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**Massachusetts Institute of Technology
Woods Hole Oceanographic Institution**



**Joint Program
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and Engineering**



DOCTORAL DISSERTATION

Comparative Analyses of Aryl Hydrocarbon Receptor
Structure, Function, and Evolution in Marine Mammals

by

Joy M. Lapsertis

February 2007

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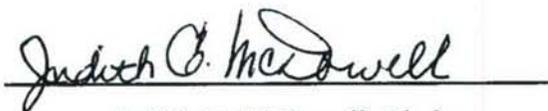
DOCTORAL DISSERTATION

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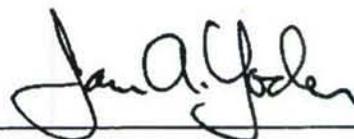


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**COMPARATIVE ANALYSES OF ARYL HYDROCARBON RECEPTOR
STRUCTURE, FUNCTION, AND EVOLUTION IN MARINE MAMMALS**

by

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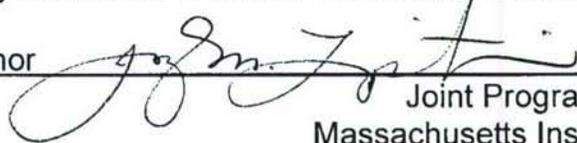
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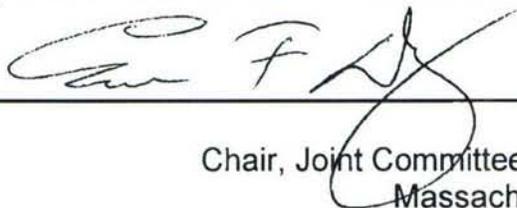
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Comparative Analyses of Aryl Hydrocarbon Receptor Structure and Function in Marine Mammals

by
Joy M. Lapsertis

ABSTRACT

Marine mammals possess high body burdens of persistent organic pollutants, including PCBs and dioxin-like compounds (DLC). Chronic environmental or dietary exposure to these chemicals can disrupt the function of reproductive and immune systems, as well as cause developmental defects in laboratory animals. The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor, mediating the expression of a suite of genes in response to exposure to DLC and structurally related chemicals. Species-specific differences in AHR structure can affect an organism's susceptibility to the effects of DLC. The structures and functions of several cetacean AHRs were investigated using *in vitro* molecular cloning and biochemical techniques. Using a novel combination of remote biopsy and molecular cloning methods, RNA was extracted from small integument samples from living North Atlantic right whales to identify the cDNA sequence for AHR and other genes of physiological importance. Biopsy-derived RNA was found to be of higher quality than RNA extracted from stranded cetaceans, and proved a good source for identifying cDNA sequences for expressed genes. The molecular sequences, binding constants, and transcriptional activities for North Atlantic right whale and humpback whale AHRs cDNAs were determined using *in vitro* and cell culture methods. Whale AHRs are capable of specifically binding dioxin and initiating transcription of reporter genes. The properties of these AHRs were compared with those from other mammalian species, including human, mouse, hamster, and guinea pig, and other novel marine mammal AHRs, using biochemical, phylogenetic, and homology modeling analyses. The relative binding affinities for some marine mammal AHRs fall between those for the high-affinity mouse AHR^{b-1} and the lower affinity human AHR. Species-specific variability in two regions of the AHR ligand binding domain were identified as having the greatest potential impact on AHR tertiary structure, yet does not sufficiently explain differences observed in ligand binding assays. Additional studies are necessary to link exposure to environmental contaminants with potential reproductive effects in marine mammals, especially via interactions with steroid hormone receptor pathways.

Thesis Supervisor: Mark E. Hahn
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If it weren't for the granting of WHOI SeaGrant funds for studying molecular mechanisms of dioxin sensitivity in marine mammals to my advisor, Mark Hahn, I probably would not have been in the Joint Program at all. Was it coincidence, providence, or the confluence of like-mindedness that resulted in Mark's submission of the proposal and my interest in that very topic? More likely, it was the timing – that the publications to date had prompted this next step in investigating marine mammal toxicology. The “weight of evidence” approach had been employed to make a case for concern for marine mammal health related to exposure to environmental contaminants. The summation of many publications had achieved critical mass, after which it was both necessary and possible to investigate mechanisms of action. And I was ready to get involved.

I should first acknowledge the mentors who helped prepare me for this adventure. Prior to commencing doctoral studies, I had studied under Professor Virginia Hayssen at Smith College where I completed a master's degree in biology. Under Ginny's encouragement, advice, and wise tutelage I gained the skills necessary to succeed as a scientist. She developed my understanding of mammalian biology, and pushed me to broaden my expertise into the realms of molecular biology. Her analytical abilities are sharp, and her perception of both the big picture and details are exquisite; I hope to live up to her role model. Also during my master's studies I came to know Professor Thomas Zoeller at the University of Massachusetts at Amherst. He introduced me to the complexities of the endocrine system, welcomed me into his lab, and stimulated my interest in endocrine disruption and toxicology. Combined with my long-term interest in marine mammals, from there it was a direct path to the Hahn lab at Woods Hole Oceanographic Institution.

From my first meeting with Mark E. Hahn, I could see that he was a great advisor. Mark comments have always been thoughtful and highly insightful. Like Ginny Hayssen, Mark is soft spoken and thoughtful. This frequently results in long pauses in conversation that I can't help but fill with my own voice, the upshot being that I often find the answers to my own questions, or spontaneously develop new ideas. This method of advising has pushed me to become more independent in my work, and boosted my confidence in my own abilities.

Mark has been an attentive advisor though consumed by so many projects, proposals, and scientific ideas. Through his success he has been an inspiring figure and mentor. While my chosen path differs from his, I have found much to learn from his approach to scientific inquiry, service, and education. Many thanks are due to him for upholding the promise of the Joint Program of providing continual funding throughout my graduate school experience. While Mark has encouraged me to apply for other funding vehicles, he has worked tirelessly to support my work through grants from NOAA's Right Whale Grants Program, the Northeast Consortium, and WHOI SeaGrant. Funding short-falls

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Dr. Ann Tarrant was also a post-doc during much of my graduate experience at WHOI, and it has been truly inspiring to watch both Rebeka and Ann develop into world-class scientists with independent lines of research. Ann has been continuously generous with her time and knowledge, and maintains a brilliant perspective on life illustrating an ability to balance research with personal growth.

While I joined the Hahn lab with a toolbox full of techniques and skills, I could not have completed my thesis without the training and assistance provided by Dr. Sibel Karchner and Diana Franks. They not only showed me the ropes of the lab, and expanded my molecular and biochemical skill-set, but they provided a warm and caring environment for learning and collegiality. The Hahn lab couldn't function without them, and I don't think anyone (including Mark) would

want to work without them either. Their sage advice has been a guiding light during uncertain times.

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ABBREVIATIONS

AHR, aryl hydrocarbon receptor
BAP, benzo[a]pyrene
 B_{max} , maximal binding constant
BW, beluga whale
cDNA, complimentary DNA
GBA, GenBank Accession number
GP, guinea pig
[³H]TCDD, tritiated 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
HAM, hamster
HS, harbor seal
HU, human
HW, humpback whale
IVTT, *in vitro* transcription and translation
 K_d , dissociation binding constant
MO, mouse
mRNA, messenger RNA
NARW, North Atlantic right whale
NUPM, nested universal primer mix
PCR, polymerase chain reaction
RACE, rapid amplification of cDNA ends
RLU, relative luciferase units
SBA/DCC, saturation binding assay with dextran-coated charcoal
SDM, site-directed mutagenesis
SDS-PAGE, polyacrylamide gel electrophoresis
TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
UPM, universal primer mix
UTR, untranslated region

Chapter 1: Introduction

1. ENVIRONMENTAL CONTAMINANTS: PERSISTENT ORGANIC POLLUTANTS

1.1 Xenobiotic Compounds

The post-industrial era environment is burdened with a variety of persistent organic pollutants (POPs), including some of the most toxic xenobiotic substances known. Innumerable xenobiotic compounds exist in the environment, and are defined simply as 'chemicals foreign to a living organism'. In most cases however, 'xenobiotics' refers specifically to anthropogenic contaminants (toxicants), excluding toxins that may be exogenously produced by other organisms or by geological means. Many of the most problematic xenobiotic compounds tend to be persistent both in fatty tissues of organisms and in marine sediments owing to their lipophilic properties and resistance to environmental and biologic degradation. Of those POPs that are subject to metabolic degradation, some can become more potent (and dangerous) than the parent compound. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) is the most toxic xenobiotic chemical, and is the benchmark compound for many experimental studies in order to classify the relative toxicity of structurally related compounds such as PAHs (polycyclic aromatic hydrocarbons), halogenated aromatic hydrocarbons (HAHs), and planar HAHs (PHAHs) such as polychlorinated biphenyls (PCBs) and dioxins. Trophic transfer contributes to bioaccumulation and biomagnification, sometimes leading to large contaminant body burdens in wildlife and humans.

1.2. Sources, Levels, & Fates

From post-World War II until approximately the 1970s, the production and use of POPs was widespread in the United States. Some POPs were intentionally manufactured as pesticides, fungicides, and herbicides, whereas others are by-products of industrial activities, such as combustion, pulp mills and

paper production, and electrical power plants. Concerns about effects on human and wildlife health helped to restrict the use of these compounds in the USA and Europe. Nevertheless, the environmental persistence of even small amounts of dioxins, dibenzofurans, PCBs, and other organochlorines remains problematic, revealing more of the sublethal effects of POPs in the absence of extreme exposures (Giesy *et al.* 1994). Unfortunately, POP production and use in less developed countries is less controlled and leads to continual inputs of xenobiotic contaminants in the global system. Global POP contamination is understood to result in mass transfer of airborne pollutants to the open oceans, especially to arctic and tropical regions, leading to the conclusion that the marine environment is a 'sink' for environmental contaminants (Ali *et al.*; MacKay 1982; Tanabe *et al.* 1994). PCBs exist in the tissues of most marine animals, including prey species such as plankton and fish (Ali *et al.*; Hoekstra *et al.* 2003). Marine mammals accumulate high concentrations of POPs through trophic transfer and biomagnification (Hoekstra *et al.* 2003) as well as via lactational transfer across generations (Tanabe *et al.* 1994).

The majority of toxicological studies of marine mammals simply measure POP residue levels; however, direct comparisons of their impacts are complicated by geographic area and species-specific factors including trophic position, metabolic rates, and seasonal changes in blubber thickness. High tissue burdens of PCBs and DDTs have been reported in many odontocete (up to 700 µg/g sum blubber PCBs; striped dolphins (Kannan *et al.* 1993), pilot whales (Weisbrod *et al.* 2000b), beluga whales (Muir *et al.* 1996), killer whales (Ross *et al.* 2000)) and pinniped species (up to 5000 µg/g sum blubber PCBs; California sea lions (DeLong *et al.* 1973), grey seals (Blomkvist *et al.* 1992)). Mysticete POP levels range from trace levels up to 27.45 µg/g PCBs and 47.70 µg/g DDT (Weisbrod *et al.* 2000a; Woodley *et al.* 1991). Analyses of blubber biopsies have revealed the presence of PCBs, DDT and other organochlorines in right whales (Weisbrod *et al.* 2000a; Woodley *et al.* 1991). Concentrations of

POPs in polar bear blubber are measurable in the mg/kg range (Norstrom and Muir 1994), and can reach incredibly high levels in the far north and east Arctic limits of their range (Norstrom *et al.* 1998).

1.3. Potential Impacts

Typical exposures of marine mammals to POPs seldom result in direct lethality. However, among the most obvious effects of POPs on wildlife is reproductive impairment, ranging from conception to early neonatal development and to gonadal maturation. Although these effects are often variable and difficult to compare due to differences in exposures and physiological status, nearly every organism studied has shown some form of reproductive dysfunction associated with elevated POP concentration. Eggshell thinning, suppressed vitellogenesis, malformed reproductive organs, reduced numbers of offspring, and complete failure to reproduce are documented in fish, birds, and mammals (Colburn and Clement 1992). Other potential impacts of POPs include suppression of immune function, increased risk of tumors, and porphyria.

Exposure to POPs has been linked to numerous sublethal effects on reproduction and immune function in marine mammals (Reijnders 2003; Reijnders 1986a; Tanabe *et al.* 1994). Reproductive impairment due to PCB and DDT exposure has been suggested in beluga whales in the St. Lawrence River population (Martineau *et al.* 1987; Masse *et al.* 1986). North Atlantic right whales (NARW) exhibit symptoms of reproductive dysfunction (low calving rate, high neonatal mortality (Knowlton *et al.* 1994)), however efforts to connect POP burdens with actual reproductive impairment have been limited by sampling techniques and other physiological factors (Woodley *et al.* 1991). Reproductive impairment by PCBs and DDTs has been hypothesized and experimentally tested in pinnipeds (Helle *et al.* 1976a; 1976b; Le Boeuf and Bonnell 1971; Reijnders 1986b). Field observations of Baltic seals have correlated high blubber

PCB burdens with pregnancy ratios and uterine pathologies (Helle *et al.* 1976a; 1976b), and high PCB levels in California sea lions are associated with premature births (DeLong *et al.* 1973).

Experimental studies of terrestrial mammals provide important links between field observations in marine mammals and quantifiable data. Mink suffer reproductive impairment and increased kit mortality from even low PCB exposures (Heaton *et al.* 1995; Resturn *et al.* 1998). Experimental work with rhesus monkeys exposed to PCBs has shown resorption, abortion, and stillbirth rates consistent with mink data (Barsotti *et al.* 1976). Fetal death and stillbirth can be induced in sows treated with low levels of PCBs (Hansen *et al.* 1975). The low concentrations of PCBs used in these studies are consistent with environmental levels.

2. A MECHANISM OF ACTION

2.1. Aryl Hydrocarbon Receptor

Although the exact mechanisms of action for these POPs are not entirely clear, dioxin-like compounds proximally bind to the aryl hydrocarbon receptor (AHR), a soluble, ligand-activated transcription factor, and a protein in the bHLH-PAS (basic helix-loop-helix Period-ARNT-Single-minded) superfamily causing alterations in gene expression and toxicity (Burbach *et al.* 1992; Poland *et al.* 1976). Upon ligand binding, AHR is transported into the nucleus to dimerize with ARNT (Ah Receptor Nuclear Translocator). In the nucleus, TCDD-AHR-ARNT activates transcription of genes with promoters containing xenobiotic response elements (XREs; Figure 1). Best known for its interaction with dioxin, the AHR participates in a number of cellular activities including induction of cytochrome P450 1A (CYP1A), cell cycle control, and teratogenesis, but an endogenous ligand has not been identified. Because the AHR has been predominantly defined by its role in xenobiotic metabolism, yet is also apparently involved with basic cell physiology, this mechanism of action provides an excellent framework for integrating toxicology with basic biology.

Studies of TCDD exposure in animals as diverse as chicken, monkey, cow, hamster, and other vertebrates have shown a common pattern of toxic effects and biochemical induction implicating an AHR-dependent mechanism of action (Hahn 1998; Poland and Knutson 1982). Species-specificity, as well as dose-dependent effects and duration of exposure, complicate direct comparison of toxic endpoints, yet many of the lesions observed in vertebrates exposed to TCDD and HAH exhibit tissue-specific pathologies including hyperplasias and altered differentiation, or hypoplasias, atrophy, and necrosis of epithelial tissues (Poland and Knutson 1982). Short of the lethality observed from acute doses, laboratory animals display a variety of short-term toxic responses ranging from wasting syndrome to immune, hepatic, and endocrine pathologies (Pohjanvirta and Tuomisto 1994). Experiments with AHR-null mice have shown that the AHR is required for TCDD- and benzo[a]pyrene-induced toxicity (Fernandez-Salguero *et al.* 1996; Mimura *et al.* 1997). These “knock-out” experiments substantiate over thirty years of work investigating the mechanism action for TCDD and related HAH.

Studies of mouse and human AHRs have helped to define the basic sequence structure and specific functional domains for AHRs in general (Burbach *et al.* 1992; Dolwick *et al.* 1993b; Fukunaga and Hankinson 1996; Fukunaga *et al.* 1995; Mimura *et al.* 1994). Ten to eleven exons compose AHR cDNAs of approximately 3.0 kb in length, encoding proteins of 850 – 950 amino acids and 95 – 110 kDaltons. DNA binding and heterodimerization with cofactors predominantly occurs in the amino-terminal portion of the protein, whereas the more centrally located PAS-B domain controls ligand binding. The carboxyl-terminus tends to include numerous glutamine residues and is critical for transcriptional activity (Figure 2). The bHLH, PAS-A and PAS-B domains are highly conserved across vertebrate species, and are easily recognizable in amino acid alignments.

3. SPECIES OF INTEREST

3.1 *Marine Mammals*

Marine mammal toxicology has been limited by numerous logistical and ethical challenges. Marine mammals are protected in the United States by the Endangered Species Act and the Marine Mammal Protection Act, and with a few notable exceptions, other nations also provide some level of protection for these species. Protection is mandated due to exploitative commercial hunting of some species to near (or total) extinction, in addition to more contemporary threats such as habitat degradation, high mortalities from ship strikes, epizootic episodes, and a plethora of health issues. Marine mammals are sensitive targets for POPs because of the large proportion of lipids per body mass, trophic position, and extensive life spans that characterize this group. Most of the data used for studying marine mammal biology and toxicology originates from stranded animals, skewing the perspective towards sick or unhealthy individuals. Healthy specimens can be collected from subsistence hunts (North America) and scientific whaling (Japan), but are a limited and undesirable means for acquiring information on biological or physiological condition of marine mammals. Notwithstanding these challenges, the dramatic physiological adaptations in marine mammals to their environment provide a unique perspective for mammalian toxicology and tempt scientific investigation. Recent advances in molecular biology and acquisition of small biopsies promise to help negotiate these issues.

These logistical, ethical, and legal challenges limit the methods available to test toxicological questions in marine mammals. *In vivo* studies can provide the most definitive results, but such work is essentially impossible due to the reasons stated above (with several exceptions, for example see Reijnders (1986)). Less invasive environmental studies using stranded (sick or dead) marine mammals are common, but research progress is constrained by the

random numbers, species, and physical condition of stranded animals. The conclusions of these analyses must be carefully derived to account for sampling bias. Live biopsy of healthy, wild marine mammals (Lambertsen 1987) has provided a means to reduce bias and improve sample quality; however, small skin and blubber samples may not be representative of total toxicant burden or other factors being measured (Woodley *et al.* 1991).

Because cetaceans have been shown to retain more PCBs than other animals, including lower chlorinated congeners that are typically rapidly metabolized, it has been suggested that cetaceans have a reduced ability to metabolize certain PCB isomers (Tanabe *et al.* 1988). Comparisons of “marine mammal” biology are complicated in that taxonomic relationships within this artificial (habitat-based) group are not monophyletic, and trophic position varies widely. Such variability among mammals can cause problems in making valid comparisons.

The AHR has been characterized in an odontocete (beluga) (Jensen and Hahn 2001) and two pinnipeds (harbor and Baikal seals) (Kim and Hahn 2002; Kim *et al.* 2002). These studies have illustrated high conservation of amino acid sequence, and beluga and harbor seal AHR have shown specificity and relatively high affinity of AHR for TCDD *in vitro*. The extent of sequence and functional conservation of AHR among marine mammal clades, and in comparison with terrestrial mammals, has yet to be examined.

3.2 *Surrogate Species*

The use of surrogate species presents an attractive alternative to the methodological challenges faced in addressing marine mammal toxicology. Human studies – limited by similar ethical and legal issues - have benefited tremendously from experimental work on rodents, for example. However in order to be truly useful, surrogate species must be carefully chosen and the results validated for applicability to the target species. Furthermore, species-

specific characteristics (either of the surrogate or target species) must be examined and experimentally controlled. Because mechanisms of PCB-related reproductive toxicity are not well understood and species-specific differences may exist, it is important to choose a surrogate species with high sensitivity to conservatively estimate toxicant effects.

The mink has been heralded as an ideal surrogate species for mammals because of its extreme sensitivity to POPs and ease of handling. Because mink enzymatic activities are more similar to marine than terrestrial mammals, mink is considered a particularly good surrogate for small cetacean species (Schwacke *et al.*). However, its applicability to these or other marine mammal species in regards to AHR sensitivity has not yet been validated.

Another alternative is the use of *in vitro* studies, which permit a reductionist approach and perhaps yield the most discrete results. Focusing on the molecular and cellular level, *in vitro* studies include assays using amplified genes, native extracted or recombinant proteins, and experimentation in cultured cell lines. For example, *in vitro* protein interactions with potential ligands or other proteins can identify functional characteristics that may be important at the organismal level, or in the least can help to formulate hypotheses for future study. Combined with careful inclusion of surrogate species and comparison with model species (such as rodents), the results of *in vitro* assays such as ligand binding studies can be used to infer *in vivo* conditions.

3.3 Potential Risks Specific to North Atlantic Right Whale

Environmental contaminants have been measured in NARW blubber biopsies, feces, and prey. Some authors caution that seasonal changes in lipid deposition and mobilization influence the concentrations of contaminants by effectively diluting measurements during periods of blubber deposition (Woodley *et al.* 1991). Others have shown fluctuations based on sampling efforts over seasons and years (Weisbrod *et al.* 2000a). Persistent organic pollutants

measured in the most superficial blubber are a record of recalcitrant chemicals or metabolic products, and may not accurately represent the recent exposures of NARW to environmental contaminants. Recent work on bottlenose dolphins shows that toxicant mobilization is dynamic and more closely associated with the deepest blubber strata (Montie 2006). Deep tissue biopsies are more likely to sample adipose tissues involved in the storage and mobilization of organochlorines in cetaceans (Aguilar 1985).

But what is the actual daily or annual exposure of an adult NARW to AHR agonists? Because profiles of blubber contaminants mirror the organochlorine signatures of specific prey and regions, it is assumed that exposure to environmental contaminants is primarily dietary (Weisbrod et al. 2000a). We estimated the daily toxicant exposure of NARW based on an adult body mass of 40,000 kg (Kenney et al. 1986), NARW ingestion rates and feeding ecology (Baumgartner and Mate 2003; Durbin et al. 2002), and measurements of prey contaminant loads (Moore et al. in preparation; Weisbrod et al. 2000a). Durbin et al. (2002) estimated two ingestion rates for NARW. Assuming a weight-specific feeding rate, they calculated a high ingestion rate of 9.41×10^8 copepods per day. By tagging NARW to track feeding dive depths and durations, and sampling zooplankton to identify life cycle, concentration, and distribution (MOCNESS and OPC), they determined a lower ingestion rate of 4.61×10^8 copepods per day, and determined that NARW specifically select stage V *Calanus finmarchicus* copepodites (CV) (Baumgartner and Mate 2003; Durbin et al. 2002). Estimating a wet weight of 1.7 mg per CV (Durbin et al. 2002), a single NARW consumes between 7.8×10^8 and 1.6×10^9 mg of CV per day. Using a mean estimate of daily consumption (1.20×10^9 mg CV per day) and mean values of total PAH and total PCBs measured in zooplankton from the Bay of Fundy, Gulf of Maine, Cape Cod Bay, and Georges Bank (Moore et al. in preparation; Weisbrod et al. 2000a), we estimate that daily dosage of PAH and PCBs for a feeding, adult NARW is 1.53×10^{-3} and 1.44×10^{-4} mg/kg, respectively. The calculated daily dosage of a

single PAH, BaP, is 9.33×10^{-6} mg/kg. The concentrations of the most potent PCBs (non-ortho-substituted PCBs 77, 126, 156 and 169) were lower than the limits of detection of the method (Weisbrod *et al.* 2000a), therefore total PCB exposure is a slight underestimate, but even very small concentrations of these PCBs may have significant effects.

Three mono-ortho-substituted PCB congeners, for which toxic equivalency factors (TEFs) exist, were found in measurable amounts in NARW biopsies: PCB 105, 114, and 118. The TEFs for these PCBs have recently been adjusted to 0.00003, representing the relative potency of these individual compounds to produce biological (or toxic) effects relative to 2,3,7,8-TCDD (van den Berg *et al.* 2006). Following the assumption of additivity, the toxic equivalency (TEQ) is calculated as the sum of the product of each congener's concentration and its TEF. For PCBs 105, 114, and 118, the mean TEQ for NARW biopsies sampled during summer and winter is 0.011, and 0.032 ng/g sample lipid content, respectively (mean summer lipid content = 14.8%, winter = 3.6%). Because the TEFs for these PCBs are equal, the higher concentration of PCB 118 accounts for the majority of the dioxin-like activity in each calculation, as well as the seasonal difference. Substituting WHO 2005 TEFs with relative potencies (REP) empirically derived from beluga whale *in vitro* AHR binding for PCB 105 and 118 (Jensen 2000) increases the TEQs above by ~2.5-fold. Although van den Berg *et al.* (2006) highlight the wide variation in reported REP values for mono-ortho-substituted PCBs, and attribute it to low-level contamination by more potent dioxin-like compounds, Jensen's estimate may be a more accurate assessment of cetacean sensitivities to dioxin-like PCBs.

Complicating the calculation of NARW exposures to environmental contaminants is the combination of this chronic low exposure and the potential for more acute (re)exposures during fasting and migration during which lipid mobilization reduces the percent lipid approximately 4-fold, and concentrations of total PCBs increases ~6-fold in the superficial blubber (Weisbrod *et al.* 2000a).

While these are indeed very small dosages, chronic low exposure may still produce biological effects. The predicted timing of ovulation for NARW occurs during the time when persistent organic pollutants are at their highest concentration in blubber, increasing the potential for reproductive disruption.

For comparison, dietary exposure to BaP at low chronic dosages can cause adverse reproductive and developmental effects in mice. Administration of as little as 10 mg/kg BaP during gestation decreased gonad weights and reduced fertility and reproductive capacity in offspring of both sexes by impairing germ cell development (Kristensen *et al.* 1995; MacKenzie and Angevine 1981). Another study found that high doses of BaP (120 mg/kg/d) during gestation also caused stillbirths, fetal resorption, and malformations regardless of AHR allelomorph (high and low affinity AH receptors) (Legraverend *et al.* 1984). Although in each of these studies exposure to BaP was much higher than our estimate of the exposures to NARW, these experimental exposures were limited to less than ten days; longer chronic exposures in NARW may have similar effects.

Because NARW population growth differs so dramatically from the southern right whale and other cetaceans, it is important to compare NARW with other Cetaceans. In comparison to NARW, sympatric HW have more diverse prey sources, and depend more heavily on schooling fish such as sand lance, herring, and mackerel, and occasionally euphausiids, in the western North Atlantic (Hain *et al.* 1982; Payne *et al.* 1990). These planktivorous fishes have different life histories, potentially exposing HW to different sources of persistent environmental contaminants. Furthermore, while it is well established that bony fishes possess mechanisms of detoxication (Andersson and Forlin 1992; Hahn *et al.* 1998) that could effectively reduce their PAH accumulation, copepods (especially in diapause) may not. Thus, HW may have both more diversity of DLC and less exposure to PAH in their diet than NARW. In contrast, BW in the Gulf of St. Lawrence suffer from heavily contaminated food sources, and themselves have high DLC burdens (Martineau *et al.* 1987). This BW population

has a high rate of disease, including a high incidence of tumors, reduced immune responses, and a low reproductive rate (Bailey and Singer 1995; DeGuise *et al.* 1995; Martineau *et al.* 1988; Martineau *et al.* 1995). Studies of BW may represent effects of extremely high and chronic exposures, whereas NARW have low daily exposures punctuated by periods of acute (re)exposure during fasting or reproductively sensitive times.

4. SPECIFIC OBJECTIVES

Given the obstacles to studying marine mammal toxicology, how can we investigate the specific factors and mechanisms involved in biological responses to POPs? Can molecular techniques typically employed for laboratory systems be applied in a minimally invasive manner to infer the responses of marine mammals? Have mysticetes evolved different mechanisms in response to dioxin like compounds that make them more or less susceptible to the effects of these chemicals? How do marine mammals compare to other mammals in their sensitivity to POPs?

We began this project with a specific interest in the reproductive health of NARW. We hypothesized that NARW may be particularly sensitive to the effects of POPs. However, the highly endangered NARW is difficult to study, and fresh tissue sources are rarely available; the exception is that integument biopsies are routinely collected for population genetic studies. We sought to determine whether RNA extracted from NARW integument biopsies could be used to clone genes of toxicological and physiological interest. We also compare biopsy-derived RNA with RNA from tissues collected from stranded cetaceans, the most frequently used and most widely available cetacean tissue. These results are described in Chapter 2.

The successful amplification of a fragment of AHR cDNA lead to further characterization of the NARW in comparison with another new mysticete AHR from humpback whale, and the previously described beluga whale (Jensen and

Hahn 2001), mouse (Burbach *et al.* 1992), and human (Dolwick *et al.* 1993a) AHRs (Chapter 3). *In vitro* biochemical and cell-culture assays were employed to describe the binding and transcriptional activities of these cetacean receptors.

We also sought to place these and other marine mammal AHRs in context with mammalian species more frequently used in laboratory experiments. While the application of the comparative approach in an *in vivo* system is cumbersome, the extension of *in vitro* binding assays, combined with sequence analysis and three-dimensional homology modeling, comprise a more tractable series of analyses for this purpose (Chapter 4).

