in Figures 2.1-2.4 with EROD activity by species and dose.

Table 2.1 shows all tested chrysene concentrations for each species and reference species mallard and Figure 2.1 shows the EROD results for these assays. All species were tested at 1.0 μM chrysene with a 24 hour dose exposure as an initial dose for consistency and comparison. Previous testing (Hollmén et al. 2013) found this dose typically elicits a CYP1A response in most species without killing the culture or causing drastic CPE changes. These results indicate differences in species EROD responses at this dose with the highest comparative response from black brant and lowest from Steller's eider. Horned and tufted puffins comparatively had a similar response to each other as did common, spectacled, and king eiders. Additionally, there was no apparent visual difference in pathological response between control wells and the 1.0 μM chrysene dose for all species. Doses higher than 2.5 μM chrysene resulted in lower EROD response and as dose concentration increased, cell coverage decreased, and cellular debris increased for all tested species.

Tested doses of BNF are listed in Table 2.1 and EROD response curve results for all tested species are shown in Figure 2.2. Again, we used a 1.0 μM dose as an initial comparison between species and to categorize species responses as high, moderate or low. For this CYP1A inducing compound, common murre had the highest EROD response and greater white-fronted goose the lowest at 1.0 μM. CPE results for all dose concentrations were similar for all tested species with little to no visual change compared to control wells. Common murre and mallard had the highest overall response to any dose concentration of BNF (1.0 μM and 2.0 μM respectably).

Steller's eiders, greater white-fronted geese and mallards were the only species we were able to test culture responses to the hydrocarbon phenanthrene. Using concentrations of 0.5-50.0 μM, there was no enzyme response to this hydrocarbon (data not shown). However, phenanthrene at higher concentrations produced a black material ("asphalting") that coated the well and obscured CPE readings of the cultures and leaving us unable to verify cell viability.

Table 2.1 summarizes ANS oil doses per species and Figure 2.3 represents EROD response curve results for five target species (tufted puffin, common eider, common murre, greater white-fronted goose and Steller's eider), and control species mallard, dosed with 0.5-20.0 μL of neat ANS oil. These results show a low degree of variation in enzyme activity over the course of the EROD assay compared to the other testing reagents. This indicates maximum cellular activity was potentially reached at these ANS oil doses. Overall, 5.0 μL ANS oil had the highest enzyme response observed in all testing reagents. Comparing the estimated response curves, tufted puffin and common murre were the highest responders of the target species and control species, mallard also showed a high EROD response to 5.0 μL ANS oil. Greater white-fronted goose had the lowest estimated EROD response to 5.0 μL ANS oil. CPE results showed that common murre had a higher amount of non-viable cells (CPE score 3-4) than other species and common eiders had a larger amount of cellular vacuolarization for all dose concentrations (CPE score 1-3). In all species, ANS oil doses had more non-viable cells than other testing reagents, and in general, a more non-viable cells were observed at the higher ANS oil doses (e.g., Steller's eider ANS oil 1.0 μL CPE score 0-2 and 10.0 μL CPE score 1-3). The only trends in slope of the response curves were for ANS oil grouping from neat doses: 0.5 μL, 1.0 μL and 10.0 μL ANS oil had slight negative slopes and 5.0 μL a slight positive slope.

To further explore cellular responses to ANS oil, we dosed Steller's eider cells with 1.0 μL of different dilutions of ANS oil and carrier reagent DMSO (1 part ANS oil to 1 part DMSO up to 10 parts ANS oil and 1 part DMSO; Table 2.1 and Figure 2.4). Tufted puffin and mallards responses were also tested at the 1:1 dose. In Steller's eiders the 1:1 ANS oil:DMSO dose had the lowest enzyme response, but the highest amount of cellular debris in the well (CPE score 2-4). Cells were also classified as unknown viability due to morphological changes (i.e., cellular membranes had a faded appearance). It is likely this dose overwhelmed the cell culture, limiting enzyme response. Mallards had similar EROD activity to the 1:1 dose as Steller's eiders while

tufted puffins had a near double response to the dose. Both mallard and tufted puffin also had a high amount of cellular debris and similar visual morphological changes and possible reduced viability. Steller's eiders EROD activity increased with increasing amounts of ANS oil, with the highest activity from 10:1 ANS oil:DMSO dose. The highest EROD responses to ANS oil:DMSO doses were still comparatively lower than doses that used neat ANS oil.

Table 2.1: Summary of chrysene, β-naphthoflavone (BNF), phenanthrene, and Alaska North Slope crude oil (ANS oil) test doses by reagent for each species including control species mallard.

Species	chrysene	BNF	phenanthrene	ANS oil (neat)	ANS oil: DMSO
black brant	1.0 µM	----	----	----	----
greater white-fronted goose	0.5, 1.0, 1.5, 2.0, 5.0 µM	0.5, 1.0, 2.0, 5.0 µM	1.0, 5.0, 10.0, 50.0 µM	0.5, 1.0, 5.0, 10.0, 20.0 µL	----
common eider	0.5, 1.0, 2.0, 3.0, 5.0 µM	0.5, 1.0, 2.0, 5.0 µM	----	1.0, 5.0, 10.0 µL	----
Steller's eider	0.5, 1.0, 1.5, 2.0, 3.0, 5.0 µM	0.5, 1.0, 2.0, 5.0 µM	0.5, 1.0, 2.0, 5.0, 10.0, 50.0 µM	0.5, 1.0, 5.0, 10.0, 20.0 µL	1:1, 3:1, 5:1, 10:1
spectacled eider	1.0, 1.5 µM	----	----	----	----
king eider	1.0 µM	----	----	----	----
long-tailed duck	0.5, 1.0, 1.5, 2.0, 3.0, 5.0 µM	0.5, 1.0, 2.0 µM	----	----	----
tufted puffin	0.5, 1.0, 2.0 µM	0.5, 1.0, 2.0 µM	----	1.0, 5.0 µL	1:1
horned puffin	1.0, 2.0, 5.0 µM	----	----	----	----
common murre	0.5, 1.0, 2.0 µM	1.0, 2.0 µM	----	1.0, 5.0, 10.0 µL	----
mallard (control)	0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 10.0 µM	0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 10.0 µM	0.5, 1.0, 2.0 µM	1.0, 5.0, 10.0 µL	1:1