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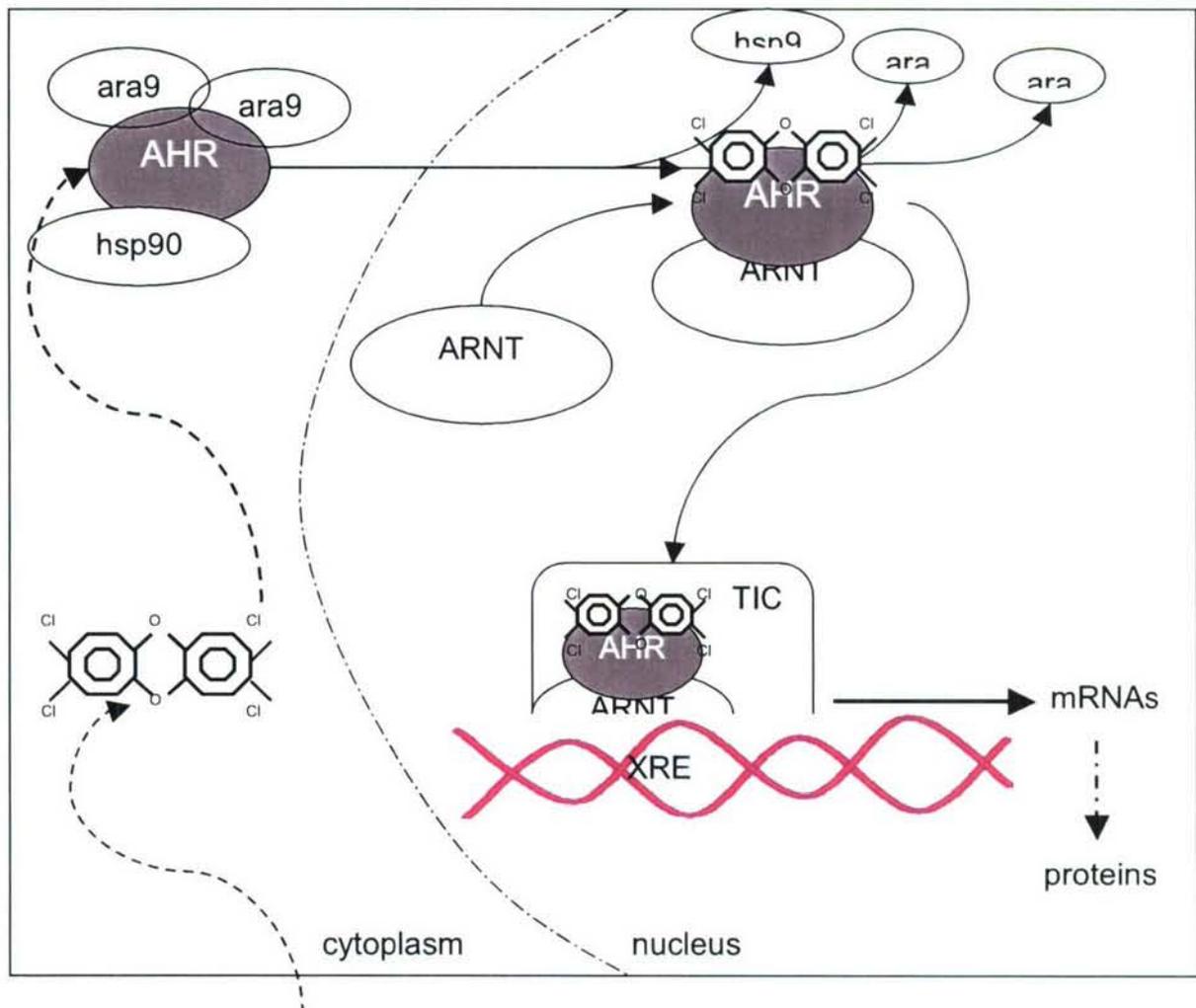


Figure 1. The intracellular mechanism of action for the aryl hydrocarbon receptor.

Once bound with ligand, the cytosolic AHR protein enters the nucleus and exchanges the Ara9 and Hsp90 cofactors for ARNT. The ligand-AHR-ARNT complex locates xenobiotic recognition elements (XRE) in the promoters of responsive genes, and recruits the transcription initiation complex (TIC). Transcription and translation of target genes including *cyp1a* and *cyp2b* follows.

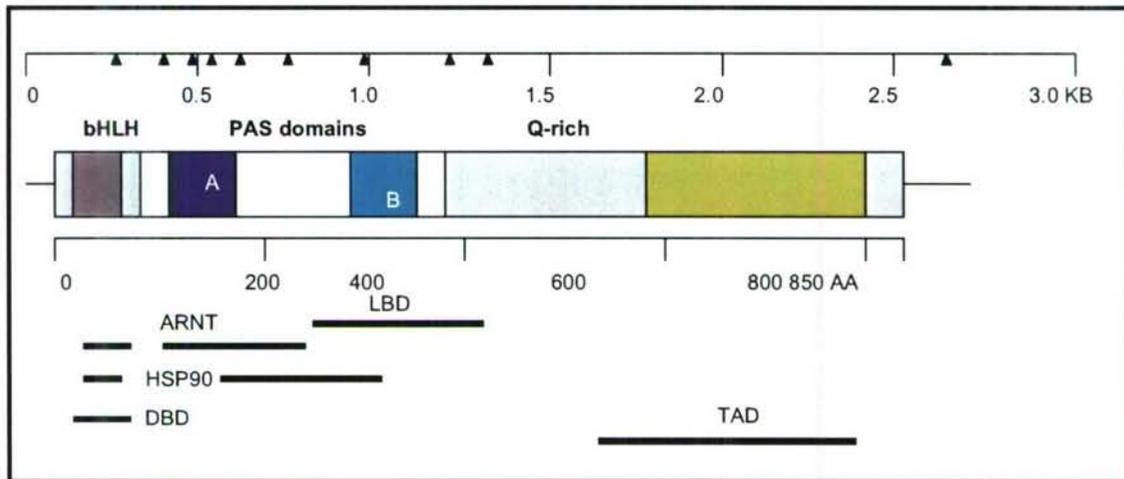


Figure 2. Structural and functional domains of the AHR.

Aryl hydrocarbon receptor cDNA (top; intron/exon boundaries = black triangles) and protein structure characteristics with PAS-family and AHR-specific structural motifs indicated, with approximate locations of functional domains (bottom) based on the murine Ahr (Burbach et al. 1992; Dolwick et al. 1993; Fukunaga et al. 1995; Mimura et al. 1994). Boxes represent protein coding regions; untranslated regions of the cDNA are shown as lines. LBD, ligand binding domain; HSP90, heat-shock protein 90 dimerization region; ARNT, ARNT dimerization binding domain; DBD, DNA binding domain; TAD, transcriptional activation domain.

Chapter 2.

Genes of toxicological response cloned from biopsies of North Atlantic right whales (*Eubalaena glacialis*): comparison with stranded cetacean samples

ABSTRACT

The North Atlantic right whale (NARW) is the most endangered large whale species with a total population size estimated at around 350 individuals. Vessel collisions and fishing gear entanglements are the leading causes of mortality, but variable reproductive success contributes to the species' failure to reach pre-whaling numbers. Physiological or toxicological stressors may play causative roles in the observed reproductive variability, but are difficult to measure in large, wild cetaceans. Polycyclic aromatic hydrocarbons induce metabolic enzymes (cytochrome P450s; CYP1A1) through an aryl hydrocarbon receptor (AHR) mediated pathway. Minimally invasive integument biopsies from live cetaceans have given researchers access to tissue samples from many individuals. Here, we isolated RNA from these samples to identify and clone biomarkers of toxicant response and nutritional status. The quality of biopsy-derived RNA was better than that isolated from necropsied skin and blubber samples of stranded Delphinids (white-beaked dolphin, Atlantic white-sided dolphin, pilot whale, common dolphin), and a humpback whale (HW). A fragment of an AHR cDNA was amplified by RT-PCR, cloned, and sequenced from NARW, HW, and the Delphinids. A CYP1A1 fragment was similarly identified from NARW RNA. Estrogen receptor-alpha and aromatase (CYP19) were also investigated using the same techniques. RNA from stranded cetaceans showed a pattern of degradation related to time of sampling post-mortem; however, AHR and leptin cDNAs were amplified and cloned from some of these tissues. Although biopsies provide the best source of RNA, necropsies conducted on cetaceans up to 24 hr post-mortem are still of value. Here we show that RNA from biopsies can be used to identify biomarkers of physiological condition and toxicological response. cDNAs derived from this source can be cloned for use in gene expression and *in vitro* functional studies to better understand the molecular structure, function, and regulation of proteins involved in reproductive success.

1. INTRODUCTION

Right whales, *Eubalaena glacialis*, were once so abundant in the North Atlantic Ocean that they were considered the preferred commercial whaling target. Because they are slow moving and float when dead, right whales were dubbed the “right whale” to hunt. Centuries of hunting, coupled with life history characteristics such as slow reproductive rates, contributed to the rapid decline and near extinction of the North Atlantic right whale (NARW). Unlike other species (e.g. grey whale), the NARW population has not recovered since the cessation of whaling. Currently the NARW population is estimated around 350 individuals, and while some molecular evidence suggests the population is slightly larger (Frasier 2005; Frasier *et al.* 2007), modeling suggests that extinction could be imminent within the next 150 to 200 years (Fujiwara and Caswell 2001). Ship strikes and entanglement in fishing gear are now the leading causes of right whale mortality; however, a recent decline in calving rate suggests the disruption of other biological factors. Curiously, twelve percent of adult female NARW have never reproduced (Brown *et al.* 1994; Kraus *et al.* 2001; Kraus *et al.* 2007). The observed reproductive variability might be explained by physiological (e.g. nutritional status) or toxicological (e.g. responses to contaminants) factors; however, measuring such factors in large whales using traditional *in vivo* methods is exceptionally difficult.

Numerous logistical, ethical, and legal issues preclude the use of standard experimental techniques in the study of marine mammal biology. Whaling collections have historically provided much of the information about cetacean physiology, anatomy, and population status. Necropsies of stranded specimens are also useful in studying anatomy, physiology, and life history of cetaceans; however, interpretation of these samples is sometimes confounded by poor health condition of stranded individuals and decomposition of carcasses. The

use of minimally invasive techniques for studying physiological parameters in living whales has increased in recent years.

The development of minimally invasive remote biopsy has permitted the sampling of integument from live, healthy cetaceans (Aguilar and Nadal 1984; Lambertson 1987). Molecular techniques were initially applied to karyotype, identify, sex, and assess paternity of individual whales (Amos and Hoelzel 1990; Arnason *et al.* 1985; Gowans *et al.* 2000; Hoelzel *et al.* 1991; Palsboll *et al.* 1992). More recently microsatellite loci have been employed to examine patterns of reproductive success and to challenge estimates of population size in NARW (Frasier 2005; Frasier *et al.* 2007). Biochemical analyses of lipid content, contaminant load, enzyme activity and localization, and stable isotope ratios have been conducted on many cetacean species through the use of biopsy collection (Aguilar and Borrell 1991; Angell *et al.* 2004; Fossi *et al.* 1992; Fossi *et al.* 2000; Gauthier *et al.* 1996; Hooker *et al.* 2001; Ross *et al.* 2000; Todd *et al.* 1997; Weisbrod *et al.* 2000a). Integumentary biopsies have also been used for tissue culture (Godard *et al.* 2004) and cell culture of odontocete fibroblasts (Godard *et al.* 2004; Marsili *et al.* 2000).

Nucleic acids in general, and ribonucleic acid (RNA) in particular, are sensitive to degradation by endogenous and environmental nucleases. Therefore, sample preservation is critical to obtaining high quality material for genetic analyses, and collection of tissue for this purpose during cetacean necropsies is often neglected except under the most optimal conditions (<6 hours post-mortem, cool temperatures, clean and convenient sampling location). When we began this work, cetacean biopsies had been used for isolation and analysis of DNA, but there were no reports of RNA isolated from biopsies. The application of existing gene cloning techniques to RNA collected from biopsies is emerging as a new approach to understand cetacean biology.

Previous studies have addressed toxicological and reproductive issues in marine mammals at the level of protein expression and activity. Variations in aryl

hydrocarbon receptor (AHR) structure and function contribute to species-specific susceptibility to polycyclic aromatic hydrocarbons (PAH) and planar halogenated aromatic hydrocarbons (PHAH). AHR molecular structure, binding capacity, and tissue-specific expression has been characterized for beluga whale (Jensen 2000; Jensen and Hahn 2000; Jensen and Hahn 2001), harbor and Baikal seals (Kim and Hahn 2002; Kim *et al.* 2002), and in several other marine mammal species (Lapseritis and Hahn 2001; Lapseritis and Hahn in preparation). Some AHR ligands induce cytochrome P450 1A (CYP1A) enzymes, a biomarker of PAH/PHAH exposure, and CYP1A protein expression and activity have been shown in many cetacean species (Angell *et al.* 2004; Fossi *et al.* 2003; Goksøyr *et al.* 1988; Goksøyr *et al.* 1986; White *et al.* 1994; White *et al.* 2000; Wilson *et al.* 2005a). Another cytochrome P450 (CYP19/aromatase) is among the enzymes responsible for conversion of androgens to estrogens, a critical aspect of reproductive function, and has been studied in white-sided dolphin (Wilson *et al.* 2005b). For cetaceans and other mammalian species, successful reproduction depends on a number of biological factors, including sufficient energy (lipid) storage (Lockyer 1986). Mammalian fat deposition and energy utilization are strongly influenced by leptin, a protein that also regulates numerous reproductive functions at least in part through CYP19 and estrogen receptor alpha (ER α) expression (Catalano *et al.* 2003; Catalano *et al.* 2004; Chehab 2000).

In order to address questions concerning the possible role of persistent organic pollutants in the decline of the NARW, we sought to test the feasibility of using integument biopsies as a source of RNA to clone genes for contaminant susceptibility and other biomarkers related to physiological condition. We extracted mRNA (polyadenylated messenger RNA) from integument biopsies of NARW, and amplified complementary DNA (cDNA) sequences for several expressed genes: AHR, CYP1A1, CYP19, ER α , leptin, and actin. We also compared the quality of RNA from biopsies to that obtained from samples

collected during necropsies of stranded delphinid cetaceans. We show that gene expression can be detected at the level of mRNA in cetaceans using minimally invasive techniques, and demonstrate a technique to better understand physiological processes active in whales, including toxicological responses.

2. METHODS

2.1 Tissue collection

Integumentary biopsies were collected using retrievable biopsy darts fired from a crossbow. Two biopsies consisting primarily of epidermal tissue were collected from live NARW in the Bay of Fundy (field identification “K” & “R”, both collected 4/6/1999) using standard techniques (Brown *et al.* 1991; Lambertson 1987) in conjunction with other sampling efforts. The samples were stored in DMSO and immediately placed in liquid nitrogen dry shippers. Samples were stored in liquid nitrogen for 34 months until processing.

Post-mortem surgical biopsies were obtained from Delphinid cetaceans found dead, or stranded live and euthanized, on the shores of Cape Cod, MA, USA, with the cooperation of the Cape Cod Stranding Network (CCSN). Integument including epidermal, dermal, and subdermal (skin and blubber) tissues were obtained from individual *Lagenorhynchus albirostris* (white beaked dolphin; WBD), *L. acutus* (Atlantic white-sided dolphin; AWSD), *Globicephalus melas* (pilot whale; PW), and *Delphinus delphis* (common dolphin; CD) during standard necropsies, replicating the type of sample obtained from biopsy darts. Integument, mammary gland, and ovary samples were collected shortly after the death of a juvenile humpback whale (HW; *Megaptera novaeangliae*) that stranded live on a Cape Cod beach. CCSN identification numbers link collected tissues with individual records including necropsy reports (Table 1). Tissues were flash frozen and stored in liquid nitrogen for one to eleven months until processed.

2.2 RNA extraction

Total RNA was extracted using RNA STAT60™ (Tel-Test, Inc.), with care taken to eliminate ribonucleases and to prevent cross-species contamination by diligent workspace and equipment cleaning, and extraction on separate days. Briefly, skin and blubber biopsies (0.3 - 1.0 g) were pulverized by hand in a mortar and pestle with continuous addition of liquid nitrogen. Tissue powders were then homogenized in glass tubes with STAT60™, extracted with chloroform, and precipitated with isopropanol/ethanol according to the manufacturer's protocol. Total RNA quality was assessed by denaturing gel electrophoresis, and concentrations were determined by spectrophotometry. mRNA was purified from North Atlantic right whale total RNA samples using the Nucleotrap mRNA Purification Kit (Clontech) or Micro Poly Purist (Ambion).

2.3 RT-PCR

Complementary DNA was generated with OmniScript reverse transcriptase (OmniScript RT kit, Qiagen) using random hexamer primers and 1 µg RNA (mRNA for NARW, total RNA for all other species). Amplification was by PCR with degenerate, gene-specific primers (Table 2) and AmpliTaq Gold DNA polymerase (50 µL reactions: 1X PCR Gold Buffer, 2 mM MgCl₂, 200 µM dNTPs, 0.2-2.0 µM each gene specific primer, 1.25 U AmpliTaq Gold; Applied Biosystems). Thermocycling conditions for the PCR step were generally: 95°C 10 min; 35 cycles: 95°C 15 sec, 50°C 30 sec, 72°C 60 sec; 72°C 7 min; rapid cooling to 4°C. PCR products were visualized by electrophoresis on a 1% agarose gel with ethidium bromide, then gel extracted for cloning (Gene Clean II, Bio101, Inc.).

Primers and PCR conditions used to successfully amplify AWSO CYP19 in a previous study were kindly supplied by J.Y. Wilson (Wilson et al. 2005b). Two pairs of primers were used in separate PCR reactions (CYP19 2-23f/CYP19

810-830r and CYP19 672-691f/CYP191353-1373r; 0.4 μ M each) in an initial PCR attempt utilizing AmpliTaq Gold polymerase, and reaction conditions as follows: 95°C 7 min; 35 cycles: 95°C 30 sec, 50°C 30 sec, 72°C 90 sec; 72°C 10 min; rapid cooling to 4°C. Following unsuccessful amplification, the reaction was repeated using Advantage 2 polymerase (50 μ L reaction: 1X PCR Buffer, 1X dNTP Mix, 1X Advantage 2 polymerase mix; Clontech Laboratories, Inc.), 1.0 μ M each primer, and reaction conditions as follows: 95°C 1 min; 30 cycles: 95°C 5 sec, 60°C 1 min; 72°C 7 min; rapid cooling to 4°C. Delphinid cDNA was used in the first PCR; NARW total RNA was used in both sets of reactions, and human placental cDNA (Clontech) was used as a positive control in the Advantage 2 reactions.

2.4 Cloning and Sequencing

Individual gene products resulting from RT-PCR in each species were handled separately and with the same general methods. Purified PCR products were cloned into the pGEM-T Easy© plasmid vector (Promega), and propagated in *E. coli* JM109 cells. Plasmid DNA was isolated from 6-12 colonies per gene using the boiling-lysis method (Sambrook *et al.* 1989). Inserts were independently verified by restriction endonuclease digestion (EcoRI; Promega), and with internal PCR using nested primers for AHR (D143F/D328R; data not shown). Two to four positive clones were sequenced bi-directionally to obtain consensus sequences. Delphinid AHR and actin were sequenced using M13 primers and Big Dye Terminator Ready Mix© with standard parameters, purified using G-50 Sephadex with Centri-Sep columns or isopropanol precipitation, and visualized on an ABI 377 DNA sequencer (Applied Biosystems); other sequencing reactions were performed by the University of Maine DNA Sequencing Facility (Orono, ME). Sequences were manually edited using Sequencher 4.2.2 (Gene Codes Corp.) and MacVector 8.1.1 (Accelrys, Inc.) software, and examined by NCBI/GenBank tBLASTx for gene identification

(Altschul *et al.* 1997). Predicted amino acid sequences were determined for each sequence, and aligned with known cDNA sequences from other species using Clustalw in MacVector 8.1.1.

3. RESULTS

3.1 RNA Extraction

We first sought to establish that RNA could be obtained from NARW integument biopsies, and to compare its quality with that collected from necropsy samples. Using standard RNA extraction methods, we obtained good total and mRNA yields from small tissue samples (0.3 - 1.0 g) (Table 1). RNA quality was determined by electrophoresing equal volumes of extracted total RNA on a formaldehyde gel (Figure 1). The total RNA extracted from biopsied NARW tissues was of higher quality than that extracted from necropsy samples, as shown by the increasing clarity and brightness of the 18S and 28S ribosomal RNA bands. A trend in RNA degradation appears related to time of sampling post-mortem, but may also reflect individual or species-specific differences.

Spectrophotometry was used to evaluate purity and determine quantities extracted from each sample. The ratio of light absorbance at 260 versus 280 nm (260/280) provides a measure of RNA purity, with an optimum ratio of 2.0; in practice, 260/280 ratios of 1.8 – 2.0 are common, indicating some protein contamination (Sambrook and Russell 2001). NARW biopsy-derived RNA samples produced an average ratio of 1.79, compared with the average necropsy-derived RNA 260/280 ratio of 1.66. The greatest yield of total RNA was extracted from one NARW biopsy (1087 $\mu\text{g/g}$ tissue). This is consistent with expected yields from fresh tissues (1000-1500 $\mu\text{g/g}$ skeletal muscle or brain, 1000-4000 $\mu\text{g/g}$ placenta; RNA Stat-60TM, Tel-Test, Inc.). Interestingly, the yield of total RNA from stranded cetaceans that were sampled less than approximately

15 hours post-mortem was similar to that acquired from live biopsy samples (Table 1). mRNA was only extracted from HW and NARW integument samples, providing yields consistent with those regularly obtained in this laboratory (~2.5%). NARW biopsies yielded better RNA than that from necropsied Delphinids, both in terms of quality and yield.

3.2 Gene Specific PCR Products & Sequence Analysis

Our next objective was to determine whether RNA from NARW integument biopsies could be used to clone cDNAs encoding proteins related to contaminant effects and physiological condition. Actin was chosen as a positive control given its ubiquitous high expression in mammalian tissues, and was successfully amplified from all samples using general PCR conditions and degenerate primers (Figure 2). Actin was sequenced from AWSD, HW, and NARW to verify specific PCR amplification (GenBank Accession #EF091836 - EF091838, respectively).

3.2.1 Aryl Hydrocarbon Receptor (AHR)

The AHR is a ligand-activated transcription factor that regulates the expression of a number of genes in response to binding PAH and PHAH, such as benzo[a]pyrene, non-*ortho*-substituted polychlorinated biphenyls (PCBs), and dioxin-like compounds (Chen and Bunce 2004). With the assistance of numerous cofactors, ligand-bound AHR binds response elements in specific gene promoters, activating transcription of CYP1A1 and other genes (Hankinson 2005; Schmidt and Bradfield 1996; Whitlock 1999).

An initial AHR cDNA fragment of approximately 690 bp was isolated and sequenced from the necropsied samples as described in Methods using general PCR conditions and degenerate AHR primers. PCR products from white-beaked dolphin, Atlantic white-sided dolphin, and pilot whale were cloned and

sequenced, and alignments and BLAST searches confirmed AHR-specific amplification (GenBank Accession #EF091831 – EF091833, respectively). Once we were convinced that our primers could amplify cetacean AHR, a larger fragment was amplified from all RNA samples (Figure 2). The NARW PCR product was cloned and sequenced (Figure 3), and subsequently the entire NARW AHR cDNA was fully cloned and sequenced (GenBank Accession #EF091829; Chapter 3). These data show that AHR is expressed in cetacean integument, including NARW superficial biopsies.

3.2.2 *Cytochrome P450 1A1 (CYP1A1)*

CYP1A1 expression is induced in an AHR-dependent manner, and is a biomarker of exposure to PAH and PHAH (Nelson *et al.* 1996). The CYP1A1 enzyme acts to hydroxylate the compounds responsible for its expression, facilitating excretion and detoxification; however, this mechanism of detoxication can proceed via reactive intermediates, and occasionally the metabolites exhibit increased toxicity (Whitlock 1999). Two fragments were amplified from NARW mRNA using primers designed for dolphin CYP1A1 (Montie 2006), and the smaller (~370 bp) was confirmed by sequencing to be CYP1A1 (Figure 4; GenBank Accession #EF091834). Expression of CYP1A1 in NARW superficial integument biopsies suggests that AHR protein is transcriptionally active in this tissue, and that AHR ligands were present.

3.2.3 *Estrogen Receptor alpha (ER α)*

Estrogen receptors are also ligand-dependent transcription factors, regulating the expression of a suite of downstream genes of reproductive function. Two forms exist in mammals, ER α and ER β , with overlapping functions (Hall *et al.* 2001). Degenerate primers for a large portion of the ER α cDNA were designed from alignment of published sequences (human, mouse, rat, cow, and pig). Although ER α can be expressed in human skin (Kwon *et al.* 2004), it is

more abundantly expressed in reproductive tissues (Kuiper *et al.* 1997). Therefore, RT-PCR was conducted using NARW RNA as well as RNA isolated from humpback whale integument, mammary, and ovary tissue. Amplification of appropriately sized product was only detected from HW mammary-derived RNA (Figure 5A), but its sequence remains unconfirmed. Although a lack of ER α expression in cetacean integument cannot be excluded, more degenerate primers designed to a more conserved region of ERs, or less stringent PCR conditions might be more successful.

3.2.4 Aromatase/Cytochrome P450 19 (CYP19)

Binding of estradiol with estrogen receptors induces expression of CYP19, an enzyme that converts androstenedione and testosterone to estrogens (Kinoshita and Chen 2003). This pathway is not only critical for hormone synthesis and regulation, but chemical cross-talk between the ER and AHR pathways provides potential for endocrine disruption along these two axes (Baba *et al.* 2005; Matthews and Gustafsson 2006).

Two attempts were made to amplify CYP19, each using two separate sets of primers previously used to amplify aromatase in cetaceans. Human placental cDNA produced bright bands of expected size for both primer pairs, whereas multiple bands were visible for the NARW reactions (Figure 5B). Two faint bands (~800 and ~900 bp) roughly equivalent to the band seen for the human positive control were recovered from one NARW Advantage 2 reaction (CYP19 2-23f/CYP19 810-830r); however, sequencing revealed non-specific products.

3.2.5 Leptin

Lastly, we attempted to amplify a cDNA for NARW leptin. Leptin is the product of the *ob/LEP* gene and a regulatory hormone secreted by many cell types including adipocytes and placental tissue (Friedman and Halaas 1998; Magarinos *et al.* 2006). Leptin plays important roles in nutrition and reproductive

physiology, controlling fat deposition, energy expenditure, pubertal onset, and fertility (Chehab 2000). Oligonucleotide primers were designed in the 5' and 3' UTR of published leptin (*ob*) cDNAs using an alignment of sequences from human, pig, mouse, rat, cat, dog, harbor seal, and grey seal (Ob-F1 & Ob-R1, Table 1). Primers used for amplification of seal leptin were also synthesized and used under the standard PCR conditions above (S2 & AS2, Table 1; Hammond *et al.* 2005). Only the latter primers yielded visible products; bands from WBD and other delphinids were of expected size, while those for NARW were larger than expected (Figure 6A). Although cloning and sequencing of the NARW products yielded non-specific sequence, the WBD band was confirmed as leptin (Figure 6B; GenBank Accession #EF091835).

4. DISCUSSION

4.1 Application of Molecular Biology Methods to Cetacean Biopsies

Establishing non-destructive methods for understanding cetacean physiology and toxicology is difficult given the obstacles of studying these large wild animals, but is particularly critical for endangered species. Applying the tools of molecular biology toward biopsy samples is an approach that can circumvent some of these obstacles by enabling the use of *in vitro* experiments ranging from gene expression to functional biochemical studies. It is hoped that biomarkers in integument samples could provide information on individual health and reproductive parameters or activity of other internal organs; this goal may be achieved through a combination of complementary approaches. Here we show that RNA from integument biopsies can be used to identify biomarkers of physiological condition and toxicological response. cDNAs derived from this source can be cloned for use in gene expression and *in vitro* assays (see

Chapter 3) to better understand the molecular structure, function, and regulation of proteins involved in reproductive success.

4.2 Cetacean Integument Characteristics

Although many general characteristics of mammalian integument are observed in cetaceans, several fundamental differences are worth noting (Berta and Sumich 1999). Sweat and sebaceous glands are not present in cetacean integument, and hair is virtually absent; sparse vibrissae are localized in the anterior sensory regions and mouth in whales, and are minimally present in dolphins. The functional replacement for thermoinsulatory hair in the aquatic environment is an expanded, specialized hypodermis (blubber). Cetacean epidermis is also relatively thick compared with that of other mammals, yet characteristically high sloughing rates reduce the depth of the topmost layer of dead cells (stratum corneum). Epidermal replacement has been calculated at 9 times that of humans (Bergstresser and Taylor 1977), with surface renewal occurring about every 2 hours (Geraci *et al.* 1986). The interface between cetacean epidermis and dermis is also unusual in the elaborate development of rete ridges and dermal papillae, which may have evolved in response to the large hydrodynamic and mechanical stresses placed on cetacean skin, but also serve to reduce the distance between the mitotically active stratum germinativum (the deepest epidermal layer) and the skin's surface. Thus, rapidly dividing and differentiating cells are positioned close to the skin's surface and are easily sampled by remote biopsy; these cells may be performing important physiological processes that can be identified using the techniques we describe here.

4.3 Higher quality RNA is found in biopsies than from strandings and can be used to clone genes expressed in skin and blubber.

Our results show that integument from biopsies of live NARW and stranded delphinids can be used to collect RNA of sufficient quality and quantity to clone expressed genes. Genes specific to chemical susceptibility are accessible using these techniques, and can be developed as reagents for *in vitro* toxicological investigation (Lapseritis and Hahn in preparation). We have shown that combining careful tissue sampling already underway with standard molecular biology techniques is a useful way to identify species-specific cDNA sequences in cetaceans.

RNA extracted from biopsies from living whales was of higher quality than RNA isolated from post-mortem tissues. RNA is particularly sensitive to tissue decomposition, and stranded cetaceans are often exposed to extreme temperatures that destroy cellular material, particularly labile nucleic acids. Attempts were not made to isolate RNA from post-mortem right whales because carcasses are typically located in remote areas that preclude sampling, or are identified at advanced stages of decomposition (and often lacking skin altogether). Because biopsies are routinely collected from NARW during annual surveys and for other study purposes, and very small subsamples can be used to support multiple uses, biopsies represent a reliable source of fresh tissue for nucleic acid analysis. Until recently, the use of biopsies for RNA had not been reported (Lapseritis and Hahn 2004; Spinsanti *et al.* 2006).

Biopsy samples provided the best RNA for RT-PCR and permitted detection of several cDNA sequences from the NARW. Ideally, biopsy samples would uniformly sample integument perpendicular to the surface of the animal, but in reality many biopsies consist of superficial tissue as the darts obliquely strike low-profile animals upon surfacing or diving. Our biopsy samples included an oblique sample with undetectable hypodermis by gross visual examination. Nonetheless, we were able to detect expressed genes in epidermal and dermal tissues indicating that living cells could be recovered from the superficial layers.

It should be noted that despite being partially degraded, RNA recovered from necropsy samples was still of sufficient quality to amplify partial and full sequences of expressed genes. Knowing the progression of RNA degradation is important for researchers dependent on this tissue source, and for individuals sampling cetaceans at necropsies. The quality of RNA appears to decrease with increasing time sampled post-mortem. Other factors may also contribute to this difference: species-specific differences; individual factors including health condition and life history; trauma associated with stranding; and environmental conditions at stranding. For species rarely observed alive or for stranding events of unusual circumstances, tissue samples collected from stranded specimens may still be useful for identifying genes related to physiological condition, even when sampling occurs as late as 18 hours post-mortem. Such samples are most likely to be useful for qualitative rather than quantitative measurements.