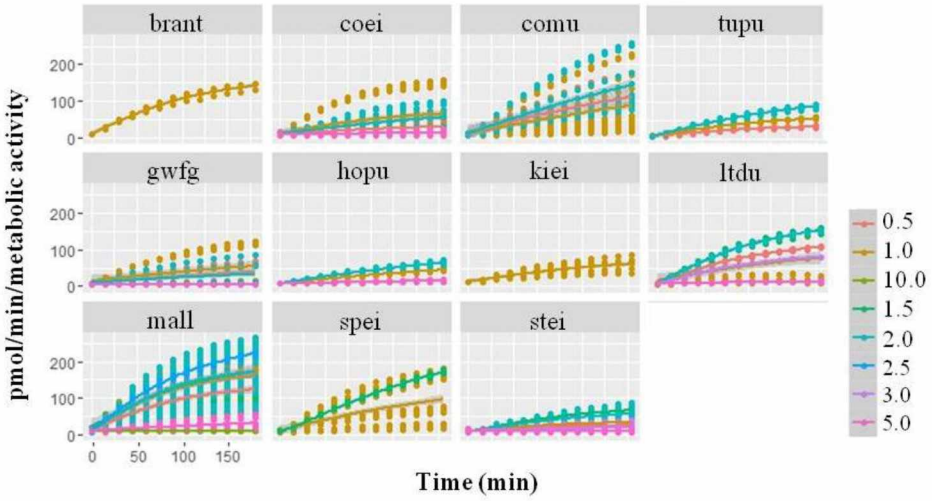

Figure 2.1: EROD results of tested species to different concentrations of chrysene doses (µM). Assay time in minutes is on the x-axis and pmol/min/metabolic activity on the y-axis. Real data (individual cell culture wells) is represented in dots and the solid line is the estimated response trend over time. Species abbreviations: coei-common eider, comu-common eider, tupu-tufted puffin, gwfg-greater white-fronted goose, hopu-horned puffin, kiei-king eider, ltdu-long-tailed duck, mall-mallard, spei-spectacled eider, and stei-Steller's eider.

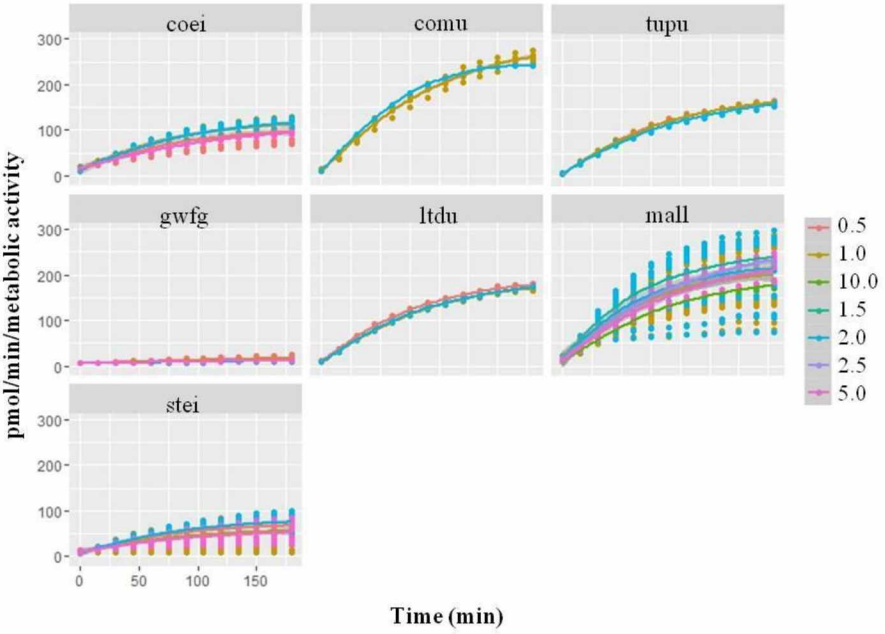

Figure 2.2: EROD results of tested species to different amount to different concentrations of BNF doses (μM). Assay time in minutes is on the x-axis and pmol/min/metabolic activity on the y-axis. Real data (individual cell culture wells) is represented in dots and the solid line is the estimated response trend over time. Species abbreviations: coei-common eider, comu-common eider, tupu-tufted puffin, gwfg-greater white-fronted goose, ltdu-long-tailed duck, mall-mallard, and stei-Steller's eider.

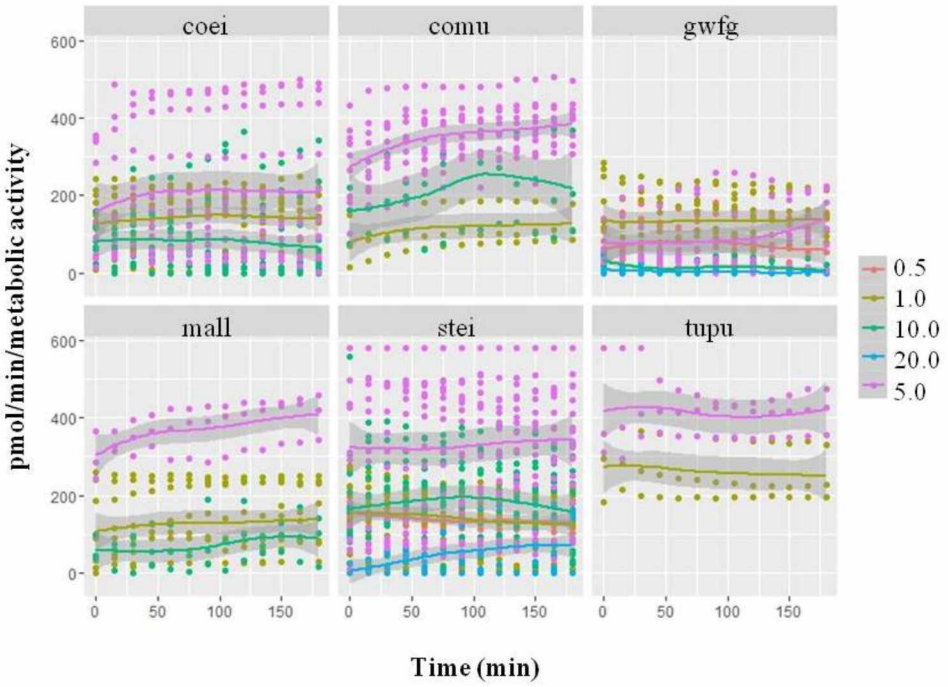

Figure 2.3: EROD results of tested species to different amount of neat Alaska North Slope crude oil doses (μL). Assay time in minutes is on the x-axis and pmol/min/metabolic activity on the y-axis. Real data (individual cell culture wells) is represented in dots and the solid line is the estimated response trend over time, and the shaded area is the 95% confidence interval. Species abbreviations: coei-common eider, comu-common eider, gwfg-greater white-fronted goose, mall-mallard, stei-Steller's eider, and tupu-tufted puffin.

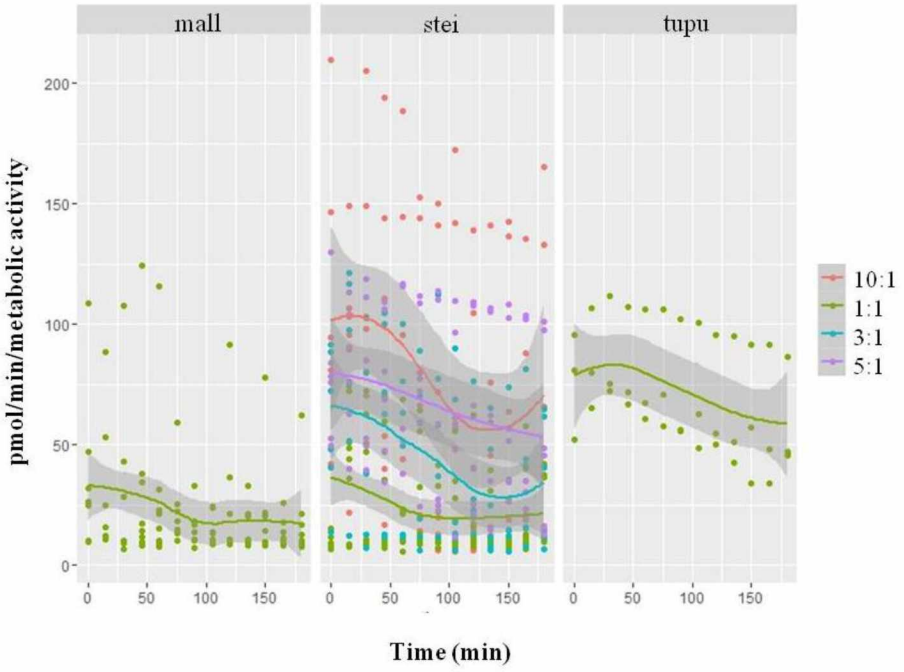

Figure 2.4: EROD results of tested species to Alaska North Slope crude oil (ANS oil) dissolved in carrier reagent dimethyl sulfoxide (DMSO) at 1:1, 3:1, 5:1 and 10:1 ANS oil:DMSO. Assay time in minutes is on the x-axis and pmol/min/metabolic activity on the y-axis. Real data (individual cell culture wells) is represented in dots and the solid line is the estimated response trend over time, and the shaded area is the 95% confidence interval. Species abbreviations: mall-mallard, stei-Steller's eider, and tupu-tufted puffin.

2.5 Discussion

2.5.1 Cell culture

Overall, tested cells from all species responded similarly to extraction and culture protocols with the exception of common murre cells. These cells required extra washings with 1X PBS 24 hours after seeding due to a large amount of red blood cells present on top of the culture. We had previously attempted to remove minimal red blood cell contamination with a Percoll/sucrose gradient step during hepatocyte extraction (Kennedy et al. 2003) for mallard but this method was unsuccessful. Future work with common murre cells could attempt this step if

red blood cell contamination becomes problematic. During the last year of our study we were able to acquire greater white-fronted goose and common eider eggs from the wild. While we do not have data from hepatocytes sourced from captive eggs from these species for comparison, Head et al. (2006) suggested that environmental levels of CYP1A inducing chemicals in eggs generally would not be expected to impact the potency of testing compounds.

2.5.2 Sensitivity Comparisons

We established reference EROD activity response curves with estimated response trends over time for many of our target species to chrysene, BNF, and ANS oil. In summary, the most sensitive responder comparatively was common murre which exhibited consistently high responses at all tested doses. The other tested alcids, tufted puffin had high BNF and ANS oil responses and low responses to chrysene, while horned puffin was only tested with chrysene and showed low EROD activity. Among the sea ducks, common eider and Steller's eider were the only sea duck species tested with all reagents. Common eiders responded high to our initial test dose of 1.0μM chrysene but had moderate enzyme response to BNF and ANS oil doses. Steller's eider responses varied depending on the testing reagent. Greater white-fronted geese had moderate to low sensitivity at all test doses. Additional testing is needed to fully assess long-tailed duck, spectacled eider, king eider, and black brant sensitivities to CYP1A inducing compounds.

Head et al. (2015) identified Pekin duck, a domestic duck species, as a relatively insensitive species to 18 PAHs including chrysene and phenanthrene (Head et al. 2015). In our testing, mallard, another domestic duck, showed sensitive EROD responses to many of our test doses, although previous work in our laboratory (Hollmén et al. 2013) showed mallard as a comparatively low, insensitive, responder to chrysene and BNF doses. We identified the variability in mallard responses was likely due to changes in egg sources. Eggs from large commercial providers had EROD responses 2-3x higher (at the final time-point 180 minutes) than small scale local private breeders. The reason for the discrepancy between sources is unknown and warrants further investigation.