North Atlantic right whales, and mysticetes in general, rarely strand alive in New England waters; they are often discovered floating at sea in advanced states of decomposition. Therefore, direct comparison of NARW biopsy samples with necropsy samples was impractical in this study; however, a sympatric mysticete species (humpback whale) was found live-stranded in Chatham, MA, and died on the beach in the evening following attempts at release. Necropsy commenced the morning after death, and samples were obtained from integument, mammary, and ovary tissues. Total RNA was extracted and mRNA purified. The quality of integument-derived total RNA was equivalent to that from other necropsied cetaceans in this study, but the ovarian RNA was badly degraded. Actin was amplified by PCR from all humpback whale tissues using methods described above, and an AHR cDNA was subsequently fully cloned and sequenced from integument-derived RNA (Chapter 3). This may be the closest approximation to RNA degradation expected in a recently stranded NARW, and is consistent with our measurements from stranded odontocetes.

Even less invasive techniques than biopsies have been developed to assess biological parameters in large whales. Sloughed skin and fecal samples have been used with success; however, the utility of such samples is limited to DNA and hormone assays (Amos *et al.* 1992; Clapham *et al.* 1993; Hunt *et al.* 2006; Parsons 2001; Parsons *et al.* 1999; Rolland *et al.* 2005; Valsecchi *et al.* 1998). DNA from these sources often cannot be reliably assigned to individuals, is of suboptimal quality and quantity, and can easily become contaminated by prey DNA (Taberlet and Waits 1998). While we have not assessed the presence of RNA in such samples, we are not optimistic in applying our approach to fecal or sloughed skin samples. The minimally invasive nature of remote live biopsy is justified for gaining access to the quality and quantity of RNA shown here, and promises to increase our knowledge of cetacean physiology.

4.4 Toxicological and Physiological Applications

While some countries continue to support so-called scientific whaling, those efforts have not advanced the understanding of cetacean physiology, in part due to poor experimental design and failure to publish results (Gales *et al.* 2005). Some have called for expansion of non-invasive methods for acquiring information on large whale physiology, specifically endocrine function (Hunt *et al.* 2006). The application of minimally invasive sampling with qualitative gene expression techniques outlined here promises to illuminate characteristics relevant to understanding toxicological response, reproductive state, and nutritional status in cetaceans. Understanding of cetacean reproductive endocrinology could be improved using gene constructs developed from biopsies for a number of potentially expressed genes such as steroid hormone receptors (currently under investigation) and their downstream gene targets. This report identifies one marker of nutritional status (*leptin/obese*); however, other biomarkers of lipid metabolism and immune response may be investigated in a

similar manner (e.g. peroxisome proliferator-activated receptors, interleukins 2, 6, & 8 (Chinetti *et al.* 2000)).

Detection of aryl hydrocarbon receptor expression in skin of living NARW is important in establishing a mechanism of action for some dioxin-like environmental contaminants and PAHs – chemicals known to be present in the habitat. Previous studies have shown that NARW carry body burdens of AHR agonists including PAHs, PCBs, and DDT derivatives (Moore *et al.* in preparation; Weisbrod *et al.* 2000b). Human keratinocytes express AHR, CYP1A1, and other AHR-regulated CYPs, and are responsive to exposure to AHR ligands (Reiners *et al.* 1998; Swanson 2004), and skin is a potential target tissue for PAH and dioxin-like compounds that activate CYP enzymes in an AHR-dependent manner (Reiners *et al.* 1998; Saarikoski *et al.* 2005; Tauchi *et al.* 2005). The presence of AHR transcripts in cetacean skin indicates the potential for activation of the CYP1A pathway; detection of CYP1A1 transcripts supports this hypothesis.

Previous studies have documented the presence of immunoreactive CYP1A1 protein in cetacean vascular endothelial cells (Godard *et al.* 2004; Wilson *et al.* 2005a), including in NARW integument biopsies (Angell *et al.* 2004). Although we did not specifically investigate the presence of endothelial tissue in our biopsy samples, the presence of vasculature is likely to be minimal given the oblique angle of sampling and from gross examination, but histological analysis will be necessary to confirm the degree of vascularization. The predominant route of exposure of cetaceans to AHR agonists is probably systemic via dietary consumption because measured PAH and PCB levels in water are very low (Moore *et al.* in preparation); however, dermal exposure cannot be excluded. Alternatively, autocrine or paracrine signaling may have induced AHR and CYP1A1 expression within adipocytes or in neighboring cells. Quantitative measurement of expressed AHR and CYP1A1 transcripts, paired with histological examination by immunohistochemistry, could help clarify which cell

types express each protein and whether the source of AHR agonists was internal or external.

Several studies of estrogen receptors and aromatase in human skin have localized expression to dermal papillae associated with hair follicles and sebaceous glands, and shown greater expression of ER β than of ER α (Kwon *et al.* 2004; Thornton *et al.* 2006; Thornton *et al.* 2003). Given the absence of these features in cetacean skin, the probability of ER expression (and notably the alpha form) is reduced. Yet both ER isoforms are expressed in human adipocytes (Pedersen *et al.* 2001), and CYP19 is abundantly expressed in human adipose stromal cells (Price and O'Brien 1993), suggesting the real possibility of identifying these proteins in cetacean integument biopsies if sampling includes hypodermis. Identification of these sequences would be important for further characterization of cetacean reproductive endocrinology, and holds potential for more directly assessing issues of reproductive failure in NARW. We are continuing efforts to amplify both ER α and ER β cDNAs, using more degenerate primers targeted to a shorter segment of the cDNA, and less stringent PCR parameters. Combined with new methods for sampling hormones from whale feces (Hunt *et al.* 2006; Rolland *et al.* 2005), characterization of steroid hormone receptors for *in vitro* studies of gene expression and biochemistry could prove to be a powerful approach for understanding NARW reproductive endocrinology, even on an individual basis.

The structure, function, and expression of leptin in NARW are of particular interest because of observed reproductive failure and poor body condition of females (Angell 2005; Pettis *et al.* 2004). Because of the regulatory interconnectedness of estrogen receptors, CYP19, and leptin (Catalano *et al.* 2003; Catalano *et al.* 2004), and their roles in lipid storage and reproduction, understanding how these proteins function (or are disrupted) in NARW is a priority for physiological investigations. We successfully amplified and cloned a segment of leptin cDNA from white-beaked dolphin integument. This is the first

indication of leptin expression in cetaceans, although a leptin sequence has been deduced from beluga genomic DNA (Doyon *et al.* 2001). Right whale leptin cDNA might be recovered from superficial biopsies under slightly different PCR conditions, or with alternative primers. That we were able to amplify a segment of leptin cDNA from a fresh necropsy sample, but not a biopsy, points to an important difference in the tissues collected. Because epidermis is much thicker in mysticetes than in odontocetes, and the total depth of sampling was equivalent (~2 cm deep), our odontocete samples contained a large portion of hypodermis, whereas the biopsy samples predominantly contained epidermis and dermis. Our inability to isolate a leptin cDNA from NARW may be due to a lack of appropriate tissue expressing leptin; full depth biopsies including more adipose tissue may be more effective. This may also be true for ER α and CYP19, which should be more highly expressed in adipose tissue. However, our ability to clone leptin from a stranded cetacean provides hope of acquiring leptin sequence from suitable NARW tissue in the future.

4.5 Summary

The isolation of good quality RNA from cetacean biopsies is best applied in combination with other methods such as cDNA cloning, functional biochemistry, immunohistochemistry, environmental contaminant data, hormone measurements, and gene expression analyses. The initial qualitative process of gene discovery illustrated here can be followed by quantitative methods, such as real-time RT-PCR (qRT-PCR). Once sequences have been identified, qRT-PCR can be used to determine expression of genes of interest. In the time since we first reported using RNA from cetacean biopsies (Lapseritis and Hahn 2004), another group has shown its use in qPCR (Spinsanti *et al.* 2006). The actin sequences shown here can be important for applying this technique to NARW, HW, and AWS, and are available to other researchers via GenBank.

Our results show that the quality of RNA recovered from biopsies is greater than from necropsied tissues, and validates the idea that gene expression can be detected in cetaceans using minimally invasive techniques. Expressed genes can be cloned from biopsy sources, and used to examine the physiological status of living cetaceans. Extension of the methods described here should include qRT-PCR, paired with complementary techniques like immunohistochemistry to determine the extent of transcript and protein expression, and tissue- or cell-specific expression. Identification of cetacean AHR and CYP1A1 sequences permits *in vitro* examination of toxicological processes otherwise unachievable in living whales because of ethical, legal and logistical obstacles. These advances offer insight at the molecular level, and promise to increase our understanding of cetacean physiology and toxicological responses.

New sequences from this study are available in GenBank, Accession #EF091829, EF091831 – EF091838.

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Table 1. Sample types and RNA yield.

Species CCSN Id.	Sample Type	Sample Mass (g)	Total RNA Yield ($\mu\text{g/g}$)	Absorbance 260/280	mRNA Yield ($\mu\text{g/g}$)
Common Dolphin (<i>Delphinus delphis</i>) CCSN-02-011-Dd	post-mortem (>24 h)	1.0	62	1.60	na
Humpback Whale (<i>Megaptera novaeangliae</i>) CCSN-02-259-Mn	post-mortem (16 – 20 h)	0.4	469	1.66	14
Pilot Whale (<i>Globicephalus melas</i>) CCSN-02-004-Gm	post-mortem (~18 h)	0.7	477	1.65	na
Atlantic White-Sided Dolphin (<i>Lagenorhynchus acutus</i>) CCSN-01-168-La	post-mortem (12-18 h)	0.7	1080	1.71	na
White-Beaked Dolphin (<i>Lagenorhynchus albirostris</i>) CCSN-01-073-La	post-mortem (< 6 h)	1.0	837	1.68	na
North Atlantic Right Whale (<i>Eubalaena glacialis</i>)	live biopsy	0.3 ^K	1087	1.81	27
		0.4 ^R	993	1.77	23

^K, Field sample "K" collected 6 April 1999 in Bay of Fundy.

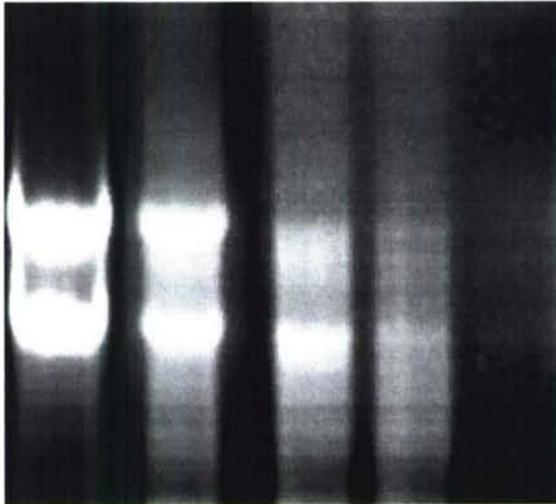
^R, Field sample "R" collected from a female 6 April 1999 in Bay of Fundy.

na – data not available

Table 2. Gene-specific, degenerate oligonucleotide primers used for polymerase chain reactions. AHR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450-1a1; CYP19, cytochrome P450/aromatase; OB, leptin; ER-a, estrogen receptor alpha.

Primer name	Sequence (5'→3')	T _m ^a
Actin Deg 5' ^b	ACA ACG GYT CSG GYA TGT GC	68°C
Actin Deg 3' ^b	GAA GCA YTT GCG RTG WAC RAT	65°C
AHR D33F ^c	GTC IAA CCC ITC IAA GMG ICA YMG	64°C
AHR D118F ^c	CTS CTS CAR GCI CTS AAY GGI TT	65°C
AHR D143F ^c	CAR GAY TAY CTI GGI TTY CAI CA	62°C
AHR D328R ^c	GCI CGC TGR ATR AAY TGR TAI CC	62°C
AHR D348R ^c	CAT ICC RCT YTC ICC IGT YTT	62°C
CYP1A1-300Fd ^{d,e}	GGA YAA GAG RCT GGA CGA GAA TGC	66°C
CYP1A1-667Rd ^{d,e}	GCC ACT GGT TYA CAA AGA CAC ARC	65°C
CYP19 2-23f ^{d,f}	TGN TTT TGG AAA TGC TGA ACC C	67°C
CYP19 672-692f ^{d,f}	GCA AGC TCT CCT YMT CAA AC	61°C
CYP19 810-830r ^{d,f}	TCC ATR BDG TCT TCC AGT TTC	60°C
CYP19 1353-1373r ^{d,f}	AAK CGT CTC ARA AGT GTR ACC	55°C
OB-F1 ^d	GYT KCN AGG YCC NAG AAG CAC ATC C	64°C
OB-R1 ^d	GGN GKC TTM TGC CTT TGG AAG AGT RG	70°C
OB S2 ^{d,g}	AGT CCA GGA TGA CAC CAA AAC C	66°C
OB AS2 ^{d,g}	GYT CAG RGC CAC CAC CTC YGT	67°C
F1-ER-a ^d	CAC ACC AAA GCV TCB GGV ATG G	65°C
R1-ER-a ^d	ATG CTC CAT GCC TTT GTT ACT CAT GTC CC	75°C

^a T_m = melting temperature; calculated: T_m = 81.5 + (0.41 * %GC) - (675/length); ^b Invitrogen Corporation, Custom DNA Oligos, Carlsbad, CA, USA; ^c Integrated DNA Technologies, Inc., Coralville, IA, USA; ^d Sigma-Genosys, The Woodlands, TX, USA; sequences provided by: ^e E.W. Montie (Woods Hole Oceanographic Institution); ^f J.Y. Wilson (McMaster University); ^g (Hammond *et al.* 2005).



NARW	WBD	AWSD	PW	CD
Live	<6 h	12-18 h	18 h	>24 h

Figure 1. Evaluation of RNA quality from post-mortem and live-biopsied cetacean integument samples.

Total RNA was assessed by denaturing gel electrophoresis. RNA recovered by biopsying live north Atlantic right whales (NARW) was found to be of highest quality. A trend in RNA degradation appears related to time of sampling post-mortem, but may also reflect individual or species-specific differences, or environmental conditions during a stranding event. Abbreviations: common dolphin (CD), pilot whale (PW), Atlantic white-sided dolphin (AWSD), and white-beaked dolphin (WBD).

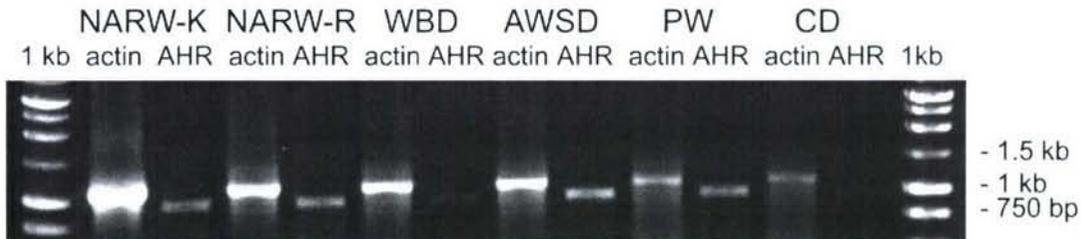


Figure 2. Actin and aryl hydrocarbon receptor are expressed in cetacean integument.

Reverse-transcriptase PCR amplified fragments of actin (1.2 kb; 1.0 μ M, 5' & 3' actin degenerate primers) and AHR (~945 bp; 1.0 μ M, D33F/D348R primers) cDNAs were visualized by agarose gel electrophoresis (1%) with 1 kb markers. PCR products were isolated from north Atlantic right whale total RNA (NARW-K) and messenger RNA (NARW-R), and from Delphinid total RNA (WBD and CD AHR bands are faint, but present). An initial AHR cDNA fragment was isolated and sequenced from Delphinid RNA samples (690 bp; 1.0 μ M primers D118F/D348R: WBD, AWS, PW).

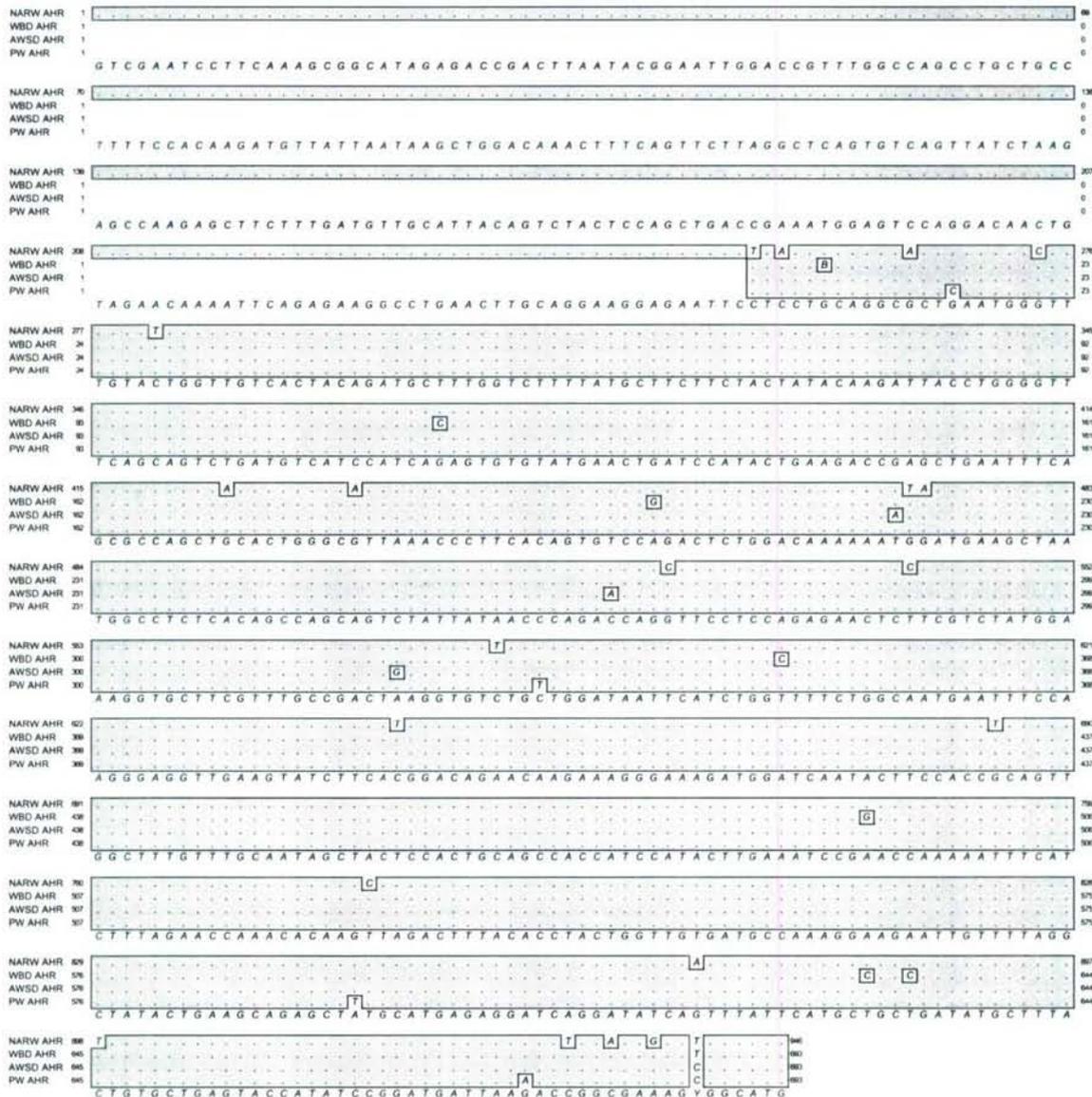
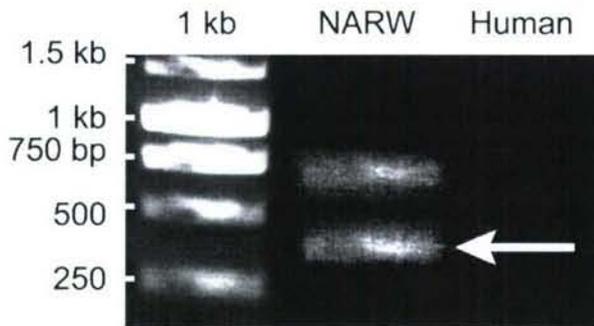


Figure 3. Nucleotide sequence alignment of partial aryl hydrocarbon receptor cDNAs from four cetaceans.

The consensus nucleotide sequence for the cetacean AHR is shown below the alignment of NARW, WBD, AWS, and PW fragments. Identical bases are indicated with "." and shaded. The NARW AHR has subsequently been fully sequenced. GenBank Accession #EF091829.

A.



B.

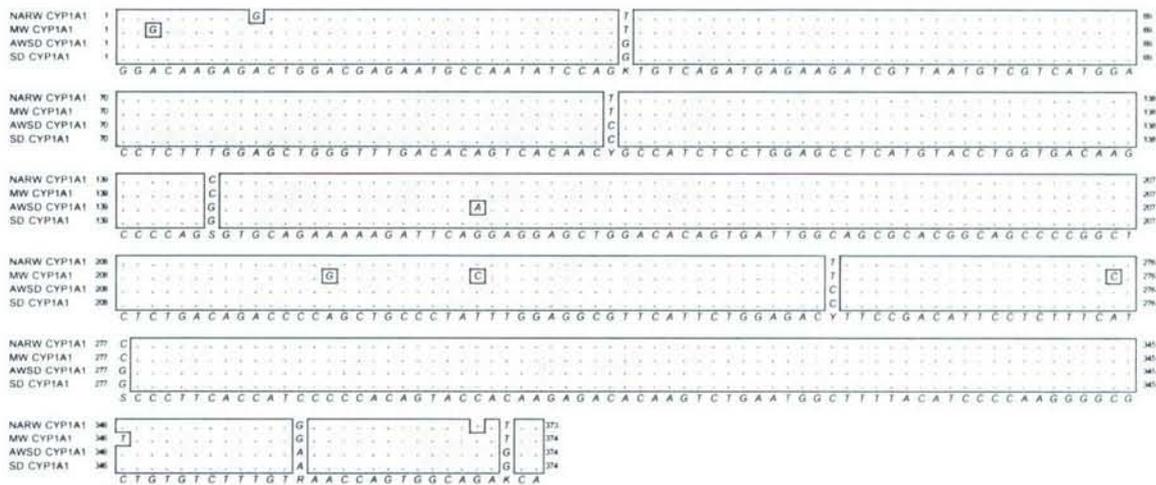


Figure 4. Partial cytochrome P450 1A1 cDNA isolated from NARW biopsy.

A. Two products were amplified from NARW mRNA using CYP1A1 primers (~370 and ~700 bp; standard PCR: 40 cycles, 0.5 μ M each primer). The smaller band is indicated by the arrow. Human placental cDNA was used as a control; however, the degeneracy of one primer precluded binding and amplification.

B. A BLAST search identified the sequence of the smaller NARW band as CYP1A1; the larger band contained an intron (Altschul *et al.* 1997). NARW CYP1A1 nucleotide sequence (GenBank Accession #EF091834) is shown aligned with partial CYP1A1 sequences from minke whale (MW), AWSD, and striped dolphin (SD); GenBank Accession #A231891, AY641536, and AF235141, respectively).

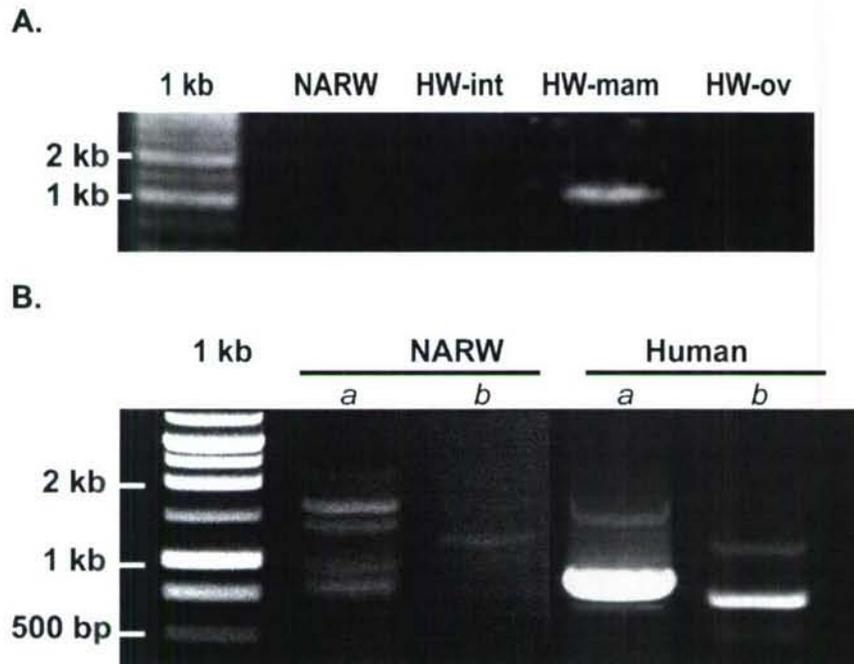


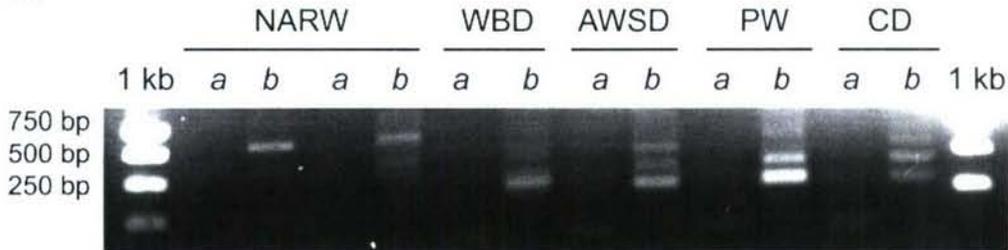
Figure 5. Estrogen receptor alpha and CYP19 PCR reactions: mysticete and human cDNA.

A. Humpback whale (HW) mRNA isolated from mammary tissue (HW-mam), but not that from integument, ovary, or NARW mRNA, produced a faintly visible PCR product using primers designed to amplify a large portion of ER α (1.5 kb expected size; 1.0 μ M, F1-ER-a/R1-ER-a). The identity of this fragment has not yet been verified, and further efforts to clone ER α and ER β from NARW and HW are in progress.

B. Two pairs of primers were used in separate PCR reactions to amplify NARW and human CYP19 cDNA using Advantage 2 polymerase (*a*: CYP19 2-23f/CYP19 810-830r, 830 bp expected size; *b*: CYP19 672-691f/CYP19 1353-1373r, 700 bp expected size; 1.0 μ M each). Sequencing of two NARW bands using the first pair of primers (~800 and ~900 bp) yielded non-specific results.

A and **B** each represent single gels, but image enhancement was used to better illustrate bands for viewing purposes in cases where bands in some lanes were much brighter than in others (i.e. CYP19: human \gg NARW), or when bands were only faintly visible (HW ER α).

A.



B.

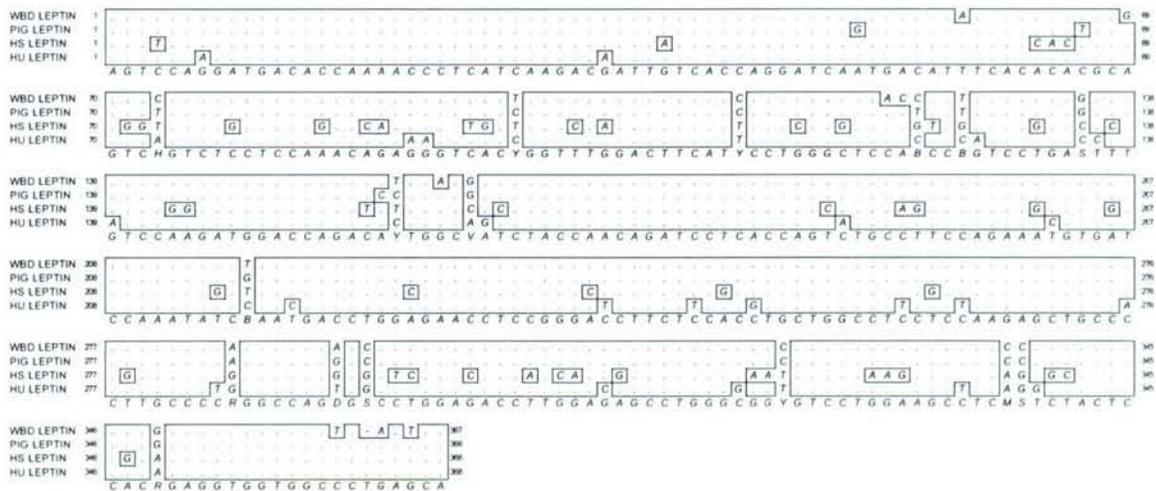


Figure 6. Leptin is expressed in white-beaked dolphin integument.

A. Two sets of primers were used to amplify leptin from cetacean cDNAs (a: OB-F1/OB-R1; b: OB S2/OB AS2; 1.0 μ M each), but only the latter pair produced visible products. NARW PCR products were larger than expected and were found to be non-specific when sequenced. Delphinid PCR products were all of expected size (~360 bp).

B. The WBD band was verified by sequencing and a BLAST search as a partial leptin cDNA (Altschul *et al.* 1997). The WBD nucleotide sequence (GenBank Accession #EF091835) is shown aligned with partial leptin sequences from pig, harbor seal (HS), and human (HU)(GenBank Accession #NM_213840, AJ618981, and NM_000230, respectively).

Chapter 3.

Molecular and Biochemical Characterization of Aryl Hydrocarbon Receptors in North Atlantic Right Whale (*Eubalaena glacialis*) and Humpback Whale (*Megaptera novaeangliae*)

ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that binds with some environmental contaminants and regulates gene expression and physiological responses. To investigate the hypothesis that North Atlantic right whales (*Eubalaena glacialis*, NARW) are highly susceptible to chemical pollutants such as polycyclic aromatic hydrocarbons (PAH) and PCBs, RNA prepared from NARW integument biopsies was used to clone and sequence a cDNA encoding AHR. A predicted 850 amino acid protein (95.8 kD) was identified as NARW AHR. An AHR cDNA was also cloned from a sympatric species, humpback whale (*Megaptera novaeangliae*; HW) using the same techniques. Pairwise ClustalW analysis of the predicted amino acid sequences revealed 94-97% identity among sequenced cetacean AHRs. Specific binding of *in vitro* expressed NARW and HW AHR proteins to a prototypical AHR ligand ($[^3\text{H}]$ TCDD; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was shown by velocity sedimentation using sucrose density gradients. Although the mean relative binding affinities of mouse and human AHRs were statistically different ($p < 0.05$; ANOVA/Tukey's post-hoc), whale AHRs did not statistically differ from that of either the high-affinity mouse AHR^{b-1} ($K_D = 0.36$ nM), or the lower affinity human AHR ($K_D = 0.97$ nM) in saturation binding assays. Whale AHRs appeared to have intermediate affinities for $[^3\text{H}]$ TCDD ($K_D = 0.64 - 0.85$ nM), but larger binding capacities (B_{max}) than mouse and human AHR proteins, which may be related to increased whale AHR protein expression in this assay. Whale AHRs could activate transcription of a reporter gene in a mammalian cell culture system transiently transfected with AHR constructs, independent of TCDD treatment. Benzo[*a*]pyrene treatment induced ligand-dependent reporter gene expression for beluga and mouse AHRs in a transient transfection assay, offering preliminary evidence for combined ligand- and species-specific activity for mammalian AHRs.

1. INTRODUCTION

North Atlantic right whales are critically endangered, with a population size considerable smaller than that of other baleen whales. Despite a 70-year respite from commercial whaling, the species has failed to recover and may be declining. Recent estimates of population growth rate range from -0.02% to 1% (Caswell *et al.* 1999); Tim Frasier, personal comm.) yet do not approach that of the southern right whale species that appears healthy (7%; source). Collisions with ships and entanglement in fishing gear cause the direct mortality of NARWs, and have proportionally larger impacts on pregnant and lactating females and their neonates. However, other causes of the species' failure to recover may include nutritional stress due to variations in prey availability, reproductive effects of exposure to marine biotoxins or anthropogenic chemical contaminants, and inbreeding suppression brought on by the extreme population reduction caused by early 20th century whaling.

Concern has grown among conservationists and scientists over the levels of persistent anthropogenic contaminants present in the marine environment, including pristine areas far from point sources of industrial waste products. Some classes of contaminants, dioxin-like compounds (DLC) such as chlorinated dibenzo-dioxins, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs), can cause a variety of non-lethal pathologies at relatively low but chronic doses. Associations between hormonal and morphological disorders at all age classes and many stages of gestation have been observed in many cetacean and pinniped species (Reijnders 2003). However, it is often difficult to clearly determine whether these pathologies in wildlife are the results of contaminant exposure because: a) many species are not well studied and the background incidence of these effects is unknown, b) species-specific differences in doses required for toxicity are unknown, and in some cases impossible to determine using standard methods, and c) interaction of the multitude of environmental and natural chemicals evades simple explanations.

Another approach is required to assess the potential impacts of DLC on endangered wildlife species.

Most vertebrate species possess a mechanism for detection and metabolism of DLC. The aryl hydrocarbon receptor is a cellular protein that binds DLC, and through its action as a transcription factor, activates the transcription of genes encoding a suite of metabolic enzymes and other genes. While in some cases this results in the detoxication of the chemical, in others the chemical's toxicity is increased via reactive intermediates. The rate of clearance and the dose and duration of exposure can also affect the degree of toxicity experienced by an organism. The genetic background, and in fact species-specific differences in AH receptor sequence, can dramatically influence the sensitivity of an organism to DLC. Mice, rats, and birds show AHR sequence-specific differences that relate to sensitivity (Karchner *et al.* 2006; Pohjanvirta *et al.* 1998; Poland *et al.* 1994). The AHR in one cetacean, the beluga whale, has been studied using *in vitro* bioassays to determine its affinity for DLC, structure-activity relationships, and tissue-specific expression patterns (Jensen 2000).

Using methods similar to those employed for the investigation of beluga whale (BW), we assess the sensitivity of the NARW by identifying and biochemically characterizing the AHR in comparison to BW and a sympatric mysticete, the humpback whale (HW; *Megaptera novaeangliae*). We establish the relative binding affinity of AHRs from these cetaceans in comparison with representative high- and low-affinity AH receptors from mouse and human, respectively. The transcriptional activities of these receptors are also examined in a cell culture system to approximate the *in vivo* condition. Given the constraints on systemically investigating contaminant effects in large, endangered cetaceans, we show the efficacy and limitations of using *in vitro* molecular biology and biochemistry techniques to approximate the toxicological effects of DLC on cetaceans.

2. METHODS

2.1 *Cloning and Sequencing Mysticete AHRs*

Biopsy samples collected from two live NARW, and dissection of a stranded HW provided integument tissue to determine AHR sequence in these species (described in detail in Chapter 2). Messenger RNA (mRNA) was isolated and purified from NARW and HW samples (RNA STAT-60; MRNA kit), and used for cDNA synthesis with oligo d(T) primers and Omniscript RT polymerase (SOURCE). Another pool of NARW cDNA was synthesized using Thermoscript RT (Invitrogen) and a gene specific primer (MM3r).

An initial fragment of NARW AHR was amplified from Omniscript cDNA by polymerase chain reaction (PCR) using degenerate primers (described in detail in Chapter 2). Gene specific primers were designed from this fragment for Rapid Amplification of cDNA Ends (RACE) (Table 1). Degenerate primers were also designed based on alignments of AHR fragments from NARW, white-beaked dolphin (GenBank Accession (GBA) # EF091831), Atlantic white-sided dolphin (GBA # EF091832), pilot whale (GBA # EF091833), mink, and polar bear (Chapters 2 and 4).

SMART RACE (Clontech) was used according to manufacturer's instructions to synthesize two separate pools of cDNA from NARW mRNA: 5' RACE Ready and 3' RACE Ready cDNAs. 5' AHR RACE product was amplified from 5' RACE Ready cDNA with a degenerate primer (MM3r, 100 μ M) and universal primer mix (UPM; supplied with the SMART RACE kit). PCR conditions were as follows: 5 cycles: 94°C/5 sec, 72°C/3 min; 5 cycles: 94°C/5 sec, 70°C/3 min; 25 cycles: 94°C/5 sec, 68°C/10 sec, 72°C/3 min; 72°C/3 min; rapid cooling to 4°C. A nested reaction was applied to this product using a specific primer (RW-1r, 10 μ M) and nested universal primer mix (NUPM; SMART RACE). Nested PCR conditions were as follows: 20 cycles: 94°C/5 sec, 68°C/10 sec, 72°C/3 min. Five additional primers were designed and used to amplify the NARW AHR 5'

sequence (not shown). Synthesis of 5' RACE Ready cDNA was repeated using a gene specific primer (MM3r) in lieu of the CDS primer supplied by the manufacturer to enrich for AHR transcripts, and this cDNA was subsequently used with RW-1r and UPM in a RACE PCR.

3' RACE was conducted under identical PCR conditions, using 3' RACE Ready cDNA, first with RW-1f (10 μ M) and UPM, followed by a nested PCR with MM-4F (10 μ M) and NUPM. A primer specific to the NARW 3' UTR was designed for full-length amplification (RW AHR-1R).

NARW cDNA synthesized with Thermoscript RT was used with the Marathon RACE kit (Clontech), following the manufacturer's instructions. RACE PCR was conducted with this cDNA using AP1 and MM3r (100 μ M) primers, and Advantage 2 polymerase with the following protocol: 94°C/30 sec; 5 cycles: 94°C/5 sec, 72°C/2 min; 5 cycles: 94°C/5 sec, 70°C/2 min; 25 cycles: 94°C/5 sec, 68°C/2 min; 68 °C/3 min; rapid cooling to 4°C. A nested PCR reaction was applied to this product using AP2 and MM2r primers: 94°C/30 sec; 20 cycles: 94°C/5 sec, 69°C/2 min; 68°C/5 min; rapid cooling to 4°C.

Full-length sequence was amplified from NARW 3' RACE Ready cDNA with a primer designed for beluga AHR (Bel-nat), RW AHR-1R, and the first PCR protocol above. Humpback whale AHR was identified in a single full-length PCR reaction with the same gene specific primers using cDNA synthesized with Omniscript RT polymerase. Thermocycling conditions were as follows: 94°C/30 sec; 5 cycles: 94°C/5 sec, 72°C/4 min; 5 cycles: 94°C/5 sec, 70°C/4 min; 30 cycles: 94°C/5 sec, 68°C/4 min; rapid cooling to 4°C.

All PCR fragments and full-length AHR cDNAs were gel purified (Gene Clean), ligated into the pGEM-T Easy plasmid vector (Promega), and propagated in *E. coli* JM109 cells. Plasmid DNA was isolated from 6 colonies per fragment using the boiling-lysis method (Sambrook *et al.* 1989). Inserts were independently verified by restriction endonuclease digestion (EcoRI; Promega). Two to six positive clones were sequenced bi-directionally to obtain consensus

sequences. RACE products were sequenced using M13 primers and Big Dye Terminator Ready Mix with standard parameters, purified using G-50 Sephadex with Centri-Sep columns or isopropanol precipitation, and visualized on an ABI 377 DNA sequencer (Applied Biosystems). Full-length products were sequenced by the University of Maine DNA Sequencing Facility (Orono, ME). Sequences were manually edited using Sequencher 4.2.2 (Gene Codes Corp.) and MacVector 8.1.1 (Accelrys, Inc.) software, and examined by NCBI/GenBank tBLASTx for gene identification (Altschul *et al.* 1997). Predicted amino acid sequences were determined for each sequence, and aligned with known cDNA sequences from other species using Clustalw in MacVector 8.1.1 (beluga whale AHR GenBank Accession #AF332999; human GenBank Accession #L19872.1; mouse AHR GenBank Accession #M94623).

2.2 *Expression Vector Construction*

Two sets of primers were designed to correct a PCR error in a NARW clone (RW5; full-length sequence) using the Quik Change XL Site-Directed Mutagenesis (SDM) kit (Stratagene). The first attempt failed to amplify plasmid DNA using 10 ng of RW5 plasmid DNA, and RW C2261A-F and RW G2261T-R primers following manufacturer's instructions and the following thermocycling conditions: 95°C/1 min, 18 cycles: 95°C/50 sec, 60°C/50 sec, 68°C/5.5 min; 68°C/7 min; rapid cooling to 4°C. Template DNA (RW5) was increased to 50 ng in a second SDM reaction and modified thermocycling conditions: 95°C/2min, 18 cycles: 95°C/1min, 60°C/50 sec, 68°C/11 min; 68°C/11 min; rapid cooling to 4°C. A final SDM reaction used RW C2261A2-F and RWG2261T2-R primers and thermocycling conditions as follows: 95°C/2min, 18 cycles: 95°C/1min, 60°C/50 sec, 68°C/15.5 min; 68°C/15.5 min; rapid cooling to 4°C. Amplified plasmids were cloned and propagated in XL10-Gold cells and sequenced by the University of Maine DNA Sequencing Facility.

Full-length PCR products of NARW AHR and HW AHR were initially cloned and sequenced in pGEM-T Easy vectors. The NARW AHR was subcloned into the *HindIII* and *NotI* sites of the pcDNA 3.1 expression vector (Invitrogen). A chimeric AHR was constructed from two HW clones by endonuclease digestion (*XmnI/HindIII* or *XmnI/ApaI*) and ligation of purified error-free fragments, then subcloned in pcDNA at the *HindIII* and *ApaI* sites. Correct orientation and sequence were confirmed by full-length sequencing of the final constructs. The beluga whale (BW) AHR expression vector (pcDNA) was previously constructed in this laboratory using similar methods (Jensen 2000; Jensen and Hahn 2001). Expression constructs for the human AHR (phuAHR; (Dolwick *et al.* 1993a) and mouse AHR (pmuAHR; (Dolwick *et al.* 1993b) were cloned in pSPORT vectors and graciously provided by Dr. C. Bradfield (McArdle Center for Cancer Research, Madison, WI). Human (HU) and mouse (MO) AHR were subcloned into pcDNA vectors by Diana Franks to assess differences in protein expression related to expression vector, but the original constructs (pSPORT vector) were used in the ligand binding assays shown here.

2.3 *In vitro* Protein Synthesis

In vitro transcription and translation (IVTT) of NARW, HW, BW, HU, and MO AHR proteins was performed using the TNT Quick Coupled Reticulocyte Lysate Systems following the manufacturer's protocols (T7: NARW, HW, BW; SP6: HU, MO; Promega). [³⁵S]-labeled methionine was added to IVTT reactions for visualization and quantification of protein synthesis by SDS-PAGE and autoradiography. [³⁵S]-labeled proteins were excised from dried gels and measured by liquid scintillation with a Beckman LS5000TD scintillation counter to quantify protein expression (~5 µL IVTT reaction per band). Unlabeled methionine was used to synthesize IVTT proteins for ligand binding assays.

2.4 *Velocity Sedimentation*

IVTT expressed AHR proteins from NARW, HW, BW, HU, and MO were analyzed by velocity sedimentation on sucrose gradients in a vertical tube rotor (Tsui and Okey 1981). IVTT reactions (50 μ L) were diluted 1:1 with MEEDMG buffer (25 mM MOPS pH 7.5 containing 1 mM dithiothreitol, 1 mM EDTA, 5 mM EGTA, 0.02% NaN₃, 20 mM Na₂MoO₄, 10% (v:v) glycerol, plus a mixture of protease inhibitors (Hahn *et al.* 1994)). AHR proteins and unprogrammed lysate (UPL) were incubated with 2 nM [³H]TCDD for 18 hours at 4°C in glass culture tubes. Final [³H]TCDD concentration was verified by sampling 5 μ L from each tube for total counts. After incubation, 95 μ L from each tube plus 3 μ L sedimentation markers ([¹⁴C]ovalbumin and [¹⁴C]catalase) were applied to 10-30% sucrose gradients prepared with MEEDMG buffer (Coombs and Watts 1985). The gradients were centrifuged at 60,000 rpm (VTi 65.2 rotor) for 140 minutes at 4°C. Fractions (~150 μ L each) were counted using a Beckman LS5000TD scintillation counter. Specific binding was calculated as the difference between total binding and non-specific binding (UPL). The sedimentation coefficient for each AHR was calculated based on the elution patterns of the markers ([¹⁴C]ovalbumin in fractions 3-4 (3.6 S) and [¹⁴C]catalase in fractions 14-16 (11.3 S)) (Martin and Ames 1961).

2.5 Saturation Binding Assays

We slightly modified the protocol developed by Jensen (2000, 2001; based on (Poland and Glover 1976)) to determine the specific binding of [³H]TCDD for IVTT-expressed AHR proteins. IVTT proteins were synthesized with unlabeled methionine in two 50 μ L reactions, combined, then diluted 1:9.5 in MEEDMG with protease inhibitors. Diluted proteins (105 μ L) were incubated in glass culture tubes with one of nine or ten concentrations of [³H]TCDD (0 – 10 nM, nominal) in DMSO at 4°C. Each tube was sampled (5 μ L) for total counts at the beginning of the incubation. After 18 hours of incubation, 28 μ L aliquots were transferred to triplicate 1.5 mL polypropylene tubes with equal volumes of 2.3

mg/mL dextran-coated charcoal (DCC). Tubes were vortexed three times for 5 seconds at 5 minute intervals. DCC was sedimented by centrifugation at 7000*g for 3 minutes to separate free ligand from protein-bound [³H]TCDD. Supernatant containing protein-bound [³H]TCDD was removed (45 μL) and measured in the Beckman LS5000TD scintillation counter. NARW, HW, BW, HU, and MO IVTT AHRs plus UPL (empty pcDNA vector) were included in each of five assays.

Radioactivity from SBA/DCC samples was detected by scintillation counting for 5 minutes in each replicate. However, we questioned the accuracy of this measurement, especially for small concentrations of [³H]TCDD for which the detection error approached 20%. We compared measurements of the same samples read for 5, 10, and 20 minutes, and determined that longer counts increased precision but did not change accuracy (data not shown).

Binding curves were plotted as free (unbound) versus bound [³H]TCDD (nM). Disintegrations per minute were all converted to nM: [(dpm – background)/specific activity of [³H]TCDD]/volume per sample. Total binding was determined by taking the mean of each triplicate counted for each concentration of [³H]TCDD. Free [³H]TCDD was calculated by subtracting the total bound [³H]TCDD from the total [³H]TCDD (total counts) for each tube. Non-specific binding for each concentration of [³H]TCDD was determined from the slope of the line fit to UPL data. Specific binding was calculated as the difference between total binding and non-specific binding. The affinity of each receptor for [³H]TCDD (equilibrium dissociation constant, K_d) and maximal binding (B_{max}) were determined by non-linear regression assuming one-site binding in Prism 4.0 (GraphPad).

2.6 Transfection Assays

COS-7 (monkey kidney) cells have been shown to lack endogenous AHR expression, and only minimally express ARNT (Ah Receptor Nuclear

Translocator) (Ema *et al.* 1994a; Ema *et al.* 1994b). We transiently transfected COS-7 cells with an AHR construct (NARW, HW, BW, HU, or MO AHR), human ARNT, a firefly luciferase reporter construct (pGudLuc6.1), and a *Renilla* luciferase control plasmid (pRL-TK). The pGudLuc6.1 plasmid contains the firefly luciferase gene with an MMTV promoter under the control of dioxin response elements derived from the mouse *Cyp1a1* gene (Long *et al.* 1998). A total of 300 ng of plasmid DNA was cotransfected: 50 ng ARNT, 20 ng pGudLuc6.1, 3 ng pRL-TK, AHR (NARW: 0.5, 1, 5, 10, or 25 ng; HW: 0.5, 1, 5, 10, or 25 ng; BW: 0.5, 1, 5, 10, or 25 ng; HU: 50 ng; MO: 25 or 10 ng), and empty vector pcDNA3.1 (177 – 227 ng).

Cell culture and transfection were performed in 48-well plates, with 40,000 cells/well in complete medium (Eagle's Medium with fetal bovine serum). After 24 hours of growth, the medium was replaced with serum-free medium and the cells transfected with Lipofectamine 2000 (Gibco) according to manufacturer's directions. Five hours later, cells were dosed with DMSO, TCDD in DMSO (10 nM final concentration), or benzo[a]pyrene in DMSO (BaP; 10 μ M final concentration), and incubated for 18 hours. Cells were collected and lysed, and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) and a Turner TD-20/20 luminometer (Turner Designs). Relative luciferase units (RLU) were determined by the ratio of emission of firefly versus *Renilla* luciferase in the presence of substrate. Data were analyzed by two-way ANOVA in Prism 4.0.

3. RESULTS

3.1 NARW and HW Aryl Hydrocarbon Receptor Cloning

An initial fragment of AHR cDNA was obtained by RT-PCR using degenerate primers and mRNA derived from NARW biopsies (Chapter 2). We acquired additional 5' coding sequence from NARW 5' RACE Ready cDNA

synthesized from integument mRNA (SMART RACE; MM3r/UPM, then nested RW1r/ NUPM); however, reactions failed to progress 5' of nucleotide 55 (coding valine 19). Other researchers have had similar difficulties with 5'-RACE extension in the same region of fish AHR cDNAs that were overcome using adaptor-ligated cDNAs made from heat-denatured RNA, and high temperature polymerases like Thermoscript RT (Karchner *et al.* 1999). However, these methods were unsuccessful for NARW AHR. The region 3' of the initial fragment, including a short UTR and polyadenylation signal, was identified using 3' RACE Ready cDNA and specific primers generated from the initial fragment (RW-2f/UPM then nested MM-4-F/NUPM). RACE reactions with Thermoscript RT-derived cDNAs and Marathon RACE did not yield any visible products.

We designed a NARW AHR-specific reverse primer (RW AHR-1R) and used it with a forward primer specific to the beluga whale AHR (Bel-nat) to amplify the full-length NARW AHR cDNA. The Bel-nat primer includes the first nine coding bases from BW AHR, all of which are highly conserved in mammalian AHRs (identical nucleotides in beluga, human, and guinea pig; a single difference in rabbit AHR). This primer combination resulted in full-length amplification of AHR from NARW 3' RACE Ready cDNA (SMART RACE), as well as from HW cDNA (Omniscrypt). Amplicons were gel extracted, purified, cloned, and multiple clones (6) were sequenced bi-directionally. Consensus sequences for NARW and HW AHR cDNAs were determined by alignment of clones. An open reading frame (ORF) encoding 850 amino acids (95.8 kD predicted mass) was identified for NARW AHR (Figure 1; GenBank Accession #EF091829). The HW AHR was similar, with an 851 amino acid ORF (95.9 kD; Figure 2; GenBank Accession #EF091830).

Amino acid sequences for NARW and HW AHRs were aligned with those from BW (Jensen and Hahn 2001), human (Dolwick *et al.* 1993a), and mouse (Burbach *et al.* 1992) using ClustalW in MacVector 8.1.1 (Figure 3). The high degree of amino acid conservation throughout the entire coding region enabled

the identification of functional domains established from studies of mouse AHR (Burbach *et al.* 1992; Dolwick *et al.* 1993b; Fukunaga *et al.* 1995; Poland *et al.* 1994). Pairwise comparisons of nucleotide and amino acid sequences for each AHR are shown in Table 2. The basic helix-loop-helix (bHLH) region is very highly conserved among these mammalian AHRs (95.6 – 100% amino acid identity), and contains a nuclear localization signal important for binding co-factors (HSP90 and ARNT) as well as DNA response elements (Ikuta *et al.* 1998). The PAS region is a defining feature of proteins in this family (Period-ARNT-Single-minded) and encompasses much of the AHR ligand binding domain (LBD). Whale AHRs share 98.3% amino acid identity in the PAS, and slightly less conservation is observed between whales and human or mouse (86.9 – 92.1%). Only three residues within the LBD differ among whales (K/R 250; M/L 347; & M/L 399). A high level of conservation is also seen among whale AHRs in the carboxy-terminus encoding the transactivation domain (defined here as amino acid 490 (rel. to mouse AHR) to the stop codon): ~89% amino acid identity in the TAD, compared with ~74% whales to human, and ~56% whales to mouse. An extensive analysis of amino acid sequence differences and their putative functional effects in marine mammal versus other mammalian AHRs are discussed in Chapter IV.

3.2 Expression Vectors

Sequence alignments from multiple clones also identified potential PCR errors in each clone. A HW AHR construct of the consensus sequence was made from two clones by endonuclease digestion, purification, and ligation of error-free sections, then subcloning in an expression vector (pcDNA).

The same method was not possible for NARW AHR because of the locations of errors relative to restriction sites. A NARW AHR clone with a single PCR error in the third position of a codon (C2261 rather than consensus A) resulted in a non-synonymous but similar amino acid (glutamine (Q) at position

749 rather than lysine (K)). Site-directed mutagenesis was attempted to alter the sequence to match the NARW AHR consensus. The first SDM reaction failed to amplify. Amplification proceeded in our second SDM, but a short inverted repeat within the primer sequence resulted in an insert of three concatenated primer sequences in the plasmid. We redesigned the primers to remove this motif, but amplification was unsuccessful.

We decided to proceed with biochemical assays using the NARW AHR construct with the single error; first, because of its location in the more variable C-terminus. Second, the side chains in lysine and glutamine are both polar and tend to be oriented on protein surfaces. Finally, lysine and glutamine have similar molecular weights (128.18 and 128.14, respectively), which in total limits the effect of this substitution. Although large-scale deletions or insertions have been shown to affect the transcriptional activity in other AHRs (Korkalainen *et al.* 2000), we do not believe the single point mutation present in our NARW AHR construct has an impact on transcriptional activation. While the possibility remains that amino acid 749 is important to recapitulate NARW AHR function, the data from transient transfections in COS-7 cells shows similar activity for NARW and HW AHRs (below, section 3.6). Further work to correct this error in the plasmid and replication of biochemical function is necessary to confirm this assertion.

3.3 Characterization of *in vitro* Expressed Whale AHR

AHR constructs were expressed using a cell-free *in vitro* transcription and translation system (IVTT). Whale AHR constructs were under the control of the T7 promoter, whereas human and mouse AHRs used the SP6 promoter. Protein expression was confirmed by autoradiography of SDS-PAGE separated IVTT reactions including [³⁵S]-labeled methionine (Figure 4A). Gel separation was consistent with predicted molecular masses of 95.8, 95.9, 95.4, 96.1, and 90.3 kDa for NARW, HW, BW, HU, and MO AHR, respectively. Bands were excised

and counted (disintegrations per minute; dpm) to quantify protein expression. Variation in protein levels was compared in four independent IVTT reactions. Whale AHRs were consistently expressed at higher levels than human or mouse AHRs; this may have been a consequence of vector or promoter differences (whale AHRs in pcDNA (T7); human and mouse in pSPORT (SP6); Figure 4B). However, when subcloned into pcDNA, expression of human and mouse AHRs remained lower than that of whale AHRs (Figure 4C). This may be explained by differences in post-translational modifications, or sequence-specific differences that affect the processivity of the IVTT system.

3.4 Specific Binding of [³H]TCDD to Whale AHRs

The ability of IVTT expressed AHRs to specifically bind [³H]TCDD (2nM) was determined by velocity sedimentation on sucrose gradients (Figure 5). Specific AHR binding was observed with peaks between fractions 10 – 19, and was differentiated from non-specific protein binding by comparison with unprogrammed lysate (UPL; IVTT with empty pcDNA vector). UPL has previously been shown to lack specific binding to [³H]TCDD in competition binding experiments (Jensen and Hahn 2001; Karchner *et al.* 1999). NARW and HW AHRs sedimented with peaks at 10.7S and 10.0S, respectively. Beluga whale, human, and mouse AHRs sedimented with peaks at 11.3S, 10.7S, 10.7S, respectively. Differences in specific binding maxima may be related to differences in the amount of protein per reaction as observed above, but also may reflect species-specific differences in ligand binding.

3.5 Relative Binding Affinities of Right Whale, Humpback Whale, Beluga Whale, Human, and Mouse AHRs by Saturation Binding Analysis

We sought to compare the relative binding affinities of NARW and HW AHRs with high and low affinity AHRs from other species. Previous studies have described the binding characteristics of the beluga, human, and mouse AHRs

(Burbach *et al.* 1992; Dolwick *et al.* 1993a; Jensen and Hahn 2001; Poland *et al.* 1994). Jensen and Hahn (2001) reported that the mouse (AH^{b-1} allele) and beluga whale have high affinity AHRs in comparison with the human. We used the modified saturation binding assay with dextran-coated charcoal (SBA/DCC) established by Jensen (Jensen and Hahn 2000; Jensen and Hahn 2001) to determine binding affinities of NARW, HW, BW, HU, and MO AHRs synthesized by IVTT (Figure 6).

Specific binding was calculated as the difference between total binding and non-specific binding (UPL). Nonlinear regression analysis, equilibrium dissociation constants (K_d), and maximum binding (B_{max}) were derived assuming one-site specific binding (Prism 4.0). To determine the optimal time of incubation we compared binding constants calculated from 2 hours and 18 hours incubation of NARW AHR with [³H]TCDD. Longer incubation time resulted in slightly higher maximal binding, but was similar to that from 2 hours incubation (Figure 7). We decided that 18 hours incubation was both logistically easier and most likely to capture the receptor behavior in the assay (whether kinetic or dynamic). Ultimately, binding constants and maximum binding derived from 2 and 18 hour incubations were within one standard deviation of the means from five replicate assays conducted at 18 hours (Table 3). These results indicated that if logistical constraints limited the length of the experiment, similar results could be achieved with shorter incubations; however, we recommend a time course of assays be conducted before using a shorter incubation time on AHRs of unknown binding function.

The means of five independent SBA/DCC, each with all five AHRs, are shown in Table 3. Differences in B_{max} are correlated with the amount of AHR protein in each assay (Figure 8; $r^2=0.86$). However, the differences in binding observed among the cetacean AHRs are not related to differences in AHR expression, and may be a function of post-translational processing, or species-specific differences in amino acid sequence and function. When normalized for

protein content, maximum binding is similar across all AHRs investigated (Table 3, B_{\max} norm).

3.6 *Transcriptional Activation of Reporter Genes by Whale AHR*

We performed transient transfection assays to investigate the transcriptional activities of NARW and HW AHRs. Mammalian cells deficient in endogenous AHR (COS-7) were cotransfected with an AHR construct, human ARNT, a firefly luciferase reporter construct (pGudLuc6.1), and a *Renilla* luciferase control plasmid (pRL-TK). TCDD induced reporter-gene activity with the MO AHR in all assays (≥ 2.5 -fold; $p < 0.001$), but HU AHR had no effect on reporter gene transcription, and luciferase activity was indiscernible from empty vector controls both with and without TCDD. Our laboratory has not observed measurable transcriptional activity with this HU AHR construct in transient transfections, but the source of this inactivity has yet to be determined. Other human AHR constructs are now being tested.

Whale AHRs were transcriptionally active in the presence and absence of TCDD. NARW, HW, and BW AHRs produced greater reporter-gene expression than MO AHR at the same plasmid concentration when treated with either DMSO or TCDD (25 ng transfected; Figure 9A). No significant increase in reporter gene expression was seen with whale AHRs.

Luciferase activity was related to the concentration of transfected AHR. AHR was titrated from 0.5 – 50 ng to establish the optimum conditions of detection to distinguish background from induced luciferase reporter expression. Luciferase activity for the highest concentration of AHR (50 ng) for whale, but not human AHR, exceeded the upper limits of detection for the luminometer. Even lower concentrations of whale AHR produced luciferase units above the upper range of measurement, necessitating a ten-fold reduction in the standard volume read on the luminometer. As little as 0.5 ng whale AHR was sufficient to induce reporter-gene transcription, independent of ligand. Still, there was no difference

in the transcriptional activation of whale AHRs with or without TCDD. Although high concentrations of transfected plasmid can cause constitutive reporter gene activation, reduction of whale AHRs did not eliminate constitutive expression.

While whale AHRs were not sensitive to TCDD under the conditions tested, BaP induced luciferase transcription 2.4-fold with BW AHR (1.0 ng transfected only; $p < 0.001$) and 1.8-fold with MO AHR ($p < 0.05$) in a single experiment (Figure 9B). The high variation observed in these data warrants additional experiments to confirm reporter gene induction by BaP; however, these preliminary data suggest that there might be ligand-specific differences in transcriptional activity of whale AHRs.

4. DISCUSSION

Direct mortality from violent interactions with ships and fishing gear remains the primary conservation focus for NARW. A population growth model suggested that saving the lives of just 2 adult females annually could reduce the risk of species extinction currently predicted in about 200 years (Fujiwara and Caswell 2001). However, there is a body of evidence suggesting that NARW may be chemically stressed, potentially contributing to the slow population growth rate by disrupting reproduction. Our principle objective was to determine if NARW possess species-specific characteristics that make them particularly sensitive to the effects of chemical contaminants in the marine environment. Specifically, we questioned whether environmental contaminants might influence the reproductive function of the species, in light of the variable fecundity observed in recent decades. To approach this question, we first sought to determine if NARW have mechanisms to detect and respond to environmental pollutants that exert toxicological effects via the AHR-mediated pathway.

In a previous study (Chapter 2), we showed that NARW express AHR and CYP1A1 in superficial integument, and are thus capable of producing a response

to AHR ligands such as PHAH and PAH. Laboratory studies inform our understanding of species-specific sensitivity to AHR agonists, showing a causal relationship between *in vitro* ligand-binding affinity and *in vivo* sensitivity (Ema *et al.* 1994b; Karchner *et al.* 2006; Poland *et al.* 1994; Ramadoss and Perdew 2004). Two early experiments found that inbred strains of mice with low affinity receptors (AH^d allele) were less sensitive to TCDD, but those with a high-affinity receptor (AH^{b-1} allele) were highly susceptible to the physiological effects of the chemical (Birnbaum *et al.* 1990; Poland *et al.* 1994). Similar types of experiments are rarely possible with marine mammals, and are impossible for large baleen whales. Jensen and Hahn (2001) established that beluga whale AHR function could be investigated using an *in vitro* biochemical assay thereby permitting controlled experimentation for a class of animals otherwise difficult to study. Given the range of species-specific responses, we sought to characterize the *in vitro* biochemical function of NARW AHR in comparison with representative high- and low-affinity AHRs to determine where it may be on the continuum of ligand binding affinity, and presumable organismal sensitivity.