Phenanthrenes are a common component in weathered crude oil (Incardona et al. 2005, Short et al. 2008) and at low concentrations ranging from 10-60 μM (similar to doses we used)

caused cardiac rhythm changes in zebrafish embryos (*Danio rerio*; Incardona et al. 2005). Phenanthrene dosed Steller's eider, greater white-fronted goose, and mallard hepatocytes did not elicit an EROD response above the background similar to other studies. Phenanthrene consistently showed no EROD activity in dosed rat (*Rattus sp.*) hepatoma cells and rainbow trout (*Oncorhynchus mykiss*) hepatocytes (Bols et al. 1999, Bosveld et al. 2002). Additionally, phenanthrene produced no EROD response in Pekin duck, greater scaup, and chicken hepatocyte cultures (Head et al. 2015). We did note a black material ("asphalting") that formed in cell culture wells at higher concentrations of phenanthrene preventing visual analysis of the culture and confirmation of cell viability. We are unsure of the mechanism that causes the formation of this material and if it could inhibit EROD activity.

Short et al. (2008) identified chrysene homologues as the primary source of CYP1A induction in Prince William Sound, AK (site of the *Exxon Valdez* oil spill) in a residual oil study using EROD responses from injected fish. While other PAHs were found in collected oil, they were less abundant, less potent, or both when compared to chrysene homologues. Chrysene, either alone or in a PAH mixture, can decrease embryo survival and cause growth retardation when applied to eggshells or injected into eggs (Hoffman and Gay 1981, Brunstrom et al. 1990). We found cellular changes at the highest tested concentrations of chrysene (>2.5 μM) which resulted in subsequent decrease in EROD activity. Several species (e.g., black brant, common eider and common murre) had comparatively high EROD responses to chrysene doses and in all species EROD activity decreased at the highest dose concentrations. The higher dose concentration likely killed cells or overwhelmed the cells' capability to process the toxin resulting in lower EROD activity.

Cytopathic changes were also found in cells dosed with neat and DMSO diluted ANS oil. Additionally, the highest EROD responses, in all tested species, were observed with neat ANS oil doses. While the exact chemical composition of our sample of ANS oil is unknown, it is clear the compound mixture has a different impact on cellular EROD response than the individual testing reagents. It is likely the cumulative effect of several PAHs is causing the elevated enzyme activity.

2.6 Conclusion

We provide the first reference EROD response curves for several arctic birds to individual PAHs and ANS oil. Based on our findings we suggest common murre and common eiders as sensitive responder candidate species for PAH monitoring programs. They both had moderate to high EROD responses to the suite of experimental doses. Additionally, as a sensitive alcid and sea duck EROD responders they add life history diversity and increase the amount of potential PAH exposure pathways to a monitoring program. Both common eider and common murre are widespread throughout Alaska (USFWS 2006, 2008) and common murre have shown they may be a sensitive indicator of ecosystem health after they experienced a large mortality event in response to prey availability changes (USGS 2016). While ideal bioindicators warn of environmental changes with a measurable, sub-lethal effect (Livingstone 1996), because of their consistently high EROD activity responses and presence throughout Alaskan waters, common murre are still a valuable option for PAH monitoring programs. However, our reference response curves also suggest that several species are possible candidate species, specifically the tufted puffin and promising sensitive EROD responders like black brant. As a group, arctic birds offer a wide diversity for PAH bioindicator species and selecting multiple species to monitor contaminants increases the likelihood an exposure event will be detected. Our EROD reference response curves provide valuable information about the sensitivity of this group to CYP1A induction from reference chemicals, PAHs in ANS oil, and ANS oil and this data will aid in the development of future monitoring plans.

2.7 Acknowledgments

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2.9 Appendix

Appendix 2

Laboratory Protocols for Harvesting Hepatocytes from Avian Sources, Media Recipes, Dosing Cell Cultures, Cytopathic Effects, and EROD Cell Culture Assay

Harvesting hepatocytes from avian sources

A. Egg maintenance

1. Before eggs arrive clean egg incubator by wiping all surfaces, internal and external, with ethanol (ETOH). Replace filter annually (filter is in a mesh holder above top egg tray). Refill reservoir with fresh distilled water (dH_2O). Turn on incubator and make sure temperature and humidity are correct and hold over time. Settings on egg incubator should not need changing except for possible fine temperature adjusting. Egg incubator should be set at: 37.5°C, 50-60% humidity, trays roll every 1.5 hour for 30 minutes, 2-30 minutes cooling periods at ~7 A.M. and ~5 P.M. (mimic incubation breaks).
2. Open egg-shipping boxes in cell culture lab and in biosafety hood when possible.
3. Brush fecal material off of eggs using kimwipe (don't rub as it could get pushed into the egg). Dip eggs in room temperature 1X chlorohexidine solution and set on kimwipe in biosafety cabinet.
4. Label each egg using a pencil and record ID number in notebook. Put eggs in egg incubator finding eggs of similar size to match up and put "pointy" ends facing each other. If need to stagger incubation start dates, fresh eggs (a few days since laid), can be kept in the hood or on counter overnight propped up at a 45° angle. If longer than

overnight needed, eggs must be manually turned 180° every ~12 hours to prevent yolk from sticking to the side of the shell.

5. Incubate eggs until day 10-15 (depending on species, type of cell harvesting, and embryo development). Livers of older embryos are not much bigger and you get more red blood cell contamination.
6. Candle eggs at day 7 and then every 3-4 days as needed. Remove eggs that are not fertile (no veins visible and light colored inside), that have died during incubation (dark with no veins), or are not needed for extraction by putting in the fridge for 24+ hours. All egg waste should be disposed of in biohazard waste containers.

B. Harvest of embryos and preparation of cell lines

1. Prepare necessary reagents and autoclave glassware and equipment (scissors, forceps, etc.) a few days before starting extraction procedure. Plastic handled knitting scissors (the best scissors to open eggs as they have a small sharp point) cannot be autoclaved but instead let sit in 95%+ ETOH before procedure. Medias can be used up to two weeks before they should be remade.
2. Record all pertinent information on cell preparation data sheet.
3. Pre-warm ~200 mL (stock bottle) collagen media at 37°C. Be careful not to overheat as this could damage the cells. Remove the bottle from the fridge in the morning and allow to come to room temperature, then 5-10 minutes before it is needed put it in a 37°C water bath.
4. Keep pre-perfusion buffer II cool (leave in the fridge).
5. Place egg in cup or tray in biosafety cabinet, air cell side up, and swab with iodine solution. Rinse iodine off with 1X phosphate-buffered saline (PBS) and wipe dry with sterile gauze.
6. Pour cold 1X PBS (enough to cover the bottom) into a small Petri dish.
7. Get a large Petri dish, scissors, knitting scissors, scalpel, and tweezers ready.
8. Score, using scalpel, a circle around the air cell. The goal is to cut open right above the bottom of the air cell. Make sure the opening is big enough to remove embryo. Use knitting scissors to cut top of egg open. Remove egg cap and discard.

9. Find embryo and grasp with tweezers and immediately remove the head (leaving it in the egg) with sterile scissors. Be careful not to drop torso into egg as it can be difficult to find. Remove torso to large, dry Petri dish. You may also remove the entire embryo to the Petri dish before removing the head if it is easier for you. If the embryo looks morphologically abnormal do not use the liver.
10. Make a longitudinal cut through the breast area with sterile scalpel or scissors and remove viscera. Remove the liver, which will be the only brown organ and one of the biggest organs. Identify the gall bladder, which is attached to the liver and is a small, green dot. Use tweezers or a new scalpel to carefully remove the gall bladder. If the gall bladder breaks and contaminates the liver, the liver cannot be used for the EROD assay. Place liver in the small Petri dish with 1X PBS.
11. Livers can be pooled or kept separate at this point depending on need and number of embryos processed.
    i. Pool up to 4 eggs (more can be pooled at later stage in protocol but add no more than 4 livers together for stirring step)
12. Pour or pipette off 1X PBS and wash liver surface with pre-perfusion buffer II (no specific amount, use enough to cover liver, ~10 mL).
13. Remove pre-perfusion buffer II and mince liver dry using sterile scissors or scalpels.
14. Add warm collagen media to minced liver and transfer liver pieces to a sterile trypsinizing flask, with a stir bar, for tissue digestion. Always make sure the stir bar is completely covered in media—better to use more media if unsure.
    a. 3-4 embryos: 48 mL
    b. 1-2 embryos: 32 mL
15. Stir flask gently at 37°C for 30 minutes on stir plate.
    a. Stir plate setting: heat 1, stir 3
    b. Rough stirring will damage cells
16. Turn on centrifuge to cool.
17. Stop the digestion with HI FBS (20%).
    a. 3-4 embryos: 12 mL
    b. 1-2 embryos: 8 mL

18. Filter cell suspension over nylon mesh (100 µm) into a 50 mL centrifuge tube containing 5 mL pre-perfusion buffer II.
19. After filtering all cell material, wash mesh with 5 mL pre-perfusion buffer II. (Sometimes the volume is too large for one 50 mL tube. In that case split digested mixture into two falcon tubes each with 5 mL buffer and a 5 mL wash. Also, may need to use two filters).
20. Centrifuge the resulting single-cell suspension at 63g (~590 rpm) and 4°C for 5 minutes.
21. Carefully remove the supernatant and discard.
22. Gently resuspend the cell pellet in 10 mL pre-perfusion buffer II. At this point if you had to use two 50 mL centrifuge tubes you can combine into one tube with 10 mL total volume.
    i. Can also combine other cell pools at this point to create one homogeneous cell suspension
23. Centrifuge suspension at 110 g (~782 rpm) and 4°C for 5 minutes.
24. Carefully remove the supernatant and discard.
25. Resuspend the cell pellet in 2 mL low glucose media with antibiotics (A/B) and 20% HI FBS. The resulting cell suspension consists of liver and blood cells including erythrocytes. Let cells sit for ~5 minutes to resuspend before count.
26. Perform a cell count on the suspension first by combine 15 µL cell suspension and 60 µL media. Add ~15 µL to 3-4 cell counting chamber wells and place under microscope. Cells will be very small and can be difficult to count. Focus up and down until cells are clear and some are darker (these are dead). Teardrop shaped cells or ones with two "pointy" ends are red blood cells, do not count these. Cells may be in clumps, do not count all cells in the clump as they are likely to die and will give you an over estimation on your count. Take average cell count between chamber wells and plug into the formula:

    (average number of cells)(10^4)(5) = X cells/mL

    If resuspended in 2 mL multiple final number by two for total cell count.

    10^4=cell chamber number

5=dilution factor

Dilute suspension if above target cell count.

C. Plating cells

1. Plate cells from the cell suspension: ~30,000 cells/well in a 96 well plate at 200 µL total volume per well. Use low glucose DMEM:MEM with A/B and 20% HI FBS.
2. Plate cell for EROD assays in black walled plates with clear bottoms.
    i. Make sure to leave three wells empty for media control

Avian Hepatocyte Culture Media

Antibiotic mix (A/B)

20 mL nystatin

40 mL penicillin-streptomycin

2.0 mL gentamycin 50 mg/mL

1. Combine the above ingredients to total 62 mL
2. Aliquot into 8 mL aliquots.
3. Freeze at -20°C until added into media.