4.1 Whale aryl hydrocarbon receptor sequences are highly conserved

NARW, HW, and BW AHRs share a high degree of amino acid sequence identity. The conservation seen among whales, human, and mouse AHRs also indicates that functional domains identified in the latter two species are likely to have similar functions in the whale AHRs. The amino-terminal end of the AHR protein contains the bHLH, PAS A, and PAS B sequences that are highly conserved in AHR proteins. Conversely, large sequence divergence has been noted for the carboxy-terminal AHR region (Dolwick *et al.* 1993a; Jensen and Hahn 2001; Ramadoss and Perdew 2004), but for the three whale species this region is exceptionally similar (~89%). While striking at first, these data conform to expectations based on evolutionary divergence of the Cetacea. The first

cetaceans evolved approximately 50 million years ago (mya) (Fordyce and Barnes 1994; Gingerich *et al.* 2001), and combined morphological and molecular data sets place the divergence of mysticetes and odontocetes between 19 and 34 mya (Arnason and Gullberg 1996; Messenger and McGuire 1998; Milinkovitch 1995); balaenopterids, including HW, only diverged in the last 7 my (Deméré *et al.* 2005). For comparison, primates and rodents diverged approximately 96 mya (Nei *et al.* 2001), and human and mouse AHRs share only 68% amino acid identity, whereas chicken and tern diverged 82-90 mya and maintain 92% identical residues throughout the AHR (Karchner *et al.* 2006). The few differences that exist between cetacean AHRs at the amino acid sequence level are analyzed in detail in Chapter IV.

4.2 Mysticete AHRs bind dioxin with high affinity

In this study we systematically compared the biochemical functions of two newly identified mysticete AHRs and a previously characterized odontocete AHR, with those from human and mouse. The mouse Ah^{b-1} allele used here has been shown to have a high ligand binding affinity (Poland *et al.* 1994). The human AHR represents a low-affinity form with an approximately 4-10 fold lower affinity than the mouse AHR (Ema *et al.* 1994b; Harper *et al.* 1988; Poland *et al.* 1994; Ramadoss and Perdew 2004). A statistical difference in K_d was only detected between human and mouse AHRs. NARW and HW AHRs both specifically bind to TCDD with a relative affinity intermediate to those of mouse and human AHRs. The beluga whale AHR was previously shown to have a TCDD-binding affinity that was significantly higher than that of the human AHR, but not different from that of the mouse (Ah^{b-1}) AHR (Jensen and Hahn 2001). In our hands, BW AHR was also statistically indistinguishable from MO AHR, but was also not different from HU AHR in saturation binding assays conducted with nearly identical methods. The high variability in K_d values among assays may be responsible for the lack of statistical differences; in particular, the variability of the human and

humpback whale AHRs in these experiments reduced our ability to identify differences among receptors. We were able to detect statistical significance in the ~3-fold difference between mouse and human AHRs, therefore the <2-fold difference between whale and mouse or human is outside the power of the analysis.

Whale AHRs can also activate gene transcription of a reporter gene in a cell system. Transcriptional activity was observed for transiently transfected whale AHRs independent of TCDD exposure, as previously shown for BW AHR (Jensen and Hahn 2001). Reduction in the amounts of whale AHR constructs transfected did reduce reporter gene expression, but did not yield differentiation of DMSO and TCDD treatments. A possible explanation is that whale AHR constructs express more protein than the mouse in the COS-7 system, resulting in higher protein concentrations with subsequent constitutive transcriptional activation. Quantification of protein expression in COS-7 lysates is required to address this hypothesis. Mouse AHR consistently activated reporter gene transcription 2.5 – 6.1 fold in a TCDD-dependent manner, but induction was only statistically significant for assays using 25 ng plasmid (2-way ANOVA, $p < 0.05 - 0.001$; three of four assays). We observed benzo[a]pyrene-dependent activation for BW AHR but not the other whales, suggesting both species- and ligand-specific differences in transcriptional activity. BaP also induced reporter gene transcription for mouse AHR. Such differences may be related to differential adaptations in cetacean suborders, or indicate that environmental contaminants may have variable effects.

4.3 Whale AHR proteins *in vitro*

The *in vitro* expression and binding capacities of whale AHRs differ dramatically from those of human and mouse AHR. Quantitative measurement of IVTT indicated that whale AHR proteins are expressed at a level ~2 – 5 fold higher than that of human or mouse AHR constructs used in saturation binding

assays (Table 3). A strong correlation exists between the amount of AHR protein and maximal binding detected in these assays (Figure 8). One explanation might be different efficiencies of transcription and translation related to different promoters in the two expression vectors (whale AHRs in pcDNA (T7), human and mouse AHR in pSPORT (SP6)). However, this difference was only minimally alleviated when mouse and human AHRs were subcloned into the same expression vectors (Figure 4C), suggesting that there is a sequence-specific influence over *in vitro* expression. The length of AHR sequence was probably not a factor limiting protein expression given that whale AHRs are long proteins (845 – 851 a.a.) and were highly expressed, while mouse AHR was the shortest protein we used (805 a.a.) and expressed the least protein in the pcDNA vector. Furthermore, the similar activities of the whale AHRs leads us to believe there is something intrinsic to whale AHR proteins such that they express better *in vitro*. It is unknown whether this factor increases transcription, translation, or the stability of the whale AHR proteins. The question remains: why are some AHR proteins more highly expressed than others *in vitro*? The answer to this question may be important in accurately applying *in vitro* biochemical methods to understand mechanisms of action of AHR agonists.

The difference in protein content may impact the results of our binding assays. *In vitro* ligand binding is sensitive to protein concentrations, and high protein concentrations may decrease the apparent binding affinities of AHRs (Bradfield *et al.* 1988). Although we show large differences in AHR expression among receptors, the total protein content of each IVTT reaction was approximately equal therefore mostly avoiding the issue developed by Bradfield *et al.* (1998). Another study illustrated problems associated with *in vitro* expression and high protein concentrations (Ramadoss and Perdew 2004). Given the high expression of our whale AHRs, we may be underestimating the ligand binding affinities of these receptors in these experiments. This also may explain the difference in beluga AHR affinity in this study compared with Jensen's

initial characterization that showed a significant difference between human and both BW and mouse AHRs. Reducing the amount of whale AHR used in the binding assays by dilution with UPL could reduce the apparent binding constants, and would perhaps allow statistical differentiation of whale AHRs from human.

Another explanation for differences in protein expression may lie in the sequences themselves. Recent work on the MDR1 in HeLa cells has suggested that codon usage may impact protein function by altering the rate of co-translational folding (Kimchi-Sarfaty *et al.* 21 December 2006). The prevalence of rare codons in a cDNA, even with conserved amino acid sequence, may further influence the rate of protein construction at a more simplistic level. A comparison of codon usage for within the constructs used here remains to be completed.

4.4 Summary

The framework developed by the methods presented in this study offers a means for comparing the primary mechanism of sensitivity to DLC in cetaceans (i.e. the AHR). We have shown that mysticete AHRs bind to TCDD as well as that from BW, and with binding constants intermediate to those of the high-affinity mouse and lower-affinity human AHRs. Cetacean AHRs are capable of transcriptional activity in a reporter-gene cell system, but further work is required to determine whether whale AHRs activate gene expression in a ligand-dependent manner. Competition binding studies are needed to determine structure-activity relationships of various AHR ligands and test whether environmentally relevant contaminants may have more important effects than studies using TCDD might indicate, especially given our results with BaP in transfection assays. Finally, we emphasize that understanding AHR activity in whales is only one small part of the complex mechanism of response to environmental contaminants, which may be influenced by many other proteins, hormones, and synergistic activities in this complex pathway.

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Table 1. Oligonucleotide primers for aryl hydrocarbon receptors.

Primer	Sequence (5' → 3')	T _m
MM-3-r	GAR GAG TTY TCT GGA GGA AGC TG	65.1°C
MM-4-F	GTT TTA GGC TAT ACT GAA GCA GAG	59.2°C
RW-1r	CAA AGC CAT TCA GTG CCT GCA GTA A	71.5°C
RW_PB-5f	GCC ACC ATC CAT ACT TGA AAT CCG	70.7°C
Bel-nat	CCC AAG CTT GGG CAC CAT GAA CAG CAG C	81.6°C
RW AHR-1R	AAC CAA GAT GAA AAG TGG GC	62.8°C
RW C2261A-F	CCT CAA AAC CAA [a]AG CAC GGA CTC AAT CCA CAG TCA GCC C	84.8°C
RW G2261T-R	GGG CTG ACT GTG GAT TGA GTC CGT GCT [t]TT GGT TTT GAG G	84.8°C
RW C2261A2-F	CCA A[a]A GCA CGG ACT CAA TCC ACA GTC AGC CC	81.6°C
RW G2261T2-R	GGG CTG ACT GTG GAT TGA GTC CGT GCT [t]TT GG	81.6°C

Table 2. Comparison of mammalian aryl hydrocarbon receptors: percent sequence identity.

Nucleotide and amino acid sequences were aligned separately using ClustalW 1.8 in MacVector 8.1.1. Pairwise comparisons are shown for full-length RW, HW, BW, HU, and MO AHRs. Percent nucleotide identity is shown above the diagonal, and amino acid identity, below.

	RW AHR	HW AHR	BW AHR	HU AHR	MO AHR
RW AHR		97.7	95.7	85.7	72.1
HW AHR	97.1		95.5	85.8	72.1
BW AHR	94.0	93.8		86.3	72.7
HU AHR	83.5	83.0	83.2		73.1
MO AHR	68.5	68.1	67.9	68.3	

Table 3. Dissociation binding constants (K_d) and maximal binding (B_{max}) determined for IVTT expressed aryl hydrocarbon receptors.

The protein content for each 100 μ L reaction (mean \pm SD) was determined by [35 S]-labeled methionine IVTT/SDS-PAGE (n=4; Fig. 4B). K_d and B_{max} (means \pm SD) were calculated from saturation binding assays of IVTT AHRs with [3 H]TCDD (n=5). In a one-way ANOVA of mean K_D s, only mouse and human AHRs were statistically different (\clubsuit , Tukey's post-hoc test, p<0.05). Maximum binding (B_{max}) was normalized taking into account differences in AHR protein content for each receptor.

	fmol AHR in 100 μ L IVTT	$K_d \pm$ SD (nM)	$B_{max} \pm$ SD (nM)	B_{max} (norm)
NARW AHR	828 \pm 160	0.64 \pm 0.19	0.41 \pm 0.14	0.42
HW AHR	852 \pm 376	0.85 \pm 0.44	0.65 \pm 0.24	0.65
BW AHR	804 \pm 362	0.78 \pm 0.22	0.49 \pm 0.09	0.52
HU AHR	172 \pm 35	0.97 \pm 0.46 \clubsuit	0.09 \pm 0.04	0.45
MO AHR	425 \pm 83	0.36 \pm 0.13 \clubsuit	0.27 \pm 0.10	0.54

1	ATGAACAGCAGC	GGGCCAACATC	ACCTACGCCAGT	CGCAAGCGGCGG	AAGCCGGTGCAG	AAAAGTGTCAAG	
	M N S S	G A N I	T Y A S	R K R R	K P V Q	K T V K	24
73	CCAGTCCCAGT	GAAGGAATCAAG	TGGAATCCTTCA	AAGCCGCATAGA	GACCGACTTAAT	ACGGAATTGGAC	
	P V P A	E G I K	S N P S	K R H R	D R L N	T E L D	48
145	CGTTTGGCCAGC	CTGTGCCTTTT	CCACAAGATGTT	ATTAATAAGCTG	GACAACTTTCA	GTTCTTAGGCTC	
	R L A S	L L P F	P Q D V	I N K L	D K L S	V L R L	72
217	AGTGTCAAGTAT	CTAAGAGCCAAG	AGCTTCTTTGAT	GTTGCATTACAG	TCTACTCCAGCT	GACCGAAATGGA	
	S V S Y	L R A K	S F F D	V A L Q	S T P A	D R N G	96
289	GTCCAGGACAAC	TGTAGAACAAAA	TTCAGAGAAGGC	CTGAACTTGCAG	GAAGGAGAATTC	TTACTGCAGGCA	
	V Q D N	C R T K	F R E G	L N L Q	E G E F	L L Q A	120
361	CTGAATGGCTTT	GTAATGGTTGTC	ACTACAGATGCT	TTGGTCTTTTAT	GCTTCTTCTACT	ATACAAGATTAC	
	L N G F	V L V V	T T D A	L V F Y	A S S T	I Q D Y	144
433	CTGGGGTTTCAG	CAGTCTGATGTC	ATCCATCAGAGT	GTTGATGAAGT	ATCCATACTGAA	GACCGAGCTGAA	
	L G F Q	Q S D V	I H Q S	V Y E L	I H T E	D R A E	168
505	TTTCAGCGCCAG	CTACTACTGGCA	TAAACCCCTTCA	CAGTGTCCAGAC	TCTGGACAAAA	ATTAATGAAGCT	
	F Q R Q	L H W A	L N P S	Q C P D	S G Q K	I N E A	192
577	AATGGCCTCTCA	CAGCAGCAGT	TATTATAACCCA	GACCGACTTCCCT	CCAGAGAACTCC	TCGTCTATGGAA	
	N G L S	Q P A V	Y Y N P	D Q L P	P E N S	S S M E	216
649	AGGTGCTTCGTT	TGCCAGTAAGG	TGTTTGTGGAT	AATTCATCTGGT	TTTCTGGCAATG	AATTTCCAAGGG	
	R C F V	C R L R	C L L D	N S S Q	F L A M	N F Q G	240
721	AGTTTGAAGTAT	CTTCATGGACAG	AACAAGAAAGGG	AAAGATGGATCA	ATACTTCCACCT	CAGTTGGCTTTG	
	R L K Y	L H G Q	N K K G	K D G S	I L P P	Q L A L	264
793	TTTGAATAGCT	ACTCCACTGCAG	CCACCATCCATA	CTTGAATCCGA	ACCAAAAATTC	ATCTTTAGAAC	
	F A I A	T P L Q	P P S I	L E I R	T K N F	I F R T	288
865	AAACACAAGCTA	GACTTTACACCT	ACTGGTTGTGAT	GCCAAAGGAAGA	ATTGTTTTAGGC	TATACTGAAGCA	
	K H K L	D F T P	T G C D	A K G R	I V L G	Y T E A	312
937	GAGCTATGCATG	AGAGGATCAGGA	TATCAATTTATT	CATGCTGCATGAT	ATGCTTTATTGT	GCTGAGTACCAT	
	E L C M	R G S G	Y Q F I	H A A D	M L Y C	A E Y H	336
1009	ATCCGGATGATT	AAGACTGGAGAG	AGTGGCATGATA	GTGTTCAAGGCT	CTTACGAAAGAC	AATCGATGAACT	
	I R M I	K T G E	S G M I	V F R L	L T K D	N R W T	360
1081	TGGGTGCAGTCA	AATGCACGCTTA	GTTTATAAGAAT	GGAAGACCAGAT	TATATCATTGCA	ACTCAGAGACCT	
	W V Q S	N A R L	V Y K N	G R P D	Y I I A	T Q R P	384
1153	CTAACAGATGAA	GAAGAAACAGAG	CATCTACGAAAA	CGAAATATGAAG	TTGCCTTTTATG	TTTACCATGGC	
	L T D E	E G T E	H L R K	R N M K	L P F M	F T T G	408
1225	GAAGCTGTTTTA	TATGAGGTAACC	AACCCTTTTCCT	CCCATAATGGAT	CCCTTACCAATA	AGGACTAAAACT	
	E A V L	Y E V T	N P F P	P I M D	P L P I	R T K T	432
1297	GGTGTGGTGGGA	AAAGTTCTGCT	ACCAAATCAACT	CTAAATAAGGAT	TCCCTCAATCCC	AGTCCCTCCTG	
	G A G G	K G S A	T K S T	L N K D	S L N P	S S L L	456
1369	AATGCCATGATG	CAACAAGATGAA	TCTATTATTCTC	TATCCCTGCTCA	AGTAGTACACCT	TTTGAAGAAAC	
	N A M M	Q Q D E	S I Y L	Y P A S	S S T P	F E R N	480
1441	TTTTTCACTGAC	TCTCAGAATGAG	TGCAGTAGTTGG	CAAAGCAATGTT	GCACCAATGGGA	AGTGACAATATC	
	F F T D	S Q N E	C S S W	Q S N V	A P M G	S D N I	504
1513	CTGAACATGAG	CAGATTGGCCAG	TCTCAGGAAATG	AACCCACACAGT	TCCGGAGATCAC	GCGGGCTCTTT	
	L K H E	Q I G Q	S Q E M	N P T V	S G D H	A G L F	528
1585	CCAGATAATAGA	AATAGTGACTTG	TACAGCATTATG	AAACACCTAGGC	ATTGATTTTGAA	GATATCAAAAC	
	P D N R	N S D L	Y S I M	K H L G	I D F E	D I K H	552
1657	ATGCAACAGAAT	GAGGAATTTTTT	AGAATGACTTTT	TCTGGTGAAGAT	GACTTCAGAGAT	ATTGACTTAACA	
	M Q Q N	E E F F	R T D F	S G E D	D F R D	I D L T	576
1729	GACCAAAATCTG	ACCTATGTCGAA	GACTCTTTAAAT	AAGTCTGCCTTT	GGCTGTTGAGT	TGCCATCAGCAA	
	D E I L	T Y V E	D S L N	K S A F	G C S G	C H Q Q	600
1801	CAGTCCATGGCT	CTGAACCCAGC	TGCATGGTACAG	GAGCACCTCCGG	TTAGAACAGCAA	GAGCAGCTACAG	
	Q S M A	L N P S	C M V Q	E H L R	L E Q Q	E Q L Q	624
1873	CACCATCAGCAG	CACACAGCAGCA	GAGCAGCAACAG	CTGTGTCAGAAA	ATGAAGCACATG	CAAGTTAACGGC	
	H H Q Q	H T A A	E Q Q Q	L C Q K	M K H M	Q V N G	648
1945	ATGTTTGCAAAC	TGGAACCTCCGAC	CAATCCGTGCCT	TTTAACTGTCTT	CAGCAAGACTTA	CAGCAGTACAGC	
	M F A N	W N S D	Q S V P	F N C P	Q Q D L	Q Q Y S	672
2017	GTCITTTTCAGAC	TTACTTGGGACC	AGTCAAGAGTTT	CCCTACAAAGCC	GAGTTTGATCCT	ATGCCTTACGCA	
	V F S D	L P G T	S Q E F	P Y K A	E F D P	M P Y A	696
2089	CAGAACTTTATC	CCCTGTAGTCAG	TCTGTGTTGCCG	CCACATTCTAAG	GGTACACAGTTA	GACTTTCCCAT	
	Q N F I	P C S Q	S V L P	P H S K	G T Q L	D F P I	720
2161	GGGGATTTCGAA	CCAGCCCCATAC	CCTACAATTTCT	TCTCATTTAGAA	GATTTTGTACA	TGTTTACAAGTT	
	G D F E	P A P Y	P T T S	S H L E	D F V T	C L Q V	744
2233	CCTCAAAACCAA	AAGCACGGACTC	AATCCACAGTCA	GCCTACTAACT	CCTCAGACATGT	TATGCGGGGCT	
	P Q N Q	K H G L	N P Q S	A L L T	P Q T C	Y A G A	768
2305	GTGTCAATGTAC	CAGTGCCAGCCG	GAAGCTCAGCAC	AGCCATGTGGCT	CAGATGCAGTAC	AACCCGACGATG	
	V S M Y	Q C Q P	E A Q H	S H V A	Q M Q Y	N P T M	792
2377	CCAGGCCCGAGC	ATGCCAGGCCCG	CAGGCAATTTTA	AACAAGTTTCAG	AACGGAGGAGTC	TTAAATGAAACC	
	P G P T	M P G P	Q A F L	N K F Q	N G G V	L N E T	816
2449	TATCCAGCTGAA	TTAAATAGCATA	AATAACACTGAC	CCTACCGCCCAT	CTTCAACCGTCA	GAAGCCAGACCT	
	Y P A E	L N S I	N N T Q	P T A H	L H P S	E A R P	840
2521	TTCCCTGACTTG	ACATCCAGTGGG	TTCCCTGTAATTC	CAAGCCCACTTT	TCATCTTGGTTT	TTTGATTAATTT	
	F P D L	T S S G	F L *				850
2593	CGTTTGTGAAAC	ATCGAAAAAATA	AAAAAATAAATA	AAAAAATAAATA			

Figure 1. Full length north Atlantic right whale aryl hydrocarbon receptor cDNA. The nucleotide and predicted amino acid sequences (numbered left and right, respectively) for the coding sequence of NARW AHR. The stop codon is indicated with an asterisk (*).

1 ATGAACAGCAGC AGCGCCAACATC ACCTACGCCAGT CGCAAGCGGGCGG AAGCCGGTGCAG AAAACTGTCAAG
M N S S S A P N I T Y A S R K R R R K P V Q K T V K 24
73 CCAGTCCCAGCT GAAGGAATCAAG TCGAATCCTTCA AAGCGGCATAGA GACCGACTTAAT ACGGAATGGAC
P V P A E G I K S N P S K R H R D R L N T E L D 48
145 CGTTTGGCCAGC CTGCTGCCTTTT CCACAAGATGTT ATTAATAAGCTG GACAAAATTCA GTTCTTAGGCTC
R L A S L L P F P Q D V I N K L D K L S V L R L 72
217 AGTGTCAAGTTAT CTAAGAGCCAAG AGCTTCTTTGAT GTTGCATTACAG TCCACTCCAGCT GACAGAAATGGA
S V S Y L R A K S F F D V A L Q S T P A D R N G 96
289 GTCCAGGACAAC TGTAGAACAAAA TGCAGAGAAGGC CTGAACTTGCAG GAAGGAGAATC TTACTGCAGGCA
V Q D N C R T K C R E G L N L Q E G E F L L Q A 120
361 CTGAATGGCTTT GTATTGGTTGTC ACTACAGATGCT TTGGTCTTTTAT GCTTCTTCTACT ATACAAGATTAC
L N G F V L V V T T D A L V F Y A S S T I Q D Y 144
433 CTGGGGTTTCC CAGTCTGATGTC ATCCATCAGAGT GTGTATGAACTG ATCCACACTGAA GACCGAGCTGAA
L G F Q Q S D V I H Q S V Y E L I H T E D R A E 168
505 TTTCAGCGCCAG CTACACTGGGCA GTAAACCCCTTCA CAGTGTCCAGAC TCTGGACAAAA ATTGTGAAGCT
F Q R Q L H W A V N P S Q C P D S G Q K I D E A 192
577 AATGGCCCTCTA CAGCCAGCAGTC TATTATAACCCA GACCCAGGTTCCCT CCAGAGAACTCT TCGTCTATTGAA
N G L S Q P A V Y Y N P D Q V P P E N S S S M E 216
649 AGGTGCTTCGTT TGCCGACTAAGG TGTCTGCTGGAT AATTCATCTGGT TTTCTGGCAATG AATTTCCAAGGG
R C F V C R L R C L L D N S S G F L A M N F Q G 240
721 AGGTGAAGTACT CTTTACGACAG AACAGGAAGGG AAGGATGGATCA ATACTCCGCCT CAGTTGGCCTTG
R L K Y L H G Q N R K G K D G S I L P P Q L A L 264
793 TTTGCAATAGCT ACTCCACTGCAG CCACCGTCCATA CTTGAAATCCGA ACCAAGAACTTC ATCTTTAGAAC
F A I A T P L Q P P S I L E I R T K N F I F R T 288
865 AAACACAAGCTA GACTTTACACCT ACCGGCTGTGAT GCCAAAGGAAGA ATGTGTTTAGGC TATACTGAAGCA
K H K L D F T P T G C D A K G R I V L G Y T E A 312
937 GAGTGTATGATC AGAGGATCAGGA TATCAATTTATT CATGCTGCTGAT ATGCTTTATTGC GCTGAGTACCAT
E L C M R G S G Y Q F I H A A D M L Y C A E Y H 336
1009 ATCCGGATGATT AAGACTGGAGAG AGTGGCATGATA GTGTTCAAGCCT CTTACGAAAAGC AATCGATGGACT
I R M I K T G E S G M I V F R L L T K D N R W T 360
1081 TGGTGCAATGCA AATGCACGCTTA GTTTATAAGAAAT GGAAGACCAGAT TATATCATTGCA ACTCAGAGACCT
W V Q S N A R L V Y K N G R P D Y I I A T Q R P 384
1153 CTAACAGATGAA GAAGGAACAGAG CATTTACGAAAA CGAAATATGAAG TTGCCTTTTATG TTTACCCTGGC
L T D E E G T E H L R K R N M K L P F M F T T G 408
1225 GAAGCTGTTTTA TATGAGGTAACC AACCCCTTTCCCT TCCATAATGGAT CCCTTACCAATA AGGACTAAAAC
E A V L Y E V T N P F P S I M D P L P I R T K T 432
1297 GGTGCTGGTGA AAAGTTCTGCT ACCAAATCAACT CTAATAAGGAT TCCTTCAATCCC AGTTCCCTCCTG
G A G G K G S A T K S T L N K D S L N P S S L L 456
1369 AATGCCATGATC CAACAAGATGAG TCTATTTATCTC TATCTGCTTCA AGTAGTACATCT TTTGAAAAGAAC
N A M M Q O D E S I Y L Y P A S S S T S F E R N 480
1441 TTTTTCAGTGAT TCTCAGAATGAG TGCAGTAGTTGG CAAAGCAATGTT GCACCAATGGGA AGTGACAATATC
F F S D S Q N E C S S W Q S N V A P M G S D N I 504
1513 CTGAAACATGAG CAGATTGGCCAG TCTCAGGAAATG AACCCACAGTC TCCGGAGATCAC GCAGGGCTCTTT
L K H E Q I G Q S Q E M N P T V S G D H A G L F 528
1585 CCAGATAATAGA AATAGTGACTTG TACAGCATTATG AACACCTAGGC ATGTATTTTGAA GATATCAACAC
P D N R N S D L Y S I M K H L G I D F E D I K H 552
1657 ATGCAACAGAAT GAGGAATTTTTT AGAAGTACTGCTT TCTGGTGAAGAT GACTTCAGAGAT ATTGACTTAACA
M Q Q N E E F F R T D F S G E D D F R D I D L T 576
1729 GATGAAATCTCT ACTTACGTCGAA GACTCTTTAAAT AAGTCTGCCTTT GGCTGTTTCAAGT TACTACCAGCAA
D E I L T Y V E D S L N K S A F G C S G Y Y Q Q 600
1801 CAGTGCATGGT CTGAACCCAGC TGCATGGTACAG GAGCACCTCCAG TTAGAACAGCAA GAGCAGCTCAG
Q S M A L N P S C M V Q E H L Q L E Q Q E Q L Q 624
1873 CACCGTCAGCAG CACATAGCAGCG GAGCAGCAGCAA CAGCTGTGTGAG AAGATGAAGCAC ATGCAGGTTAAN
H R Q Q H I A A E Q Q Q Q L C Q K M K H M Q V N 648
1945 GGCATGTTTGCA AACTGGAATCC GACCAATCCGTG CCTTTAACTGT CCTCAGCAAGAC TTACAGCAGTAC
G M F A N W N S D Q S V P F N C P Q Q D L Q Q Y 672
2017 AATGTCTTTTCA GACTTACCTGGG ACCAGTCAAGAG TTTCCCTACAAA GCTGAGATTGAC ACTCTGCCTTAC
N V F S D L P G T S Q E F P Y K A E I D T L P Y 696
2089 GCACAGAACTTT ATCCCTGTAGT CAGTCTGTGTTG CCGCCACATCT GAGGTTACACAG TTAGACTTTCCC
A Q N F I P C S Q S V L P P H S E G T Q L D F P 720
2161 ATTGGGCATTTT GAACCCAGCCCA TACCCTACACCT TCTTCTAATTTA GAAGATTTTGTG ACATGTTTACAA
I G H F E P A P Y P T A C P S S N L E D F V T C L Q 744
2233 GTTCTCTAAAA CAAAAGCAGGGA CTCAATCCACAG TCAGCCCTAGTA ACTCCTCAGACG TGTATGCGGGG
V P Q N Q K H G L N P Q S A L V T P Q T C Y A G 768
2305 GCTGTGTCATG TACCAGTCCAG CCGGAAGCTCAG CACAGCCATGTG GCTCAGATGCAG TACAACCCACG
A V S M Y Q C Q P E A Q H S H V A Q M Q Y N P T 792
2377 ATGCCAGGCCCA ACGGTGCCAGGC CCGCAGGCGTTT TTAACAAGTTT CAGAACGGAGGA GTCTTAAATGAA
M P G P T V P G P Q A F L N K F Q N G G V L N E 816
2449 ACCTATCCAGCT GAATTAATAGC ATAATAACACT CAGCCTACCGCC CATCTTACCCG TCAGAAGCCAGA
T Y P A E L N S I N N T Q P T A H L H P S E A R 840
2521 CCTTCCCTGAC TTGACATCCAGT GGATTCCTGTAA
P F P D L T S S G F L * 851

Figure 2. Full length humpback whale aryl hydrocarbon receptor cDNA.
The nucleotide and predicted amino acid sequences (numbered left and right, respectively) for the coding sequence of HW AHR. The stop codon is indicated with an asterisk (*).