Low glucose DMEM:MEM Media

55.55 mL high glucose DMEM

194.45 mL MEM

4 mL A/B mix

2.5 mL L-glutamine 200 mM

Pre-Perfusion Buffer II (pH 7.4)

2 mL HEPES buffer

1 mL 0.1 M EGTA

197 mL HBSS without CaCl, MgCl, and $MgSO_4$

Perfusion Medium II/Collagen Media

475 mL MEM

25 mL trypsin 2.5%

192 mL collagenase type IV

1 M NaOH

Bring 5 g NaOH up to 125 mL in dH_20

0.1 M EGTA

1.9 g EGTA

50 mL 1 M NaOH

Dosing hepatocyte cultures

1. Grow cells 24 hours.
    a. Check cell culture my microscopy
        i. Cells should be attached over 50-100% of the dish surface area and creating colonies. Do not use cells if they look abnormal.
2. Change media after 24 hours.
    a. Either before dosing or continuing to let cells grow 24 hours longer
    b. Remove old media
    c. Gently wash cells with 1X PBS
        i. Add ~300 µL 1X PBS to the well, pipette it off, and repeat 2-3 times.
    d. For cultures continuing to grow add 200 µL low glucose media with 20% FBS and A/B
    e. For cultures to be immediately dosed add 100 µL low glucose media without FBS to all wells.
3. Doses.
    a. Cell only: 100 µL media
    b. Media only: 100 µL media
    c. Vortex chemical doses well before adding to wells
    d. DMSO control (chrysene carrier solvent control): 4 µL to well of 1:3 DMSO: media stock
    e. Chrysene example: 1.0 µMm=4 µL to well of 25 µM stock

f. ETOH control (BNF and phenanthrene carrier solvent control): 2 µL of 1:1 ETHO: media stock
g. BNF example: 1.0 µM=2 µL to well of 50 µM stock.
h. Phenanthrene example: 1 µM=2 µL to well of 50 µM stock
i. AK North Slope crude oil is used straight

Cytopathic Effects (CPE) protocol

1. After dose exposure (typically 24 hours) evaluate culture using microscopy.
    a. general cell morphology and cytopathic effects (CPE)
2. Characterize and describe types of CPE observed.
    a. CPE should be read by a qualified person familiar with the cell type as changes can be subtle.
3. Score on a semiquantitative response scale from 0-4 (0=none to 4=100 % effect) for a suite of CPE responses.
    a. e.g., vacuolarization, cytoplasmic swelling, granularization, non-viability, cellular debris, and other abnormalities

Protocol for 96-well plate cell culture EROD assay

1. Turn on Spectramax Gemini plate reader and laptop (runs at ambient temp).
2. Set up experiment to read at 530-590 nm (excitation/emission); read for 3 hours every 15 minutes.
3. Mix 5 mL 6 µM 7-ER stock, place on ice.
    a. Mix 300 µL 100 uM 7-ethoxyresorufin (7-ER; in -80 in aliquots of 1 mL or 2 mL; is yellow and stored in methanol so does not freeze) to 4.7 mL $NaPO_4$ buffer (in fridge) in a glass beaker or flask. Vortex slightly to mix. Mixture should be a peach color, if it is pink toss and remake.
    b. Always use freshly made 6 µM 7-ER stock
4. Add 1.5 mL $NaPO_4$ buffer to one 10 mg vial NADPH (two vials is enough for three plates); mix very well on vortexer.
    a. Always use freshly made NADPH

5. Load 25 µL of 6 µM 7-ER to plates using multiwell pipettor. Toss extra mixture.
6. Confirm plate reader is ready to go.
7. Add 10 µL NADPH per well using multiwell pipettor. Toss extra mixture.
    a. At this point the reaction starts
8. Quickly replace cover, place in plate reader drawer and immediately hit "read". Plate will shake for 5 seconds before reading.
9. Once reader data appears start timer for the 15 minutes break period. Once timer rings hit read. Repeat every 15 minutes for 3 hours.
10. You can read more than one plate at a time. Read second plate immediately after first plate is done. Make sure to note/label which plate reads belongs to which cell culture plate (e.g., odd number reads to cell plate one and even number reads to cell plate two)
11. If whole plate or several wells read as "saturated" pull off 100 µL and add it to a new black plate. Add 100 µL $NaPO_4$ to original plate and 100 µL to new plate with the 100 µL pulled off solution. Read both plates.
12. Once reads are complete copy data into *Excel* and calculate mean, standard deviation, and standard error for each dose at each time-point.

Conclusion

This study is the first to report reference levels of CYP1A activity and assess hydrocarbon sensitivity for arctic birds to provide information prior to projected enhanced oil and gas resource development and vessel traffic in the Alaska Arctic. I measured CYP1A induction using EROD activity and validated opportunistic collection protocols for CYP1A analysis in three arctic bird species. I also compared species-specific EROD responses as a measure of sensitivity to PAHs and ANS oil in primary hepatocyte cultures for ten species of arctic birds found in the Beaufort and Chukchi seas. Based on results from field and laboratory sample analysis, I made recommendations for PAH bioindicator species.

In Chapter 1 I provided methods for collecting and sampling livers for greater white-fronted geese, common eider, and king eider from hunter-killed birds. I measured EROD activity in liver samples frozen at varying lengths postmortem to evaluate enzyme degradation and explore if the recommended sample collection window (with-in 10 minutes postmortem) could be expanded to facilitate field logistics including opportunistic collections. Enzyme results indicated a high degree of individual variability leading to a recommendation to maintain the currently used 10 minute postmortem collection window for consistency. I found that opportunistic sampling in the 10 minute window may be challenging, but it can be a feasible option when working in partnership with a subsistence hunter. There may be good opportunities for opportunistic collections of king eider and common eider near Utqiaġvik, AK during both spring and fall. At spring whaling camps eiders are hunted from the ice edge and during fall many hunters gather in a small area near town. Spring hunts, near Utqiaġvik and at remote field camps, are potentially the only time to acquire greater white-fronted geese opportunistic samples since most hunters prefer to target eiders during fall hunts.

Additionally, in Chapter 1 I measured CYP1A induction, using EROD activity, for greater white-fronted geese, common eiders, and king eiders sampled over three years near Utqiaġvik. While the majority of the results were within EROD activity ranges previously reported for sea ducks in un-oiled or non-industrialized habitats in Alaska, fall 2014 birds had high enzyme activity. All three species had significantly higher EROD activity in fall of 2014 when compared to samples collected in all other collection periods. The method of exposure and

toxin causing the elevated activity are unknown but the birds likely were exposed while on the North Slope since at the time of sampling they had been in the area for several months. Knowing the CYP1A half-life in birds (discussed in more detail below) would help narrow identification of potential exposure windows. EROD results for spring greater white-fronted geese also detected potential exposure in some individual geese. This may be residual enzyme activity from previous exposure or could indicate individual variation in CYP1A induction due to genetic polymorphisms in CYP1A coding genes (Courtenay at al. 1994).

Future work should involve further investigations into the cause of elevated EROD activity in fall 2014. Archived liver and kidney samples could be used in toxicology testing to directly identify which CYP1A inducing contaminant is causing the high response and if it is the same toxin among species. Since elevated EROD activity only indicates exposure, additional biomarkers, besides evaluating body condition postmortem, could be used to gain further insight into the health of the birds. For example, abnormalities in histopathology tissues could be compared to EROD activity levels to assess if they correlate with high enzyme response.

Sex hormones could also be investigated to see if they play a role in EROD activity between sexes and season. For analysis in Chapter 1, I combined all sampled birds of a species together by season due to small sample sizes in some sampling periods. Esler et al. (2010) found sex, in addition to age and body mass, of harlequin ducks had no influence on EROD activity in liver biopsies from oiled and un-oiled habitats for birds sampled in winter (March, April, and November). Limited research has investigated the role of sex hormones and PAH exposure in wildlife, with most of the research on fish. Navas and Segner (2001) found that while EROD activity decreased with increasing female sex hormone 17β-estradiol (E2), when rainbow trout (*Oncorhynchus mykiss*) hepatocyte cultures were exposed to CYP1A inducer BNF, EROD activity didn't change with the addition of E2.

Chapter 2 details primary hepatocyte culture methods and hydrocarbon EROD sensitivity results for ten arctic bird species: Steller's eider, spectacled eider, common eider, king eider, long-tailed duck, greater white-fronted goose, black brant, common murre, tufted puffin and horned puffin. Using eggs from captive and wild sources, I dosed hepatocyte cultures with single hydrocarbons and ANS oil, measured EROD activity, and noted cytopathic effects. Cellular morphological changes were found at the highest doses of chrysene (>2.5 μM) and doses with

neat and DMSO diluted ANS oil. I identified common murre as the most sensitive responder within the tested species based on comparatively high EROD activity to test doses. Several other species such as long-tailed duck, tufted puffin, and common eider had moderate or high enzyme activity responses. Greater white-fronted geese had low EROD responses and Steller's eiders' EROD responses were variable. I was not able to acquire enough cell culture material to do the full dosing regimen on black brant, spectacled eider, king eider, and horned puffin and these species require further EROD testing to fully assess their hydrocarbon sensitivity. Based on the hepatocyte culture results in Chapter 2, I recommend the most sensitive alcid, common murre, and the most sensitive sea duck, common eider, as potential bioindicator species for hydrocarbon monitoring. However, several tested species are promising PAH bioindicators (e.g., tufted puffin). Common eider and common murre differences in life histories (e.g., nesting locations) and foraging strategies (piscivorous vs. benthic) allows for monitoring of different PAH exposure pathways and increases the chances an exposure event will captured.

Greater white-fronted geese had the highest EROD activity reported from field liver collections but it was one of the least sensitive hepatocyte EROD responders comparatively to our other tested species. There could be several reasons for this discrepancy. First, greater white-fronted geese may have been exposed to a CYP1A inducing toxin that was not part of our cell culture testing. As mentioned previously, direct contaminant measurement from archived liver samples will help answer this question. The birds could have been exposed to different concentrations of PAH(s) than were used in culture dosing. However, greater white-fronted geese, along with all other tested species, showed lower EROD responses as concentrations of chrysene and ANS oil increased. Also, wild birds are under additional physiological stressors, not reflected in a cell culture, which could cause a different EROD response. While the greater white-fronted geese in this study appeared healthy on postmortem examination, without additional biomarkers it is unknown if there was some underling health condition.

The results from hepatocyte EROD sensitivity testing could also be applied to monitoring birds on wintering grounds and along migration routes, in addition to the Beaufort and Chukchi sea regions. Many of the species tested in this study winter in areas with industrial activities, for example, Miles et al. (2007) showed elevated EROD activity in Steller's eiders wintering near industrialized sea ports. Traditional sampling methods, e.g., direct-take or non-lethal surgery,

would need to be employed to get liver samples from species outside of subsistence hunting seasons or for species not targeted by hunters. Monitoring throughout the year could help identify if birds have chronic or acute exposure to CYP1A inducing compounds over the course of their annual cycle.

Future marine bird and waterfowl CYP1A monitoring programs and studies would benefit from knowledge about the half-life of the CYP1A enzyme. This information could help identify if exposure was chronic or acute, potentially where or when exposure occurred, and influence timing of field sampling. Studies mentioned in Chapter 1 found the CYP1A half-life in rats (*Rattus sp.*) to be ~19 hours (Chen et al. 2010) and CYP1A activity continued for 4-10 days after toxin exposure ceased in fish (Kloepper-Sams 1989, Fragoso et al. 1998). My hepatocyte culture data showed the arctic birds I tested had different enzyme responses to dosing reagents (Chapter 2) leading me to suspect CYP1A half-life may also change with different species. Since I used primary hepatocyte culture, cells start to naturally die 3-4 days after harvest limiting the inferences I can make about CYP1A activity beyond the life of the culture. I did complete three replicates of a 1.0 μM chrysene dose exposure experiment with Steller's eider cells recording EROD activity at 12, 24, and 36 hours post-dose (Appendix Figure A-1). In general, the highest enzyme activity response was at 12 hours with the lowest responses at 36 hours with similar CPE over the course of the experiment. This may indicate a half-life of <24 hours but caution should be used when interpreting these results since cells were dosed with one PAH at one concentration. It would be beneficial to repeat this experiment with other species, such as the common murre, that are more sensitive than the Steller's eider, with more reagents, including ANS oil, and at various concentrations. Future hepatocyte culture work could also determine EROD responses by increasing EROD assay time to capture enzyme activity decreases. I measured cellular activity for 3 hours, but EROD activity rarely declined during that time. Lastly, the use of species-specific immortalized cell lines may be another cell culture tool to investigate longer time frames of dose exposure and repeated dose exposure and subsequent EROD responses that can't be investigated within the limited time frame that primary cell culture provides.