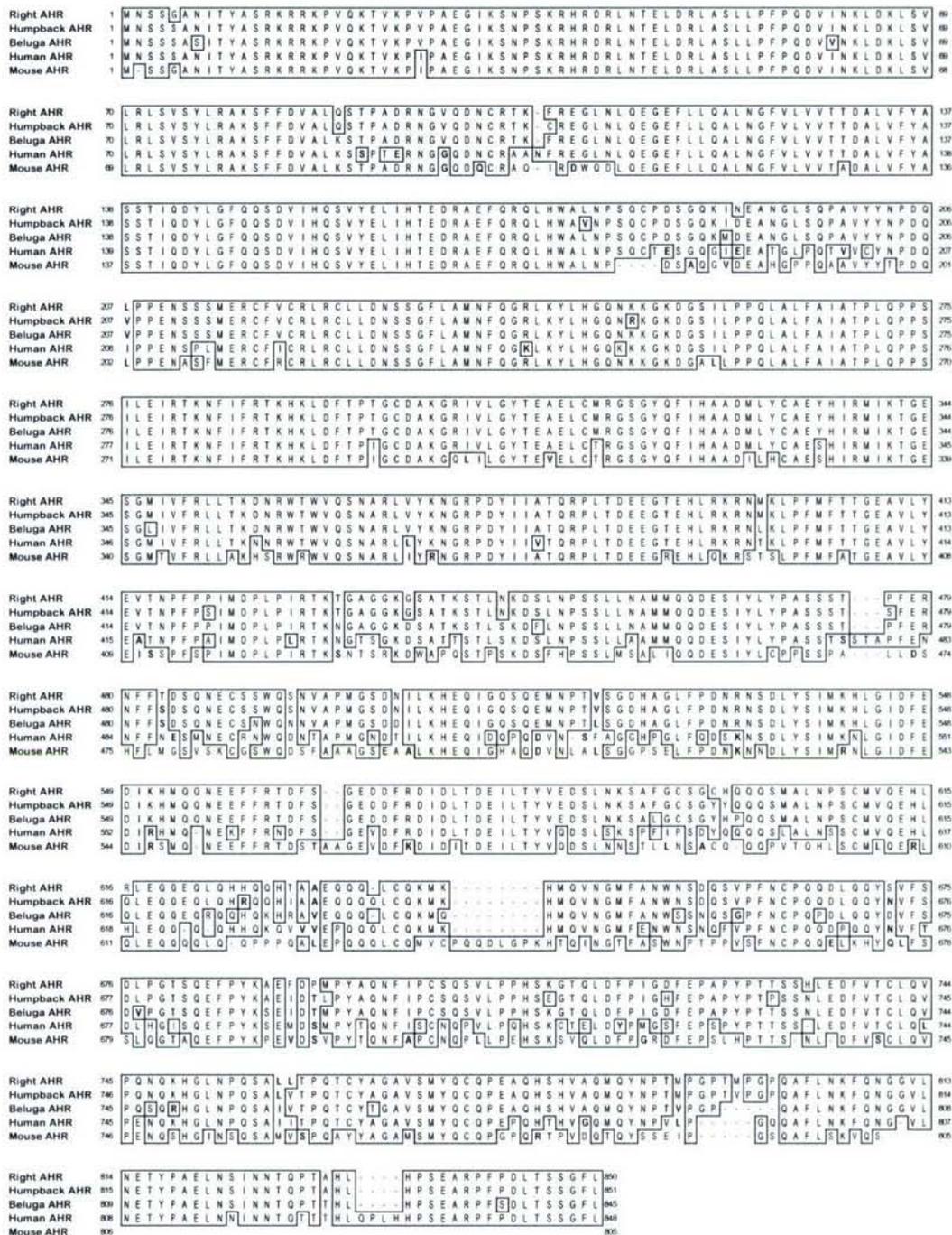


Figure 3. Alignment of aryl hydrocarbon receptor amino acid sequences.

Predicted amino acid sequences of whale, human, and mouse AHR coding regions used in this study were aligned using ClustalW (1.8) in MacVector 8.1.1. Identical residues in three or more sequences are outlined and shaded; similarities are in bold.

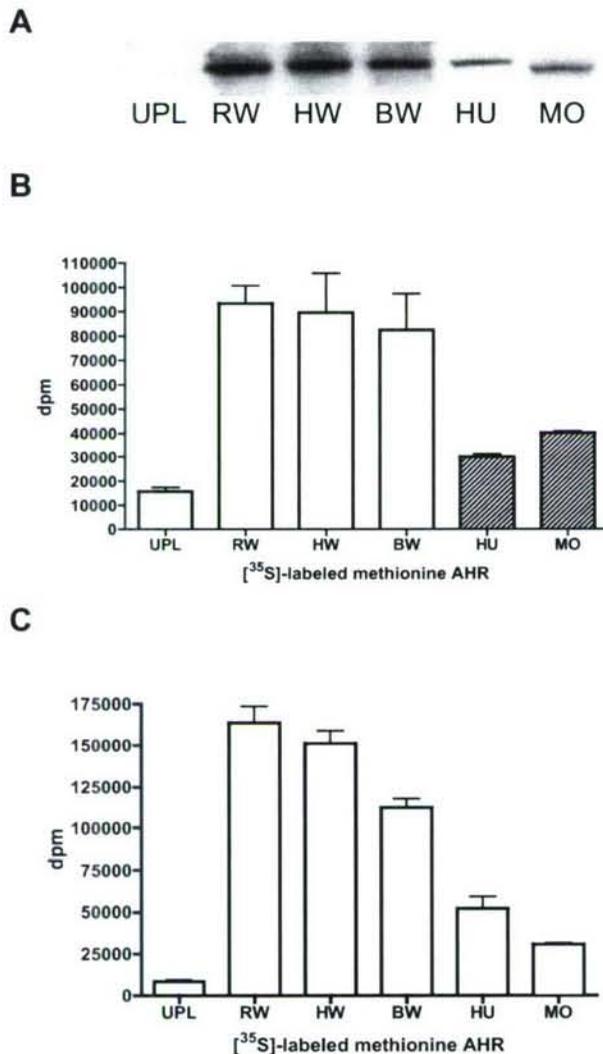


Figure 4. Whale AHR constructs express protein by *in vitro* transcription and translation (IVTT).

A, Right whale and humpback whale AHR coding sequences were ligated into pcDNA expression vectors (T7 promoter) and expressed by IVTT in the presence of [³⁵S]-labeled methionine; unprogrammed lysate (UPL; empty pcDNA vector), and beluga (pcDNA), human, and mouse AHR constructs (pSPORT vector; SP6 promoter) were also expressed. Protein was detected and quantified using SDS-PAGE and autoradiography. **B**, Variability in the amount of protein expressed in individual IVTT reactions was assessed in replicate experiments (means \pm SD, n=4). **C**, Higher expression of whale AHRs was not completely explained by different expression vectors; when subcloned into pcDNA, the pattern of lower expression of human and mouse AHR expression remained (means \pm SD, n=3).

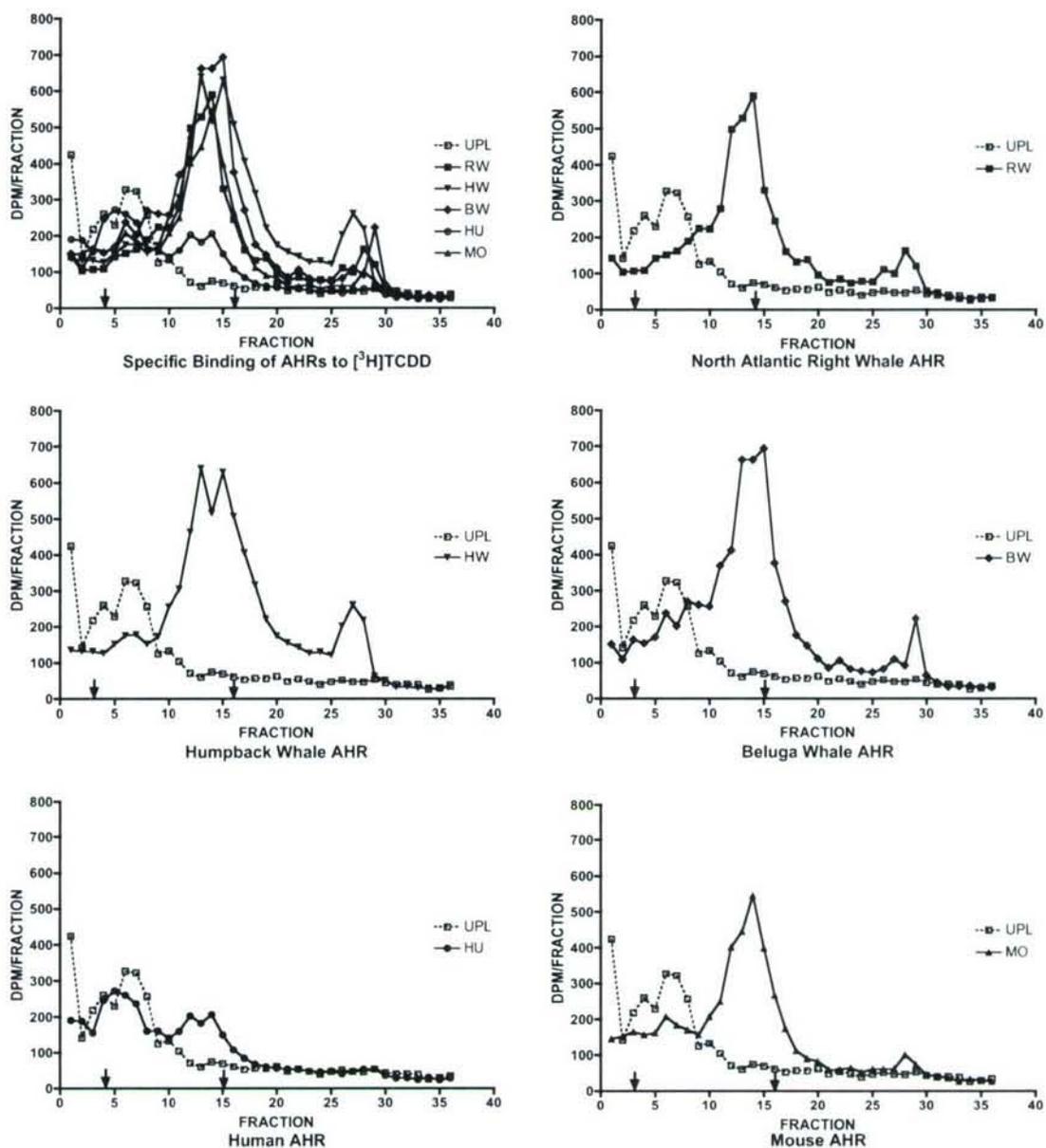


Figure 5. *In vitro* expressed AHR proteins show specific ligand binding by velocity sedimentation.

Specific ligand binding was assessed by velocity sedimentation analysis on sucrose gradients after incubation of $[^3\text{H}]\text{TCDD}$ (2 nM) with IVTT expressed AHR proteins. Specific binding was calculated for each receptor as the difference in total binding and non-specific binding (as measured by unprogrammed lysate, UPL). The elution positions of sedimentation markers $[^{14}\text{C}]\text{ovalbumin}$ (3.6 S) and $[^{14}\text{C}]\text{catalase}$ (11.3 S) are shown with left and right arrows, respectively.

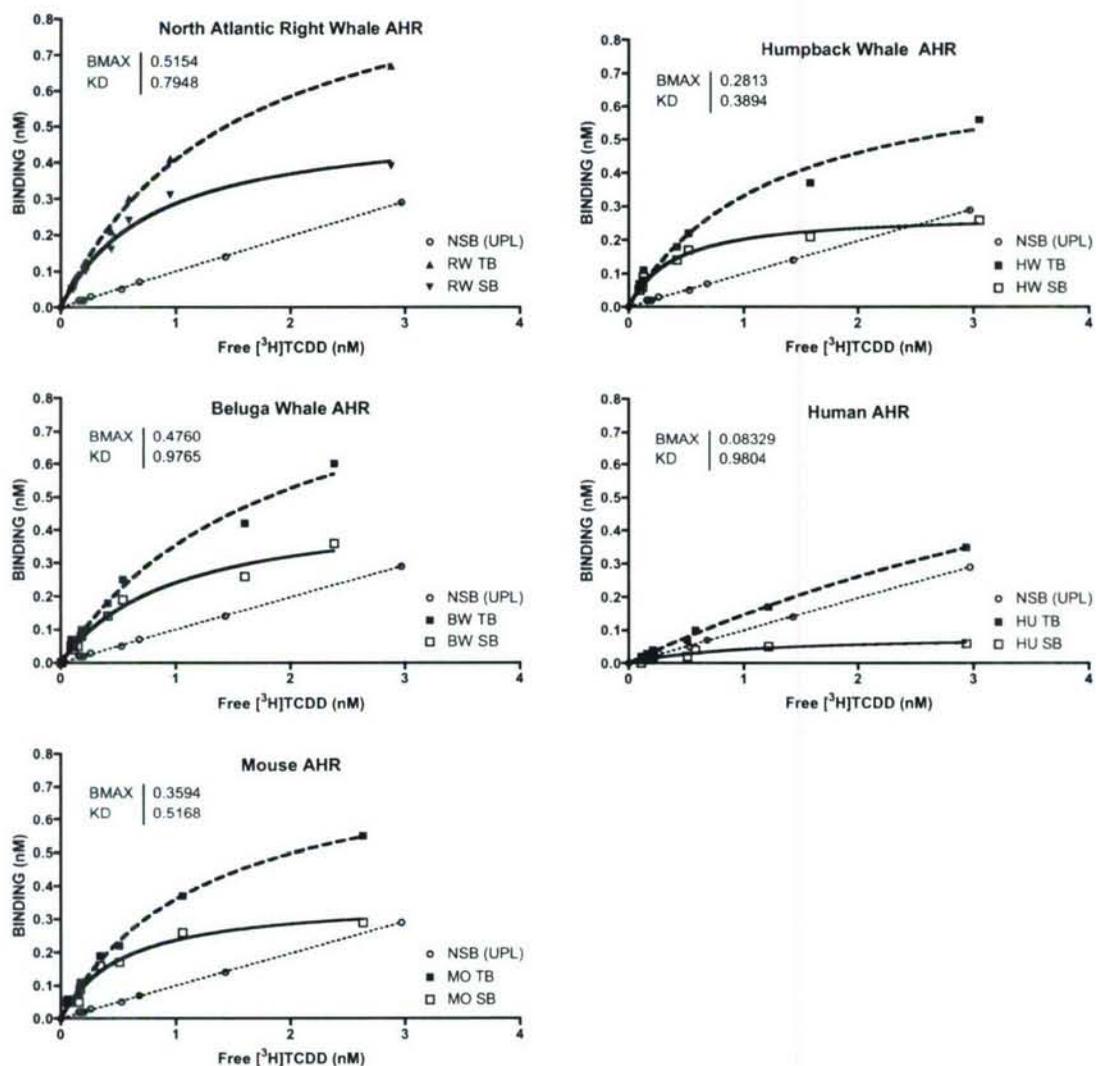


Figure 6. Representative binding curves for aryl hydrocarbon receptors.

Binding curves from one of five replicate saturation binding assays are shown for North Atlantic right, humpback and beluga whale, human, and mouse AHR. IVTT expressed AHR proteins were diluted in buffer and incubated with one of nine or ten concentrations of [³H]TCDD. Specific binding (open squares) was calculated as the difference between total binding (closed squares) and non-specific binding (open circles; UPL, unprogrammed lysate). Nonlinear regression analysis and binding constants were derived assuming one-site binding in Prism 4.0. ($Y=B_{max} \cdot X / (K_d + X)$; GraphPad).

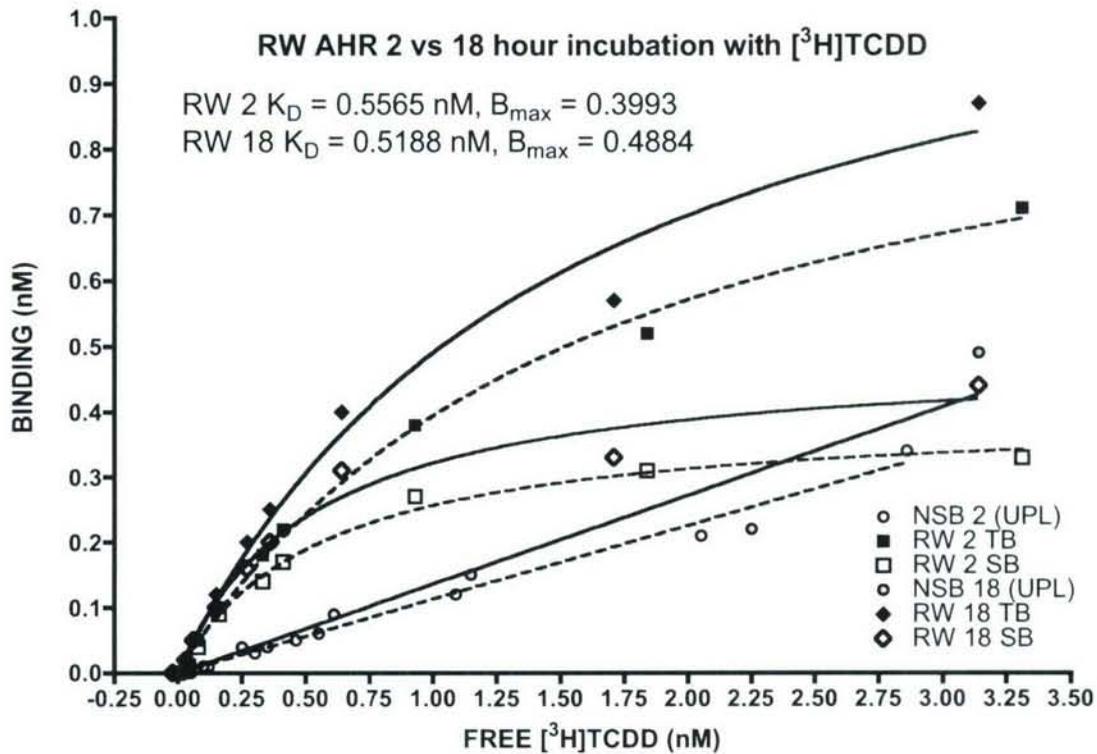


Figure 7. Saturation binding assay incubation time does not affect binding constants.

NARW IVTT AHR protein was incubated with ten concentrations of [³H]TCDD for 2 or 18 hours to determine optimum assay conditions. Greater maximal binding was achieved from the longer incubation, but measures of K_d and B_{max} are within one standard deviation of the means of 5 other assays.

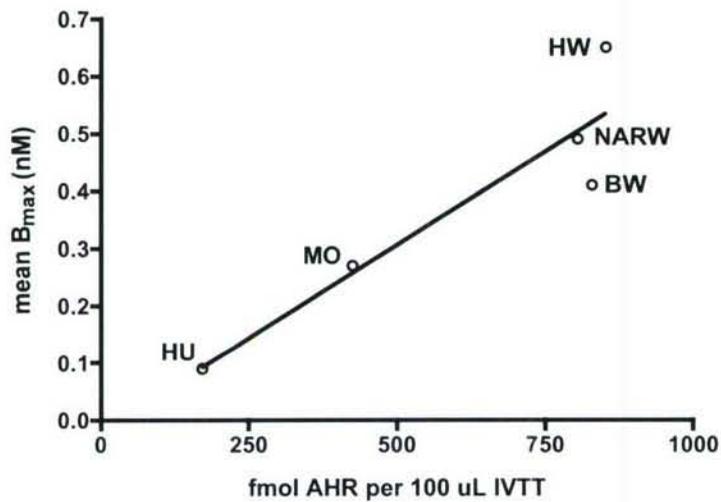


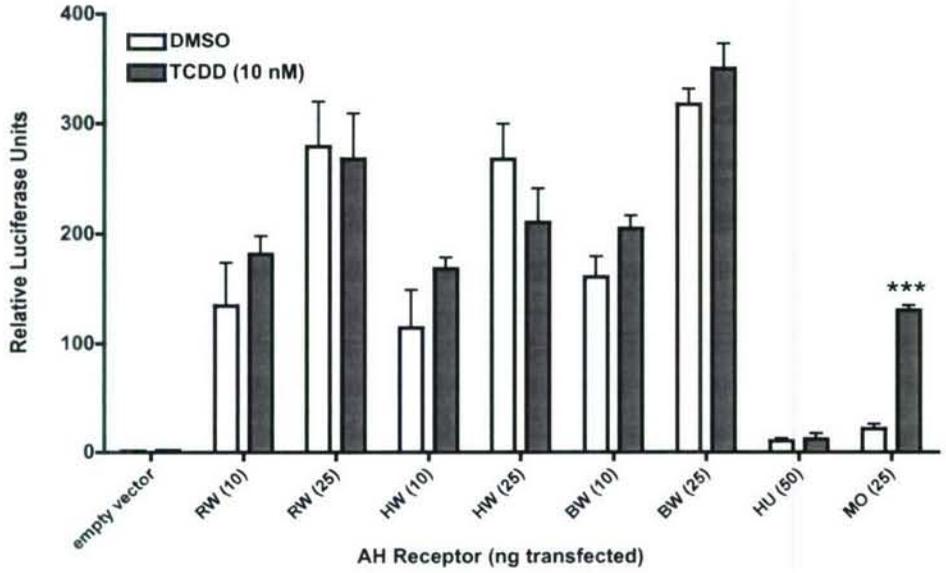
Figure 8. Maximal binding is correlated with the amount of AHR protein.

Differences in B_{max} are correlated with the amount of AHR protein in each binding assay ($r^2 = 0.86$). The same volume of IVTT AHR (100 μ L) was used for each saturation binding assay. However, each construct does not express the same amount of AHR protein (Table 3); whale AHR expression constructs consistently express greater amounts of specific protein.

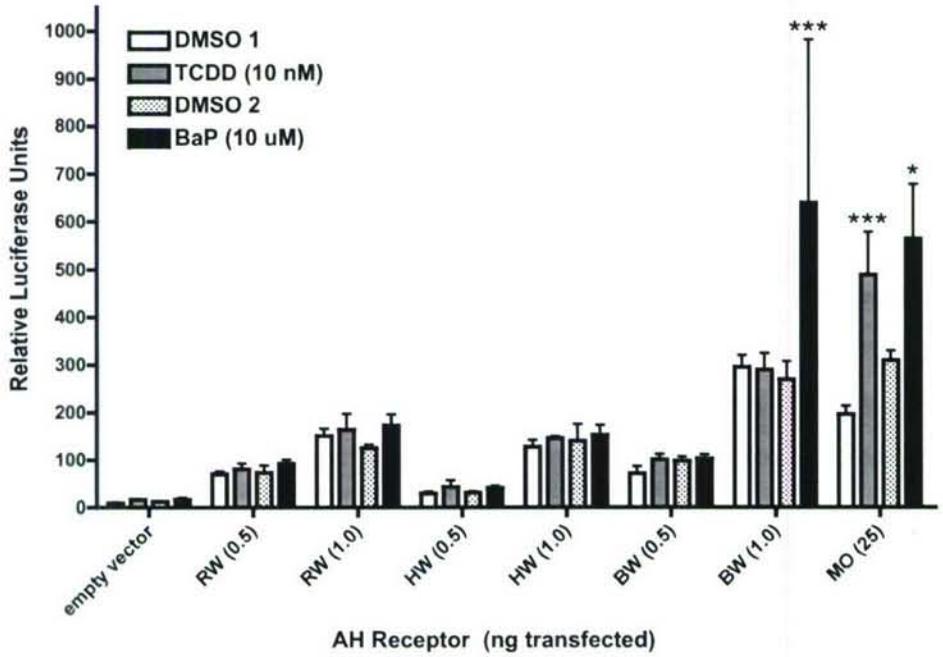
Figure 9. Whale aryl hydrocarbon receptors are transcriptionally active.

Mammalian cells deficient in endogenous AHR expression (COS-7) were cotransfected with 0.5 – 50 ng NARW, HW, BW, HU, or MO AHR expression plasmids, plus human ARNT, a reporter-gene (pGudLuc6.1), and a transfection control (pRL-TK) in 48-well culture plates. We compared transcriptional activation of each AHR with DMSO or 10 nM TCDD in four experiments (representative results shown in **A**). MO AHR consistently induced reporter-gene expression (2.5-fold) in the presence of TCDD, whereas whale AHRs were transcriptionally active with and without ligand. A single assay (**B**) consisting of two plates, one dosed with TCDD (10 nM), the other with benzo[a]pyrene (10 μ M), and each with separate DMSO controls, produced similar results for TCDD (MO AHR: 2.5-fold induction); BaP induced reporter-gene expression with BW AHR (2.4-fold) and MO (1.8-fold) AHRs. Two-way ANOVA, with Bonferroni post-hoc tests; $p < 0.05$ (*); $p < 0.001$ (***)

A.



B.



Chapter 4: Species-Specific Differences in Mammalian Aryl Hydrocarbon Receptor Ligand Binding Function

ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor best known for regulating expression of genes encoding biotransformation enzymes (e.g. cytochrome P450s) and other genes important in development, cell cycle control, and reproduction. Subtle species-specific differences in AHR proteins can affect the cellular response to chemical exposure, and subsequently, organismal sensitivity to environmental contaminants. Species-specific differences in AHR structure and function were compared to investigate variations in sensitivity of aquatic and marine mammals to persistent organic pollutants, relative to other model mammalian species. Partial AHR cDNAs were cloned and sequenced from mink and polar bear, and were compared with the ligand binding domains of other marine and terrestrial mammalian AHRs using amino acid alignment and phylogenetic analysis. The relative binding affinities of several marine and terrestrial mammal AHRs were compared in saturation binding assays using *in vitro* expressed proteins. Among these were guinea pig and hamster AHRs, species commonly believed to have high and low affinity receptors (respectively) responsible for observed differences in adult dioxin lethality, but with diverse pathologies resulting from sub-lethal exposures. Homology modeling was used to infer the tertiary structures of AHRs for representative cetartiodactyl (whale + cow), carnivoran (seal + dog), and euarchontoglires (human + guinea pig + rodent) AHR ligand binding domains. While the models illustrated potential structural differences that might influence the AHR ligand binding pocket, the major differences among clades did not explain differences in binding observed by saturation binding assays. Other regions of the AHR protein likely play a role both in the tertiary conformation of the protein and its overall function.

1. INTRODUCTION

While it is important to consider the ligand binding and gene activation functions of aryl hydrocarbon receptors in North Atlantic right whales for reasons specific to the species' recovery, placing it in context with other mammalian species helps in the interpretation of those results. The study of marine mammal toxicology is relatively young, whereas model organisms such as rodents have been extensively studied. Integrating these lines of research using a comparative approach increases the power of individual analyses, and provides a more in-depth portrait of aryl hydrocarbon receptor function in mammals.

Laboratory studies inform our understanding of species-specific responses to toxicant exposure. Rodent studies dominate the toxicology literature, providing basic information on lethal doses and phenotypes associated with controlled toxicant exposures. Acute toxicity, wasting syndrome, endocrine imbalances, immune suppression, lesions, developmental abnormalities, and behavioral changes among other pathologies have been observed in rats, mice, guinea pigs, and hamsters following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and dioxin-like compounds (DLC) (Pohjanvirta and Tuomisto 1994; Poland and Knutson 1982). More limited data exist for unconventional and wildlife species due to ethical and logistical concerns governing toxicological experimentation, and anecdotal evidence or accidental releases often prevail in this arena. Toxicological experimentation in marine mammals has primarily been conducted on pinnipeds. Several experiments have shown that harbor seals exposed to dietary polychlorinated biphenyls (PCBs) suffer reproductive and endocrine effects (Brouwer *et al.* 1989; Reijnders 2003; Reijnders 1986). Reproductive dysfunction and morphological disorders have also been possibly or probably associated with organochlorine exposure in other pinnipeds and several cetacean species (Reijnders 2003). More recently

mink has emerged as a popular subject of toxicological studies owing to its susceptibility to dioxin toxicity, closer physiological similarities to humans than those of rodents, and ease of handling (Calabrese *et al.* 1992; Heaton *et al.* 1995; Hochstein *et al.* 1988); such studies add breadth to our understanding of phylogenetic relationships salient to toxicological sensitivity.

These studies show the importance of investigating individual species' sensitivities, but classical toxicological methods, such as dosing a set of individuals and measuring specific endpoints, are not easily transferable to endangered wildlife or large animals. There is a need for indirect measurements to infer susceptibility to environmental toxicants. Characterization of the structures and functions of different species' AHRs can help to guide the choice of model species and facilitates the extrapolation of results from one species to another.

Halogenated aromatic hydrocarbons (HAH), polycyclic aromatic hydrocarbons (PAH), and DLC can exert toxicological effects in vertebrates via the aryl hydrocarbon receptor (AHR). The AHR is a cellular transcription factor and a member of the PAS family of proteins. Sources of species- and tissue-specific differences in organismal sensitivity to environmental toxicants can include ligand pharmacokinetics, pharmacodynamics, and metabolism; AHR functionality; differences in AHR cofactor function, expression and concentration; and other biochemical and physiological factors. However, in some cases, AHR binding characteristics can be directly related to organismal sensitivity.

Two issues underlie the need for more diverse representation of toxicological sensitivity. First, model organisms (typically rodents) are often studied as proxies for human biomedical applications. While this approach is sensible, it overlooks species-specific differences that can be critically important in predicting the effects of drugs or toxicants in humans. It is vital to understand such differences in order to appropriately extrapolate findings from one organism to another. Second, and related, is the application of the same principles to

wildlife health. Although often discounted as unimportant by governing bodies and funding agencies, wildlife health is important for maintaining an ecosystem able to support human populations, and is sometimes a sentinel for human health issues. Understanding the relationships of model or surrogate species to difficult-to-study wildlife species (especially endangered ones) is necessary if we are to apply appropriate and timely tools for conservation and resource management.

AHR acts in concert with many other cellular proteins, hormones, and pathways. Because of this, it is not always possible to make definitive connections between AHR functional characteristics and organismal sensitivity to DLC. However, studies of AHR knock-out mice have shown that binding of DLC to AHR is the necessary proximal step for most chemical effects (Fernandez-Salguero *et al.* 1996; Mimura *et al.* 1997). Yet any number of downstream factors may be responsible for conferring species-specific susceptibility to DLC. It remains difficult to determine whether species-specific variations in response to toxicant exposure result from these potential downstream differences, or from differences in AHR function.

In this chapter we use a comparative approach to examine AHR ligand binding domain sequences to understand species that exhibit a range of sensitivities to dioxin-like compounds (DLC). We expand the range of species from the traditionally studied (mouse, human, guinea pig, hamster) to the wild, marine realm (whales and seals). Through the use of a biochemical assay, primary protein structural analysis, and prediction of tertiary protein folding, we attempt to infer the range of sensitivity to dioxin along a continuum of low- and high-affinity AH receptors. These analyses are admittedly ambitious, and inherently fraught with errors; however we hope to put forth a model that may be tested with more rigorous *in vitro* methods or field data in the future.

METHODS

2.1 Aryl Hydrocarbon Receptor Sequences

In order to examine marine mammal AHRs in the context of those in other mammals, we compiled full and partial AHR amino acid sequences from this (Chapter 3) and other studies (Table 1). In addition, several of these AHR cDNAs were acquired as expression plasmids, kindly provided by the researchers who developed them: mouse (Burbach *et al.* 1992), hamster (Korkalainen *et al.* 2000), guinea pig (Korkalainen *et al.* 2001), human (Dolwick *et al.* 1993), beluga (Jensen and Hahn 2001), and harbor seal (Kim and Hahn 2002). Each of these constructs was subcloned into the pcDNA vector in order to standardize the conditions under which each protein was expressed in the *in vitro* system.