Combining results from field collection and hepatocyte culture sensitivity data, multiple species can be considered for future monitoring programs. Since species move in and out of

different areas of the Beaufort and Chukchi seas at different times, use of multiple species in a monitoring program increases the chance that a selected PAH bioindicator species would be in the area of an accidental oil release event. Common eider had moderate to high responses to hepatocyte culture testing reagents and it is a species often targeted by subsistence hunters. Opportunistic collections from common eiders could be collected seasonally with arrival onto the North Slope in spring and departure after breeding in the fall. Additionally, due to their wide range throughout Alaska and the Arctic, they represent a potentially useful bioindicator for many areas. While I was unable to complete the entire cell culture testing regime for king eider, and despite the fact that the species showed low responses to chrysene, field CYP1A results showed significant differences between fall 2014 and fall 2015. Reasons for this may be similar to those discussed in the previous paragraph for greater white-fronted geese. Additional EROD hepatocyte culture testing, including ANS oil, would offer more insight into king eider sensitivity responses. Since king eiders are one of the most commonly hunted bird in the Arctic, the chance to obtain samples through opportunistic sampling is greater and it also should be considered a species to include in monitoring programs. Adding black brant, a marine associated goose, to a monitoring program would provide further foraging and habitat use diversity and additional exposure pathways. Black brant are a promising indicator species because they are already targeted by subsistence hunters and the limited testing I was able to complete on them (1.0 μM chrysene) showed a high EROD response. Further cell culture testing is necessary confirm they have a consistent and measureable EROD responses to our testing reagents.

Similar to the common eider, common murre are abundant throughout many coastal areas in Alaska. Common murre was comparatively the most sensitive responder to hepatocyte culture dosing of the species tested for this project. While they are not often hunted by subsistence hunters in the North Slope area, they are hunted for meat and eggs further south on St. Lawrence Island in the Bering Sea (USFWS 2006). Recently, common murre have shown they may be a sensitive indicator of ecosystem health when they experienced a large mortality event in winter 2015-2016, potentially due to warmer ocean temperatures and subsequent changes in prey availability (USGS 2016). While ideal bioindicators warn of environmental changes with a measurable, sub-lethal effect (Livingstone 1996), because of their wide range and high EROD activity responses common murre are still a valuable option for PAH monitoring programs.

Future Arctic PAH monitoring programs should include sampling: 1) male and female birds 2) adult and juvenile birds 3) spring (pre-breeding) and fall (post-breeding) 4) multiple species. Sample sizes should be large enough to determine if sex/age are influencing EROD activity. Programs should include several bioindicator species, using recommendations from our field and laboratory EROD results, to represent species diversity and coverage in time and space in the Beaufort and Chukchi seas region. Opportunistic sampling is a viable option for CYP1A monitoring depending on selected species. Besides seasonal Arctic sampling, as discussed previously expansion to wintering grounds and migration routes would also benefit a PAH monitoring program. When possible, additional liver or kidney tissues should be archived for toxicology testing should elevated EROD activities be detected. Lastly, because elevated EROD activity doesn't directly indicate toxic effects (Lee and Anderson 2005), inclusion of health assessments and biomarkers are essential to determine if PAH exposure is impacting the birds' health.

In summary, using both EROD activity levels in wild birds and hepatocyte culture sensitivity assessments are valuable tools for measuring and understanding CYP1A responses in arctic birds. The EROD activity results provide valuable reference information for assessing potential future exposure, and field methods validated in this study assist in future collections of opportunistic liver samples from subsistence hunters. Additionally, this study contributes to the design of future monitoring programs by providing information about the sensitivity of a suite of arctic species to CYP1A induction to compounds found in crude oil and crude oil. Lastly, the arctic birds studied in this thesis represent a variety of life histories and thus possible hydrocarbon exposure pathways, providing a wide scope of potential bioindicators species that could be used in monitoring plans.

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Appendix

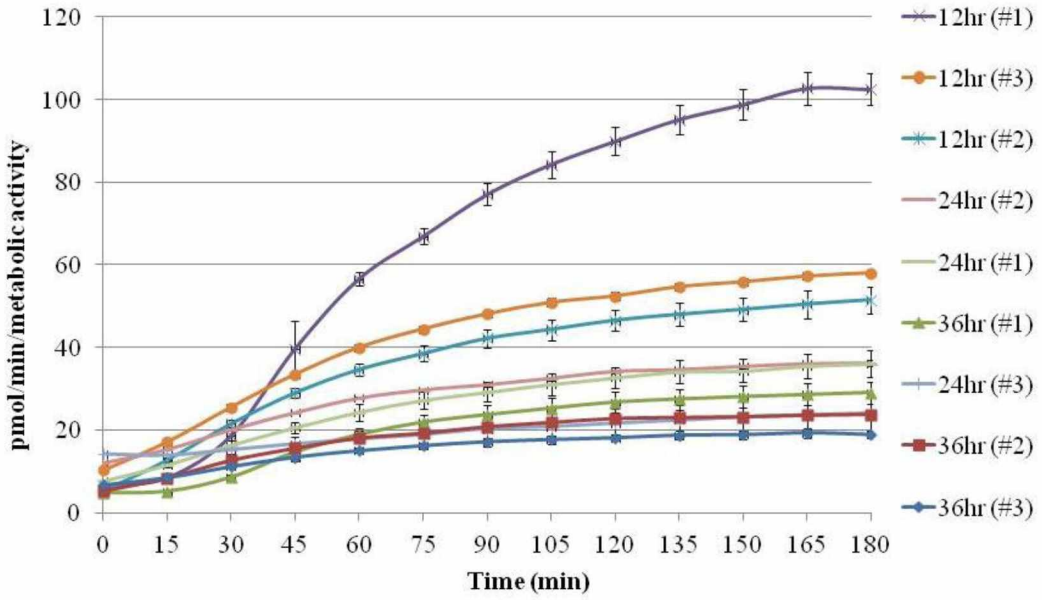

Figure A-1: EROD responses with standard error in Steller's eider to 1.0 µM chrysene with 12, 24, and 36 hour dose exposure. Each of three replicates (#1, #2, #3) is labeled next to the exposure time.