2.2 RT-PCR & Sequencing of AHR in Mink and Polar Bear

Polyadenylated RNA was isolated and purified from liver tissues from mink and polar bear samples. Tissues had been collected and stored 15 months (mink) to 6 years (polar bear) at -80°C prior to processing. Complementary DNA (cDNA) was synthesized using random/oligo d(T) primers and Omniscript reverse transcriptase. PCR was performed with degenerate primers D33F and D328R (Table 1) with the following thermocycling protocol with AmpliTaq Gold polymerase: 94°C 10 min; 35 cycles: 94°C 15 sec, 50°C 30 sec, 72°C 30 sec; 72°C 7 min; 4°C hold. Bands of expected size were excised following separation by agarose gel electrophoresis, then ligated in pGEM-T Easy cloning vector. Positive clones were again size selected by endonuclease digestion and gel electrophoresis, then sequenced using Big Dye Terminator cycle sequencing with isopropanol purification. Sequences were edited using Sequencher 4.2.2 and MacVector 8.1.1.

2.3 Sequence Alignment and Analysis

Aryl hydrocarbon receptor amino acid sequences from Table 1, plus new partial sequences from mink and polar bear AHRs, were aligned using the ClustalW algorithm in MacVector 8.1.1. Phylogenetic trees were constructed using PAUP* 4.0.

2.4 Biochemical Characterization: Ligand Binding

In vitro transcription and translation (IVTT) produced AHR proteins from cDNAs indicated with an asterisk (*) in Table 2. The amounts and sizes of each AHR were assessed by conducting additional IVTT in the presence of [³⁵S]-labeled methionine, separating 5 µl of each reaction by SDS-PAGE, performing fluorography of the dried gels, then cutting and counting each band in a scintillation counter.

Unlabeled IVTT AHRs were subjected to saturation binding analysis with [³H]TCDD as described in Chapter 3. Briefly, proteins were diluted in buffer and aliquoted into glass vials containing one of 8 concentrations of [³H]TCDD, and incubated overnight at 4°C. Incubations were separated into duplicate 1.5 mL polypropylene tubes with equal volumes of buffer plus dextran coated charcoal (DCC). Each tube was then vortexed three times for 5 seconds at 5 minute intervals. Following sedimentation of DCC by centrifugation, supernatant was removed and counted for presence of [³H]TCDD. Specific binding was determined as detailed in Chapter 3, using a series of tubes containing UPL (unprogrammed lysate) to account for non-specific binding. Binding curves and constants were calculated using Prism 4.0.

2.5 Homology Modeling

AHR LBD amino acid sequences for the species listed in Table 2 were submitted directly to the SWISS-MODEL database employing the First Approach method (<http://www.expasy.ch/swissmod/SWISS-MODEL.html>). HIF2 α was manually

selected as the template, but was also chosen by the automated SWISS-MODEL server as the best template. Models were viewed using the free software Swiss-Pdbviewer for Mac OSX (<http://www.expasy.ch/spdbv/mainpage.html>) (Guex and Peitsch 1997; Peitsch 1995; Schwede *et al.* 2003). RMS deviations were calculated before and after each three-dimensional model was adjusted with Best Structural Fit relative to the mouse AHR model to assess improvements in alignment. Previous studies have shown the PAS structures are useful for predicting functional site locations and general features of unknown mechanisms (Denison *et al.* 2002; Pandini and Bonati 2005; Procopio *et al.* 2002).

3. RESULTS

3.1 Mink and Polar Bear AHR

Unique nucleotide sequences were obtained from mink and polar bear RNAs (Figure 1), sharing high identity with other AHR sequences published in GenBank in BLASTn searches (mink: 93% identity to Carnivore and 88% identity to Cetacean AHRs; polar bear: 97% identity to Primate, and 91-95% identity to Cetacean and Carnivore AHRs). BLASTp searches of GenBank revealed 94-98% amino acid identity to known AHRs included in this study. The top three database matches for mink were dog, harbor seal, and Baikal seal AHRs; for polar bear they were white-beaked dolphin, Atlantic white-sided dolphin, and pilot whale AHRs. These sequences correspond to a highly conserved portion of the AHR protein, the PAS region, and degenerate primers have been successful in amplifying this region from many vertebrate species including cetaceans (Chapter 2), fish, a turtle, and amphibians (Karchner *et al.* 2000; Karchner *et al.* 1999; Lavine *et al.* 2005). Repeated attempts at extending the sequence information by rapid amplification of cDNA ends were unsuccessful for both mink and polar bear (data not shown). The presence of introns impeded our ability to obtain additional coding sequence, and may have been the result of incompletely

spliced RNA. Genomic DNA contamination in the RNA solutions may have reduced amplification efficiency. Contamination with genomic DNA was combated with DNase treatment and further mRNA purification, but these procedures did not yield better results.

3.2 Mammalian AHR Amino Acid Sequence Alignment

Functional domains have been determined experimentally for mouse and human AHRs. The ligand binding domain (LBD) roughly corresponds to the PAS-B domain, and includes amino acids 230-402 in mouse and 236-408 in human. Alignment of full-length AHR sequences listed in Table 2 illustrates the high conservation of amino acid sequences among mammals (Figure 2), and therefore we used the mouse and human AHRs as templates for identifying functional domains in new sequences. The variability in the carboxy-terminus noted by other researchers is less pronounced in this alignment, probably due to the increased taxa sampling within the eutherian mammals. Ligand binding domains from full-length and partial AHR sequences were aligned using the functionally determined mouse and human LBDs as guides (Figure 3). The high degree of amino acid identity among LBDs (84 – 99% within mammals, 76-82% to killifish) suggests conservation of function among vertebrate AHRs.

Maximum parsimony analysis of the LBD alignment identified 22 parsimony informative characters among the 173 amino acid, ungapped data set (indicated in Figure 2B with arrows and green shading). The most parsimonious phylogenetic tree was determined with branch and bound analysis. The resulting tree, rooted with killifish AHR1, illustrates the number of similarities among these species (Figure 4A). In particular, the cetacean branches are completely unresolved and essentially identical. Similar analysis of full-length AHR amino acid sequences from these same species introduces more areas of variability (229 parsimony informative characters of 769 residues, excluding gaps), permitting differentiation of each taxon. The tree derived from full-length

sequence more closely approximates the accepted mammalian phylogeny (Figure 4B; (Murphy *et al.* 2001)). These results indicate that the LBD contains limited information for determining phylogenetic relationships of each AHR, and may be more suitable for identifying similarities among species.

3.3 Biochemical Characterization

At this point, assessments of ligand binding activity cannot rely solely on sequence similarities to predict the binding characteristics of AHRs from unstudied species. Experimental data are required to define ligand binding affinities, and ideally the experimental design should include all species of interest within the same assay for comparison. In the previous chapter, extensive comparisons were made between North Atlantic right whale, humpback whale, beluga whale, mouse, and human AHRs to determine specific binding and binding constants. The approach here is similar, but includes more species, on a smaller scale.

Two independent saturation binding assays were conducted, the first using all 8 concentrations of [³H]TCDD, the other with a single high concentration (5.5 nM, nominal, 2-3 nM final) plus a DMSO control. The same volume of diluted IVTT AHR (100 μL) was used for each receptor in the saturation binding assay. The humpback whale and hamster AHR constructs using pcDNA vector were only available for the latter experiment. Specific binding was calculated as the difference between total binding and non-specific binding (UPL). Nonlinear regression analysis, equilibrium dissociation constants (K_d), and maximum binding (B_{max}) were derived for the first experiment assuming one-site specific binding (Figure 5).

A single concentration of [³H]TCDD was incubated with IVTT expressed AHR proteins to determine the maximum specific binding capacity of each species' AHR. Whale and hamster AHRs exhibited the greatest binding capacities (>0.9 nM; Figure 6), but also produced higher amount of protein than

the other constructs. In general, greater concentrations of IVTT AHR resulted in higher specific binding when incubated with a single high concentration of [³H]TCDD ($r^2 = 0.59$; Figure 7). However, even in the same expression vector, each construct does not express the same amount of AHR protein. We suggest that future experiments account for the difference in AHR expression by modifying the proportion of specific protein.

Previous work (e.g. Chapter 3) has shown variability among replicate saturation binding assays, complicating the statistical differentiation of individual AHRs within a narrow range of relative binding affinity (K_D) values. In order to place the single K_D value obtained in this study in context with such variation, we included the relative binding affinities calculated from multiple saturation binding assays, including the one represented in Figure 4; these data are shown in Figure 8. The K_D for the harbor seal AHR is similar to that obtained for the initial characterization of that protein (Kim and Hahn 2002). While some receptors exhibit a greater range of values than others in these assays, the distributions of K_D s are balanced above and below the means. The single values for harbor seal and guinea pig AHRs are within the expected range of values, but may not fully represent the range of binding affinities specific to each species.

3.4 Homology Modeling

Tertiary structures of the ligand binding domain were predicted using the SwissModel PDB First Approach method for right whale, humpback whale, beluga whale, cow, dog, harbor seal, Baikal seal, mouse, hamster, and guinea pig aryl hydrocarbon receptors. All structures were based on the HIF2 α (PDB# 1p97a) peptide characterized by NMR (Erbel *et al.* 2003). Fragments from mink, polar bear, and other cetaceans (sequences from Chapter 2) were too small for modeling. Computations for energy minimization of each model were accomplished with GROMOS96 implementation, and each structure was aligned relative to the mouse AHR model by Best Structural Fit in the Swiss-PDBViewer.

Similarity of tertiary structure was the overwhelming result of these first approximations. All models possess a large six-stranded, anti-parallel beta-sheet formed by a combination of amino- and carboxy-ends of the LBD. One major alpha-helix occurs at the approximate mid-point in the LBD, and two other smaller helices (or a single “broken” or twisted helix) flank the beta-sheet. An irregular loop connects these helices and sometimes includes a beta-turn or partial helix, depending on the species. Together, these structures define a hollow cylindrical space that is the likely ligand binding “pocket” (Figure 9, Figure 10A).

The morphology of the predicted tertiary structure of AHR LBDs differs at two irregularly structured regions. A Minor Loop within the beta-sheet shows slight variation among some species (e.g. it is the site of the only apparent differences in human versus rodent models), and may be dependent on the overall beta-sheet construction specific to each sequence. A second irregular feature (Major Loop) varies from an undifferentiated loop to a loop with a beta-turn. This feature spans the region between the major alpha-helix and the small helices, occupies a larger relative space than the other variable loop in the beta-sheet, and as such may be capable of greater flexibility. The latter site is found to be the site of the greatest variation among models.

The predicted tertiary structures of all three whale AHR LBDs were identical, as expected from the paucity of primary sequence variation in the LBD seen in the alignment and accentuated in the phylogenetic tree. Cow, dog, and harbor seal also share identical tertiary structures with the whale LBD, but the Baikal seal differs noticeably in one location. Among the Cetartiodactyls and Carnivora, the Major Loop contains a more rigid predicted structure that may be considered a beta-turn, with the exception of the Baikal seal, which lacks this feature and remains an irregular and highly flexible loop (Figure 10B). The functional relevance of this potential mobility cannot be inferred in the absence of

in vitro AHR binding data or *in vivo* studies for the Baikal seal. This Major Loop structure is otherwise unique to Cetartiodactyla and Carnivora.

Interestingly, the mouse and hamster AHR LBDs are identical to one another, and both differ from the human model only slightly at the Minor Loop (Figure 10C). Although large differences in K_D were shown experimentally, the B_{max} values for these species were very similar and this may be related to the similar structure of the ligand binding pockets.

The model for guinea pig AHR LBD is decidedly different (Figure 9D). A much larger portion of the protein lacks tertiary structure, including the loss of one beta strand and a truncated second beta-strand at the amino-end of the sequence, an abbreviated minor alpha-helix, and the absence of the Major Loop beta-turn (the latter as in the other Euarchontoglires). The major alpha-helix is more loosely coiled. The end result is a very unstructured LBD that may be more susceptible to the influences of other parts of the full-length protein, and may perhaps have consequences for ligand binding.

4. DISCUSSION

While others have shown that certain differences in the amino acid sequence of AHR result in changes in ligand binding affinity, none have questioned whether these differences can be used to infer a species-level predilection to POP sensitivity and increased risk of biological effects. This approach could become a first-approximation tool for regulating human activities and improving environmental conditions for endangered species.

4.1 New AHR Sequence Acquisition

As seen in the alignment of full-length AHR sequences, an expanded series of closely related taxa improves the overall alignment. Specifically, the

carboxy-terminal region of the protein appears much less variable than other authors have observed when comparing more distantly related species. While our knowledge of full-length AHR sequences is still limited, the partial sequences recently acquired for odontocete cetaceans (Chapter 2), mink and polar bear do provide more information on the diversity of AHRs within Mammalia. Subtle differences in binding characteristics and inferred tertiary structure may not be fully explained by these partial sequences; therefore, continued efforts to clone and sequence full-length AHR cDNAs remains worthwhile to better understand this pathway.

4.2 Relative Binding Affinities of Mammalian AHRs

Although individual species are often studied in depth, few studies have directly compared the binding characteristics of multiple species in order to understand the relative differences in organismal sensitivity to DLC. Here we show the relative binding affinities of AHRs from both endangered wildlife species and model organisms, but replicate assays are necessary to increase confidence in the mean binding constants and account for assay variability. A single experiment was conducted using the hamster AHR cDNA in an alternative expression vector; however, the results were ambiguous (data not shown). We have found that consistent use a single expression vector reduces intra-assay variability and eliminates a factor of uncertainty; future experiments should include the use of hamster AHR in pcDNA.

4.3 Predicting Sensitivity by Amino Acid Analysis: functionally significant polymorphisms and critical residues

The significance of any one amino acid in AHR function has been explored primarily in rodent and human studies (Okey *et al.* 2005). Eight point mutations are known among four strains of mice, two of which occur in the PAS regions

causing single amino acid changes. The switch from alanine to valine at position 375 (A375V) results in a reduction in affinity for ligand; accompanied by a L348F change, binding is reduced to a “dioxin-resistant” phenotype (Poland *et al.* 1994). A similar situation was found in the comparison of the high-affinity mouse AHR with low-affinity human AHR. The mouse amino acid 375 (A) corresponds to residue 381 (V) in human, and a V381D mutation in the human AHR abolishes ligand binding (Ema *et al.* 1994; Ramadoss and Perdew 2004).

Han/Wistar rats are among the most dioxin-resistant mammals tested, and possess a truncated AHR protein resulting from a single point mutation at the start of intron 10, which disrupts proper splicing and eliminates approximately 40 amino acids from the carboxy-terminus (Pohjanvirta *et al.* 1998). While the rat AHR mutation is outside the range of the LBD, it illustrates the power of a single site to influence the structure of the protein. Hamsters are similar to Han/Wistar rats in exhibiting a more dioxin-resistant phenotype, whereas another popular rodent model, the guinea pig, is among the most dioxin-sensitive. In hamsters, the glutamine (Q) rich carboxy-terminus is substantially expanded and enriched with Q compared with other AHRs (Korkalainen *et al.* 2000). In contrast, the same region in the guinea pig AHR is only half the size of that from hamster. A distinct correlation can be seen across published mammalian AHR sequences between the number of glutamine residues in the carboxy-terminal region and sensitivity to acute dioxin lethality (Korkalainen *et al.* 2001). These studies illustrate the potential importance of regions besides the LBD in the function of AHR in mammals.

A recent study in birds, however, addresses the specific importance of single amino acid differences the AHR LBD (Karchner *et al.* 2006). Chicken and common tern are closely related species that have highly similar AHR amino acid sequences, yet terns are 80- to 250-fold less sensitive than chickens to the effects of HAH (Brunstrom and Halldin 1998; Hoffman *et al.* 1998; Lorenzen *et al.* 1997). Only three amino acids differ in the LBD of these AHRs. Site-directed

mutagenesis experiments show that two of these sites in the chicken AHR (I324 and S380) are responsible for its high-affinity dioxin binding, as well as its ability to activate gene expression in a reporter gene system (Karchner *et al.* 2006). In contrast, the common tern AHR LBD possesses valine and alanine residues at the analogous sites and has reduced binding and transactivation functions. This study succinctly illustrates the relationship between a few amino acids in the AHR LBD and dramatic differences in organismal sensitivity to dioxin. Additionally, the chimeric chicken-tern AHR constructs used in this study show that not only differences in the carboxy-terminal “transactivation domain” impact transcriptional activity but that the LBD may influence this function as well.

4.4 Homology Modeling AHR

Homology models are only as good as the original structural data used as a template. Because neither an NMR structure nor an x-ray crystal structure exists for any species' AHR, we based our models on a related PAS-family protein, hypoxia-inducible factor 2-alpha (HIF2 α ; PDB # 1P97). Although PAS-family proteins do not share a large amount of direct amino acid sequence identity, certain regions – namely the PAS regions – contain regular motifs suitable for inferring structural elements. Appropriate templates for homology models need only share only 20-25% amino acid sequence identity with the target sequence (Gale Rhodes, website, unreferenced). The structure for the PAS-B region of HIF2 α is based on NMR spectroscopy, and is the synthesis of 26 models (Erbel *et al.* 2003). The HIF2 α PAS-B model may be the best template available until crystal structures for an AHR become available. In the meantime, attempts at homology modeling AHRs are only approximations of reality, and serve best as illustrations of hypothetical conditions, or for guiding experimental design.

Our goal was to compare the relative structures of AHRs from different mammalian species. In pursuit of this goal, whether or not the model accurately represents the true structure of AHR is less relevant. As long as all the AHR

models here were constructed in like fashion (as they indeed were), comparisons among and between species should remain valid approximations of species-specific variations. Combined with the experimental work of many others, these comparisons contribute to the hypothesis that discrete residues in the LBD are critical for AHR function. Refinement of homology modeling techniques or the addition of AHR crystal structures to existing databases could advance the accuracy of our comparative models.

Others have recently attempted to use homology modeling techniques to infer the tertiary structure of the aryl hydrocarbon receptor based on the crystal structures of HERG and FixL (Denison *et al.* 2002; Procopio *et al.* 2002). The AHR models presented here are consistent with, and only subtly differ from, these models. Ours is the only AHR structure modeled after HIF2 α , and may represent a more accurate representation as both the template and AHR dimerize with ARNT via the beta-sheet in the LBD. We expect that the remainder of the AHR protein influences the final folding conformation and consequently the function; thus, there remains a need for additional work including crystallization of the AHR. While our results are approximations, this data set increases the availability of sequence information and increases confidence in previous homology models for AHR by approaching the model via a new template.

The combination of sequence analysis, biochemical characterization, and homology modeling presents another tool for explaining differences in AHRs. We suggest that this approach provides a good framework for presenting testable hypotheses using increasingly available sequence data in public databases, and could be extended for use in other systems. Several questions have emerged from this work thus far for which we could imagine developing experiments. First, for species that show high conservation of amino acid sequence and whose tertiary structural models indicate identical LBDs, differences in ligand binding may not be related to the PAS-B region. What is the impact of the few, specifically different residues on ligand binding? Could these

variable sites confer differences in ligand specificity that are not seen in experiments with TCDD? Alternatively, other portions of the protein may influence the accessibility of the binding pocket. Furthermore, does the size and accessibility of the binding pocket alter the probability of ligand insertion? Could a wider potential binding pocket relax constraints on ligand docking, and if so, would there be consequences for binding efficacy? These questions could be addressed first with virtual mutagenesis and ligand docking studies using the existing models, followed by site-directed mutagenesis and chimeric or truncated AHR constructs in functional bioassays.

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Table 1. Degenerate aryl hydrocarbon receptor primers.

Name	Sequence (5' → 3')	T _m (°C)
D33F	GTC IAA CCC ITC IAA GMG ICA YMG	64
D143F	CAR GAY TAY CTI GGI TTY CAI CA	62
D328R	GCI GCG TGR ATR AAY TGR TAI CC	63
D348R	CAT ICC RCT YTC ICC IGT YTT	62

Designed by Karchner/Hahn; synthesized by Integrated DNA Technologies.

Table 2. Aryl hydrocarbon receptor sequences used for multiple alignment and homology modeling. Asterisks indicate those sequences used for in vitro assays.

Species	GenBank Accession #
North Atlantic right whale (NARW; <i>Eubalaena glacialis</i>)	EF091829 *
Humpback whale (<i>Megaptera novaeangliae</i>)	EF091830 *
Beluga whale (<i>Delphinapterus leucas</i>)	AF332999 *
Pilot whale (<i>Globicephalus melas</i>)	EF091833
Atlantic white-sided dolphin (AWSD; <i>Lagenorhynchus acutus</i>)	EF091832
White-beaked dolphin (WBD; <i>L. albirostris</i>)	EF091831
Cow (<i>Bos taurus</i>)	XP612996; predicted
Harbor seal (<i>Phoca vitulina</i>)	AB056700 *
Baikal seal (<i>Phoca siberica</i>)	AB072432
Mink (<i>Mustela vison</i>)	this study; not yet submitted
Polar Bear (<i>Ursus maritimus</i>)	this study; not yet submitted
Dog (<i>Canis familiaris</i>)	XP532485; predicted
Human (<i>Homo sapiens</i>)	L19872 *
Mouse (<i>Mus musculus</i>)	M94623 *
Guinea Pig (<i>Cavia porcellus</i>)	AY028947 *
Hamster (<i>Mesocricetus auratus</i>)	AF275721 *
Killifish AHR1 (<i>Fundulus heteroclitus</i>)	AF024591 *
HIF2 α (PDB #1P97)	

mink 1 C A G G A T T A T C T G G G G T T T C A G C A G T C T G A T G T C A T A C A T C A G A G C G T A T A T G A A C T T A T T 60
polar bear 1 C A G G A T T A T C T G G G G T T T C A G C A G T C T G A T G T C A T A C A T C A G A G T G T A T A T G A A C T T A T C 60

mink 61 C A C A C T G C A A G A C C G A G G T G A G T T T C A G C G T C A G C T A C A C T G G A C A T T A A A C C C T T C A C C 120
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mink 121 A T G T A C A G A C T C T G G A C A A A G A A T T G A T G A G G C T A A T G G C C T G C C A C A A C A G A T A G G C T G 180
polar bear 120 G T G T A C A G A G T C T G G A C A A G A A T T G A A G A G C C A G T G G T C T G C C C A G A C A G A T A G G C T G 179

mink 181 T T A T A C C C A G A C C A G C T T C C T C C A G A A A A C T C T T C C T T A T G G A A A G G A G C T T C G T G T G 240
polar bear 180 T T A T A C C C A G A C C A G A T T C C T C C A G A A A A C T C T C C T T A T G G A A G G A T G C T T C A T G T G 239

mink 241 C C G A C T A A G A T G T C T G C T G G A C A A T T C A T C T G G T T T T C T G G C A A T G A A T T T C C A A G G G A G 300
polar bear 240 T C G T C T A A G G T G T C T G C T G G A T A A T T C A T C T G G T T T T C T G G C A A T G A A T T T C C A A G G G A G 299

mink 301 G T T A A A G T A T C T T C A T G G A C A G A A C A A G A A A G G G A A A G A T G G A T C G C T A C T T C C C C C G C A 360
polar bear 300 G T T G A A G T A T C T T C A T G G A C A G A A C A A G A A A G G G A A A G A T G G A T C A A T A C T T C C A C C T C A 359

mink 361 G C T G G C T T T G T T C G C A A T A G C A A C T C C C C T T C A G C C A C G T C C A T C C T T G A G A T T C G G A C 420
polar bear 360 G T T G G C T T T G T T T G C A A T A G C T A C T C C A C T G C A G C C A C C A T C C A T A C T T G A A T C C G A A C 419

mink 421 C A A A A A T T T C A T C T T T A G A A C C A A A C A C A A A C T A G A C T T T A C G C C C A C T G C T T G T G A T G C 480
polar bear 420 C A A A A A T T T C A T C T T T A G A A C C A A A C A C A A A C T A G A C T T T A C A C C T A C T G T T G T G A T G C 479

mink 481 C A A A G G A A A A C T T G T T T T A G G C T A T A C T G A A G C A G A G T T G T G C A T G A A G G A T C A G G C T A 540
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mink 541 C C A A T T C A T C C A C G C C G C 558
polar bear 540 C C A G T T C A T C C A C G C C G C A 558

Figure 1. Partial cDNA sequences of mink and polar bear aryl hydrocarbon receptor.

Sequences derived from nested RT-PCR yielded partial aryl hydrocarbon receptor cDNAs (from polyadenylated RNA, primers D33F/D348R followed by D143F/D328R).

Figure 2. Alignment of full-length mammalian AHR amino acid sequences.

Aryl hydrocarbon receptor amino acid sequences for which the entire cDNA is known from cloning or genome analysis were aligned using ClustalW in MacVector 8.1.1. Killifish (*Fundulus heteroclitus*) AHR1 is shown in the bottom row. Identical residues are outlined and shaded grey. The full alignment spans two pages.

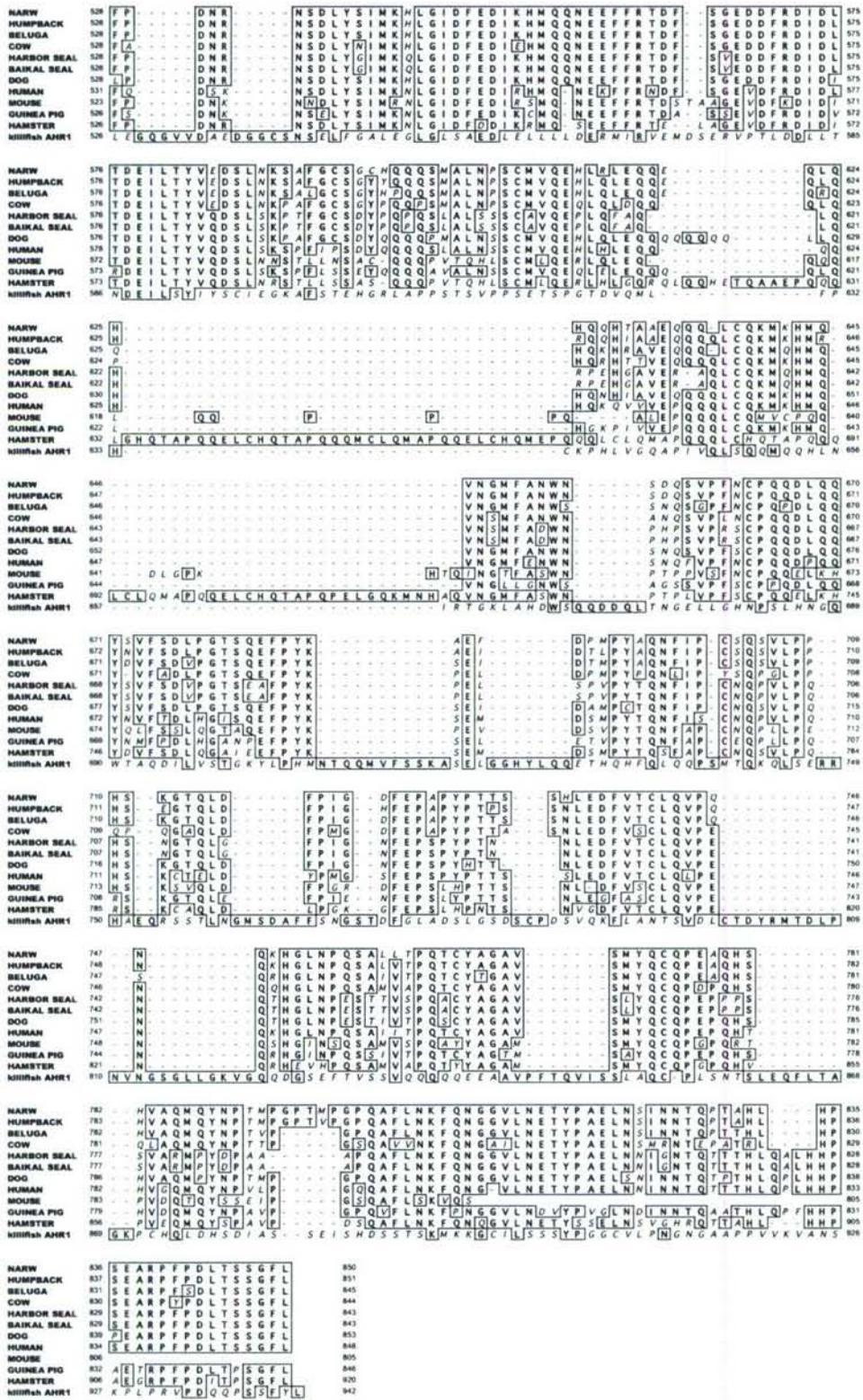
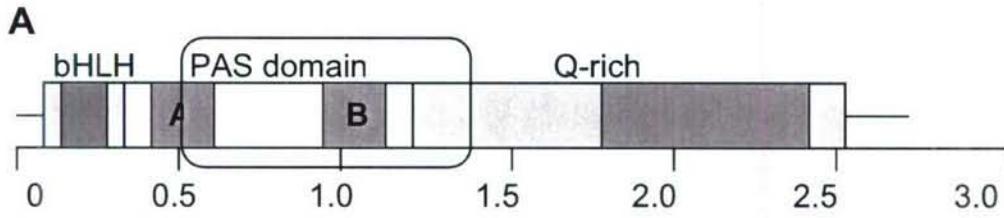


Figure 2 continued.

Figure 3. Aryl hydrocarbon receptor ligand binding domains are well conserved in mammals.

(A.) A general schematic of a prototypical AHR is shown; lower scale bar indicates cDNA length in kilobases, and boxes represent protein sequence with the basic-helix-loop-helix (bHLH), PAS, and glutamine-rich (Q-rich) regions shaded. Based on functional studies of mouse and human AHRs, the ligand binding domain (LBD; boxed in A) encompasses the PAS B domain; homologous regions of other mammalian AHRs were selected for alignment. Amino acid sequences of 16 mammalian and one fish AHR LBD were aligned using ClustalW in MacVector 8.1.1 (B). Identical residues are outlined and shaded grey. The high degree of amino acid similarity suggests conservation of function among vertebrate AHRs. Parsimony informative characters identified using PAUP*4.0 are indicated with arrows and green shading. A single amino acid responsible for much of the binding difference in human and mouse AHRs is indicated with the upward-pointing, dashed arrow. Irregular structural regions or loops showing the greatest variation in homology models are identified in aqua and orange boxes, corresponding to the sequential secondary structure coloring in Figure 9.

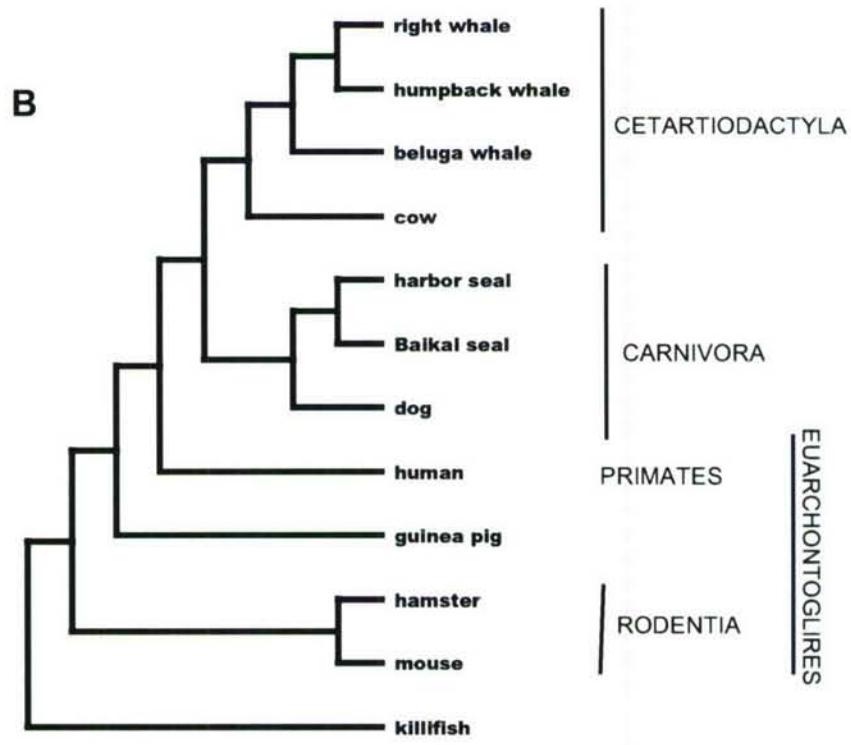
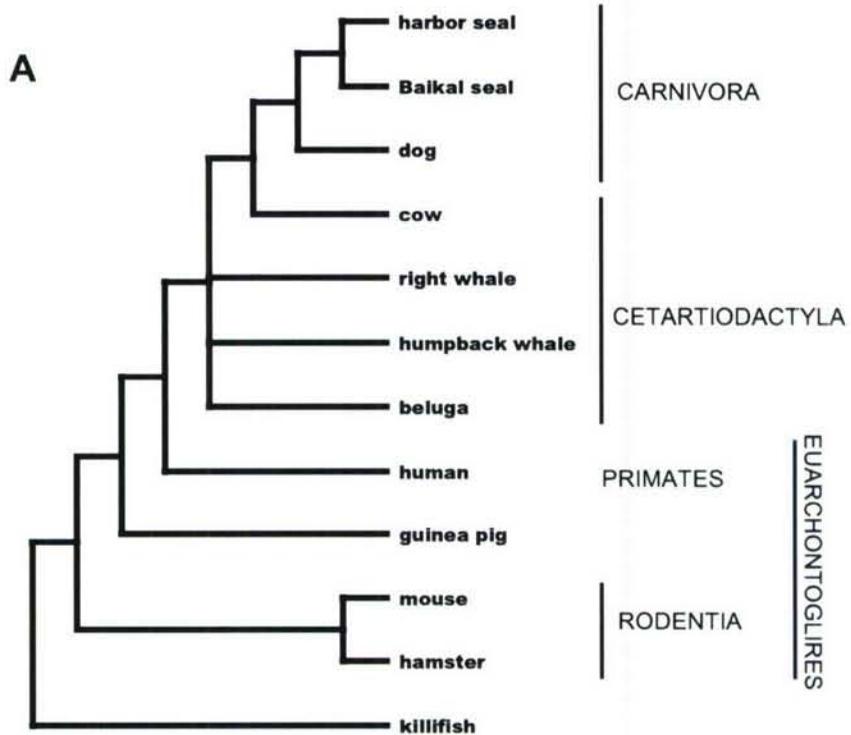


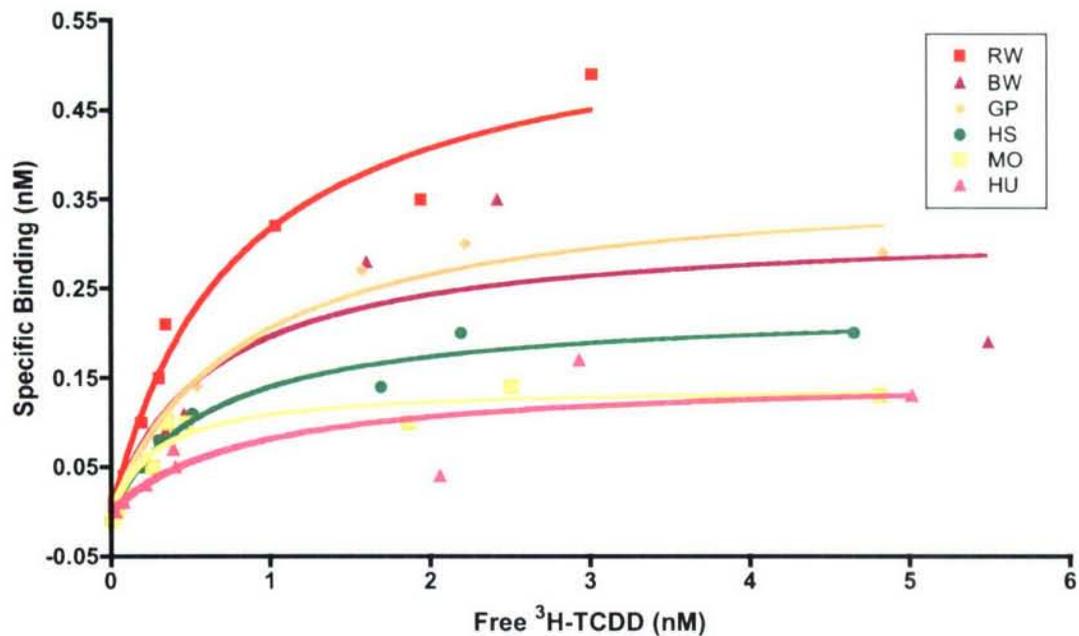
B

NARW	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
HUMPBACK	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
BELUGA	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
PILOT WHALE	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
AWSD	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
WBD	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
COW	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
HARBOR SEAL	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
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MINK	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
POLAR BEAR	1	AMN	FQGR	LKYL	HGQN	KKGG	DGS	LPP	QAL	FAI	ATP	LQPP	SILE	IR	TKN	FIF	R	T	K	H	K	L	D	F	60																																					
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killifish AHR1	1	A	L	N	T	Q	G	R	L	K	F	L	H	G	Q	N	D	Q	R	E	N	G	G	K	A	T	P	Q	L	A	L	F	A	I	A	T	P	L	Q	P	P	S	I	L	E	I	R	T	K	N	F	I	F	R	T	K	H	K	L	D	F	60
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HUMPBACK	61	T	P	T	G	C	D	A	K	G	R	I	V	L	G	Y	T	E	A	E	C	M	R	G	S	G	Y	Q	F	H	A	A	D	M	L	Y	C	A	E	Y	H	I	R	M	I	K	T	G	E	S	G	M	I	V	F	R	L	L	T	120		
BELUGA	61	T	P	T	G	C	D	A	K	G	R	I	V	L	G	Y	T	E	A	E	C	M	R	G	S	G	Y	Q	F	H	A	A	D	M	L	Y	C	A	E	Y	H	I	R	M	I	K	T	G	E	S	G	L	I	V	F	R	L	L	T	120		
PILOT WHALE	61	T	P	T	G	C	D	A	K	G	R	I	V	L	G	Y	T	E	A	E	C	M	R	G	S	G	Y	Q	F	H	A	A	D	M	L	Y	C	A	E	Y	H	I	R	M	I	K	T	G	E	S	G	M	I	V	F	R	L	L	T	113		
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MINK	61	T	P	T	A	C	D	A	K	G	R	I	V	L	G	Y	T	E	A	E	C	M	R	G	S	G	Y	Q	F	H	A	A	D	M	L	Y	C	A	E	Y	H	I	R	M	I	K	T	G	E	S	G	M	I	V	F	R	L	L	T	93		
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DOG	61	T	P	T	A	C	D	A	K	G	R	I	V	L	G	Y	T	E	A	E	C	M	R	G	S	G	Y	Q	F	H	A	A	D	M	L	Y	C	A	E	Y	H	I	R	M	I	K	T	G	E	S	G	M	I	V	F	R	L	L	T	120		
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killifish AHR1	61	T	P	I	G	C	D	A	K	G	R	I	V	L	G	Y	T	E	A	E	C	M	R	G	S	G	Y	Q	F	H	A	A	D	M	L	Y	C	A	E	Y	H	I	R	M	I	K	T	G	E	S	G	M	I	V	F	R	L	L	T	120		
NARW	121	K	D	N	R	W	T	V	Q	S	N	A	R	L	V	Y	K	N	G	R	P	D	Y	I	I	A	T	Q	R	P	L	T	D	E	E	G	T	E	H	L	R	K	R	N	M	K	L	P	F	M	F	T	173									
HUMPBACK	121	K	D	N	R	W	T	V	Q	S	N	A	R	L	V	Y	K	N	G	R	P	D	Y	I	I	A	T	Q	R	P	L	T	D	E	E	G	T	E	H	L	R	K	R	N	M	K	L	P	F	M	F	T	173									
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PILOT WHALE	114	K	D	N	R	W	T	V	Q	S	N	A	R	L	V	Y	K	N	G	R	P	D	Y	I	I	A	T	Q	R	P	L	T	D	E	E	G	T	E	H	L	R	K	R	N	M	K	L	P	F	M	F	T	113									
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COW	121	K	D	N	R	W	T	V</																																																						

Figure 4. Phylogenetic analysis of mammalian AHR.

Maximum parsimony analysis of either the LBD (A) or the full length AHRs (B) generally recapitulated the accepted view of mammalian phylogeny (Murphy *et al.* 2001). Analysis of just the LBD identified 22 informative residues of the 173 ungapped amino acid characters (as highlighted in Figure 3), but was unable to differentiate between the whale AHRs, as shown by the collapsed branches. The LBD alone is not sufficient to decipher phylogenetic relationships. Outgroup was killifish (*Fundulus heteroclitus*) AHR1.





	RW	BW	GP	HS	MO	HU
BMAX	0.5709	0.3199	0.3748	0.2291	0.1414	0.1527
KD	0.8028	0.6292	0.8222	0.6337	0.2998	0.8692

Figure 5. Saturation binding analysis of IVTT AHRs from marine mammals and rodents.

Specific binding curves were derived from incubation of IVTT AHRs with nine concentrations of [³H]TCDD in a saturation binding assay. Maximal binding (B_{max}) and relative binding affinities (K_D) specific to this experiment are shown below.

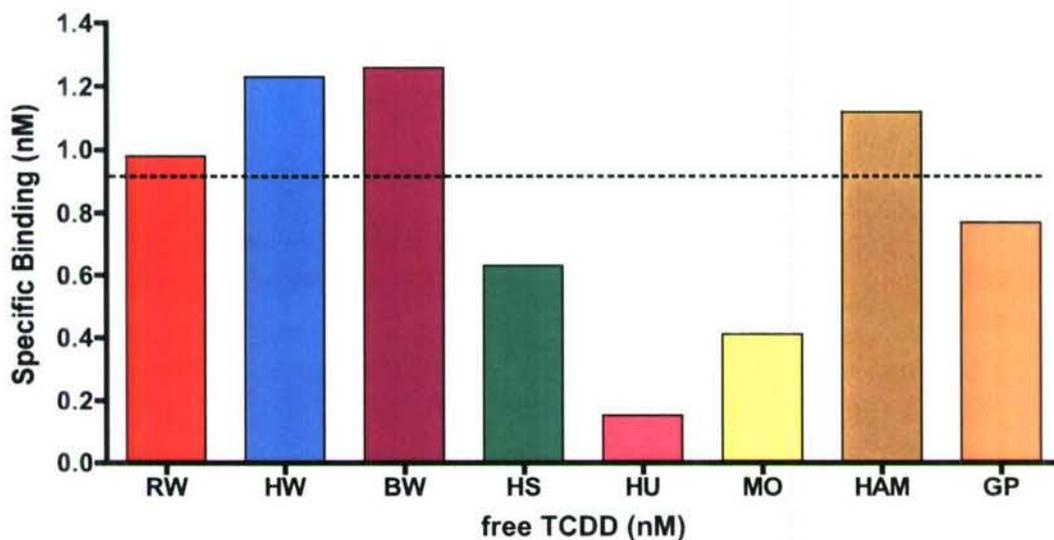


Figure 6. Maximum binding capacity of mammalian AHRs *in vitro*.

A single concentration of [³H]TCDD was incubated with IVTT expressed AHR proteins to determine the maximum specific binding capacity of each species' AHR. Whale and hamster AHRs exhibited the greatest binding capacities (>0.9 nM), but also produced higher amount of protein than the other constructs.

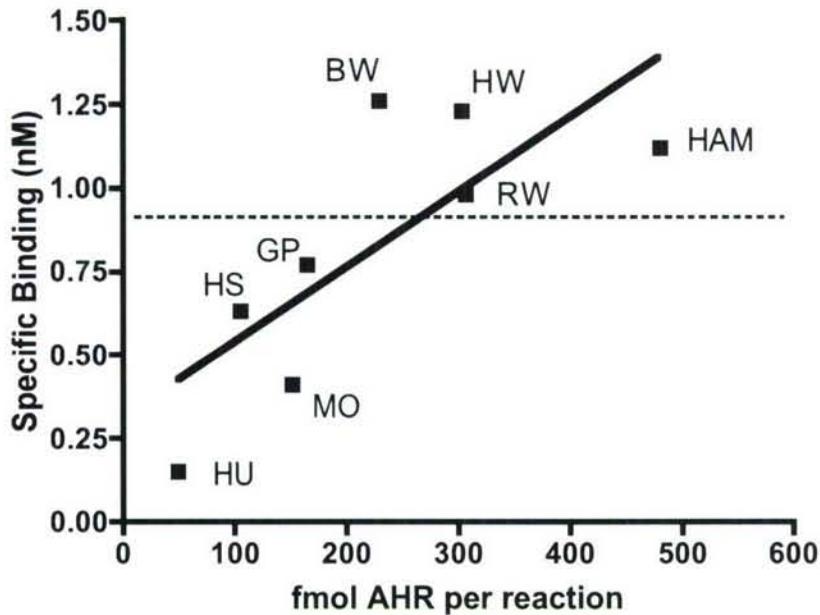


Figure 7. Specific binding is related to AHR protein concentration.

In general, greater concentrations of IVTT AHR resulted in higher specific binding when incubated with a single high concentration of [³H]TCDD (5.5 nM nominal; $r^2 = 0.59$). The same volume of IVTT AHR (100 μ L) was used for each receptor in the maximum binding capacity assay. However, even in the same expression vector, each construct does not express the same amount of AHR protein.

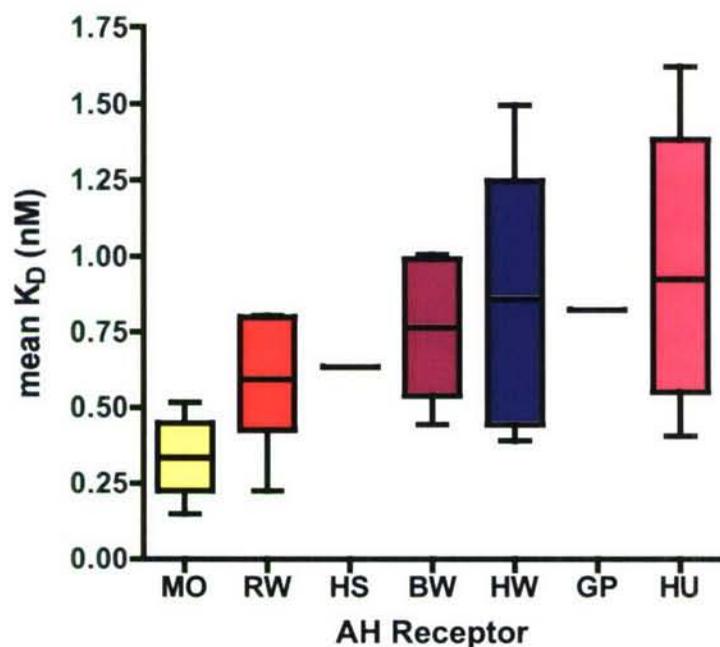


Figure 8. Relative binding affinities for IVTT expressed AHRs in saturation binding assays.

Relative binding affinities of multiple saturation binding assays with [³H]TCDD are combined in a boxplot to illustrate the range of values acquired. Sample sizes vary: MO n = 6, RW n = 9, HS n = 1, BW n = 6, HW n = 5, GP n = 1, HU n = 6.

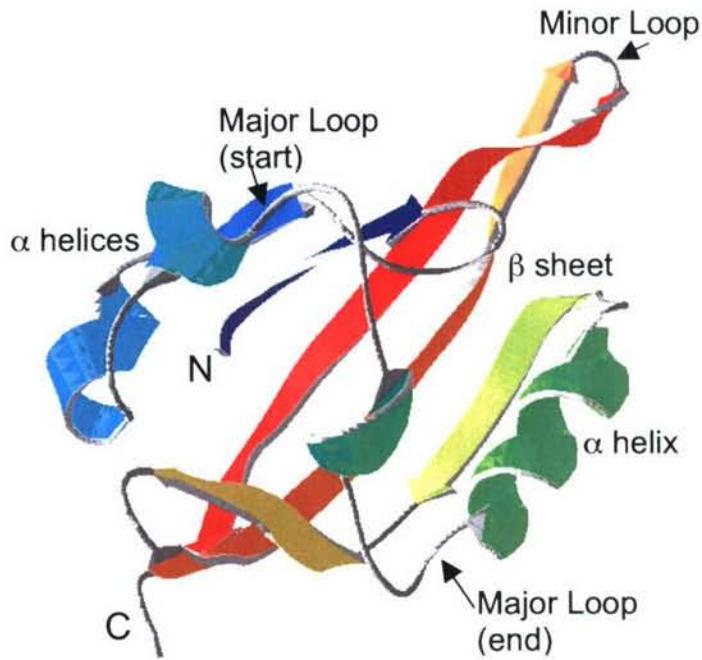


Figure 9. Predicted tertiary structure of AHR LBD as represented by the NARW homology model.

The basic morphology of the AHR LBD is illustrated by with the homology model of the north Atlantic right whale sequence. Sequential secondary structure coloring begins at the amino terminal end (N) in blue, and concludes at the carboxy terminal end of the LBD (C) in red. All AHR LBDs modeled after the HIF2 α PAS domain share major structural features including a six-stranded beta sheet, a highly conserved structure among PAS family proteins important for cofactor binding. The beta sheet is flanked by a large alpha helix on one side (green), and several smaller helices on the other (aqua shades). Pairwise comparison of homology models revealed two regions of variability: a Minor Loop between beta strands, and a Major Loop between the helices.

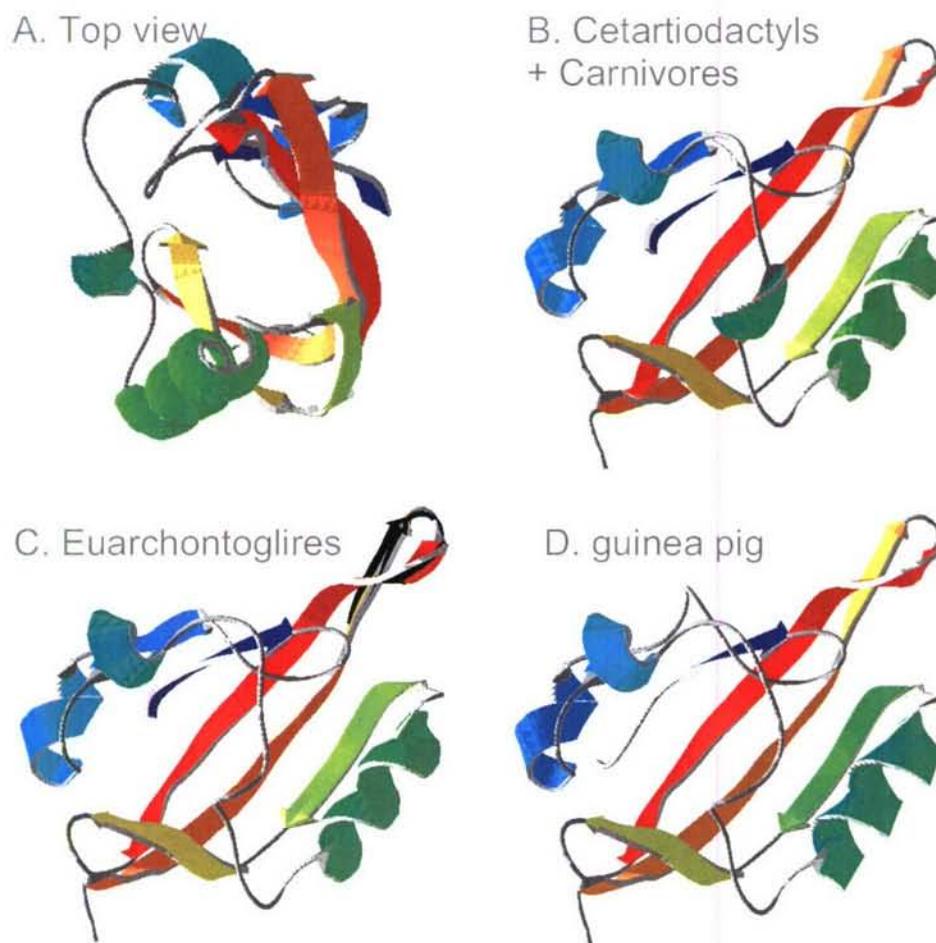


Figure 10. Comparisons of AHR LBD predicted tertiary structure among mammals by homology modeling.

An alternative perspective (A, top view) of the NARW AHR model suggests the space in which a ligand might fit. With the exception of the Baikal seal, all Cetartiodactyl and Carnivore models were identical, and possess a small beta-turn or partial helix in the midst of the Major Loop (B). In contrast, human and rodent models had no such structural element in the Major Loop, and were virtually identical except for subtle deviations in the Minor Loop (C). Additional differences are seen in the guinea pig model (D), such as truncated beta strands, and more undifferentiated loops. These models cannot resolve the differences observed in binding properties, such as those seen in mouse and human, but offer an interesting perspective on the relative importance of individual amino acid differences on receptor activity, and help to generate many more questions about the relationship between AHR structure and function.

Chapter 5: Summary and Future Research

Extensive environmental contamination combined with the inherent complexity of the natural world together present a difficult problem to people concerned with regulating and reducing harmful inputs, and for mitigating effects on both wildlife and humans. Multiple, complementary approaches are necessary to connect environmental contaminant exposures with biological effects. Progress has been slow, in particular in terms of wildlife health, and the “weight of evidence” approach (Ross 2000) has been the most powerful, but weakly effective, means of producing a change in industrial practices and human behavior. Some attempts to link environmental exposure with systematic responses have begun to emerge recently, specifically addressing the health of marine mammals (Wilson *et al.* 2005). However, much remains to be done. Projects involving pinnipeds or small cetaceans that can be repeatedly captured alive and sampled extensively (for example, the HERA Project) may provide the best information on individual, population, and temporal factors important for assessing pollutant exposures and risks to marine mammals. In the absence of such data sets for endangered species and large whales, the use of *in vitro* systems and model organisms is a more accessible means of predicting or inferring the biological effects of environmental contaminants. The work presented in this thesis takes advantage of available molecular and biochemical techniques, as well as tools from model species, to learn more about marine mammal toxicology through the examination of the aryl hydrocarbon receptor function and diversity.

1. Major Conclusions

1.1 Integument biopsies from North Atlantic right whale may be used to clone genes of toxicological and physiological significance.

We first showed that the application of two established techniques – minimally invasive integument sampling from living whales, and cDNA cloning – could be combined to successfully amplify fragments of several genes of

toxicological and physiological significance from the North Atlantic right whale. While these methods have existed for some time, the use of this tissue source for RNA extraction had not been performed (Lapseritis and Hahn 2004). From this RNA we acquired initial cDNA sequence information for AHR and CYP1A1. The quality of the biopsy-derived RNA was compared with that from stranded cetaceans at various time points post-mortem; the former was of higher quality and yield, but the latter was also useful for amplifying these sequences, including a sequence for leptin. The use of biopsied tissues to identify expressed genes opens a new avenue of research to explore the physiological status of living whales.

1.2 First characterization of aryl hydrocarbon receptor in mysticetes.

The initial fragments of AHR reported in Chapter 2 ultimately yielded full-length cDNA sequences for North Atlantic right whale and humpback whale (Chapter 3). These sequences were ligated in expression vectors for a variety of in vitro assays to determine biochemical functions relative to previously characterized beluga whale, human, and mouse AHRs. Through radiolabeled ligand binding assays, we showed that NARW and HW AHRs specifically bind dioxin. The relative binding affinities of cetacean AHRs are intermediate to those of the high-affinity mouse AHR and the low-affinity human AHR. The biological activity of whale AHRs was demonstrated by reporter gene activation in transfected mammalian cells. While TCDD-dependent activation was not observed, some ligand specificity was suggested by differential reporter-gene activation by benzo[a]pyrene.

1.3 Ligand binding domain sequences may be used to infer binding characteristics in mammalian AHRs.

While many researchers have investigated the effects of different ligands on the binding and function of individual AHRs, few have compared the

differential behavior of multiple AHRs. Here, we compared the sequences of AHRs from many mammalian species to identify areas of similarities and unique sites that might suggest their function (Chapter 4). We contributed new partial sequences for mink and polar bear AHR cDNAs. Preliminary results from a ligand binding assay provided relative binding affinities for marine mammal and rodent species in comparison with human AHR, and provide a context for predicting the sensitivity of these AHRs to dioxin *in vitro*. From these analyses we suggested a set of amino acids within the LBD that may be critical for differences in dioxin binding and subsequent AHR function. Homology models were produced for AHR LBDs that illustrate the similarities and differences in tertiary structure that may help to infer binding characteristics attributable to specific amino acids. Some of these residues appear more important than others in determining the shape and tertiary structure of the LBD in homology models of these AHRs.

2. Future Research

At this stage it is almost ironic that there remains so much more left to learn about the topics addressed in the six years of work that were put into this thesis. However, in reality it seems only natural that a thesis should inspire at least as much future work as it entailed. As has been said many times, “the more we know, the more we know we don’t know.” I know so little! And the list of projects to continue is extensive.

In sum, the work contained in this thesis contributes many details of the mechanism of AHR action in marine mammals. However, a link between environmental contaminants, AHR function, and reproductive effects remains elusive. This is necessary to connect north Atlantic right whale (NARW) reproductive failure with an AHR-dependent (or –independent) mechanism of action. Several current lines of research may shed light on this relationship. Among these are efforts to better measure the environmental contaminants

present in baleen whale diets (Moore *et al.* in preparation), to sample NARW steroid hormone levels (Hunt *et al.* 2006; Rolland *et al.* 2005), to culture cetacean oocytes (Iwayama *et al.* 2005), and to characterize whale steroid hormone receptors and their affinities for hormones versus the contaminants measured in their diets (see below).

First, I plan to aggressively pursue amplifying sequences for NARW estrogen receptors. This is an important goal as other techniques for addressing endocrine function in cetaceans are emerging (Hunt *et al.* 2006; Rolland *et al.* 2005), and the development of *in vitro* tools for characterizing reproductive function will complement hormone profiles collected in the field. New, more degenerate primers have been designed to amplify either the alpha or beta forms of the ER, and will increase our chances of acquiring sequence information if transcripts are present. We will apply these primers both to existing RNA preparations as well as to fresh tissues. We hope to obtain samples of deep blubber and liver archived at the NIST from a NARW calf that was necropsied rapidly post-mortem in January 2006. We will also collect any new tissue that becomes available during the 2007 calving season when most vessel strikes claim the lives of calves and females near shore. These same deep blubber tissues will also be used to continue efforts at leptin characterization in NARW.

In order to complete the work of Chapter 3, we intend to perform two additional experiments. The initial transactivation experiments (Chapter 3, Figure 9) must be confirmed with additional replicates, and have shown that 10 ng of whale AHR construct are likely to produce the most unambiguous results. The surprising differences in responses to B[a]P compared with TCDD in a single transactivation experiment allude to an interesting tale of two compounds with different activities in related cetacean species. We will continue to investigate this observation.

The second experiment contributing to the results in Chapter 3 will relate *in vitro* biochemical activity of NARW AHR to specific PCBs and PAHs measured

in blubber and NARW prey. A filter-based hydroxyapatite assay has been designed to measure competitive binding of 5 PAHs (phenanthrene, naphthalene, dibenzofuran, benzo[a]pyrene, and anthracene) and 5 PCBs (PCB: 101, 118, 126, 153, and 170) shown to be common in copepods collected from Georges Bank to Cape Cod Bay and the Gulf of Maine (Moore *et al.* in preparation; Weisbrod *et al.* 2000). We will also include astaxanthin, a potential AHR agonist naturally present in copepods. This experiment will more directly address the hypothesis that NARW AHR is more sensitive to ligands more prevalent in its ecological niche.

An additional set of ligand binding assays is required to improve the results given in Chapter 4. Following the subcloning of hamster AHR in the pcDNA vector, only a single binding assay was conducted with a single high dose of [³H]TCDD. In order to more fully develop the relative binding affinities for marine mammal and model rodent AHRs, several replicate saturation binding assays will be conducted. These assays will help account for differences in AHR expression observed in the quantitative IVTT that influence maximal binding capacity (Chapter 4 Figure 6). In the absence of x-ray crystallography-based determination of AHR LBD tertiary structure, the homology models must suffice to illustrate potential structural consequences of subtle differences in this region. Improvements can be made, however to refine these models, perhaps incorporating or comparing models generated from other templates such as ARNT (Erbel *et al.* 2003) or FixL (Pandini and Bonati 2005). Virtual mutagenesis experiments with these models will also benefit the overall analysis of the importance of individual amino acids on AHR ligand binding function.

Those last experiments would be greatly improved with the addition of mink and polar bear AHR sequences and constructs. A tremendous amount of effort was invested towards this end, with many pages in notebooks as the only tangible outcome. Completion of the LBD sequence would at least permit homology modeling and sequence analysis to infer binding characteristics.

Efforts at increasing the sequence data for these two species may only yield results if new RNA is prepared from fresh tissues.

These continuing projects promise to provide a wealth of research opportunities as well as to maintain my collaboration with the Hahn laboratory at Woods Hole Oceanographic Institution for several years to come. More importantly, the work submitted in this thesis has been, and continues to be, the passionate pursuit of something greater. Amidst the terrible environmental circumstances that humans have created, I remain hopeful that this and any future work, will contribute to the improvement of life for all creatures on this planet.

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