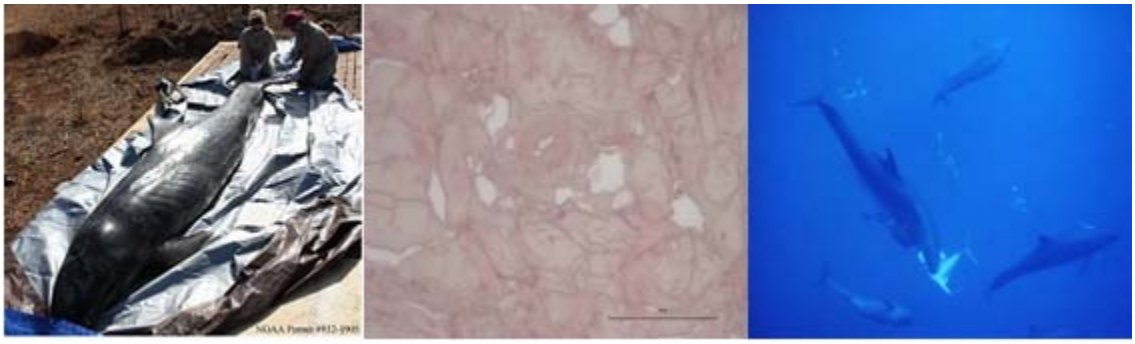
Hawai'i Pacific University

Cytochrome P4501A1 in the Blubber of Free-ranging and Stranded Hawaiian Odontocetes



by**: Kerry McDonald Foltz**

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This thesis is submitted in partial fulfillment of the requirements for the degree of Masters of Science in Marine Science at Hawaii Pacific University. We the undersigned have examined this document and have found that it is complete and satisfactory in all respects, and all revisions required by the final examining committee have been made.

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Cover photos:

(Left): stranded false killer whale (*Pseudorca crassidens*), Hawai‘i Pacific University Stranding Response Team, NOAA Permit #932-1905.
(Middle): CYP1A1 expression in false killer whale sample, © Kerry Foltz, Hawai‘i Pacific University.
(Right): free-ranging false killer whales, © Dan McSweeney, Wild Whale Research Foundation.

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1.1. The Threat of Persistent Organic Pollutants to Cetacean Populations Worldwide

In the past thirty years, a higher overall incidence of mass mortalities, epizootics, pathologies, and reproductive failures have been reported in marine mammal populations (Colborn & Smolen 1996). This increased incidence has been linked to the presence of persistent organic pollutants (POPs) that include pesticides, industrial chemicals, and industrial by-products of production. POPs are ubiquitous in the world's oceans, highly lipophilic, and not easily metabolized or degraded. These characteristics make them a significant threat to apex predators such as marine mammals, because they bioaccumulate in lipid-rich tissues and biomagnify up the food web. Marine mammals are particularly susceptible because of their high position in food webs, large fatty tissue reserves, and long life spans (Boon et al. 1992).

Exposure to POPs in mammals has been linked to endocrine disruption (O'Hara and O'Shea 2001), developmental abnormalities (Guiney et al. 1997), inflammation (Hennig et al. 2002), and mutagenesis (Shimada and Fujii-Kuriyama 2004). In marine mammals in particular, exposure to POPs has been linked to reproductive impairment (Delong et al. 1973, Subramanian et al. 1987), reduced reproductive success (Wells et al. 2005), and decreased immune function (Lahvis et al. 1995). A class of POPs, the planar halogenated aromatic hydrocarbons (PHAHs), has been linked to high incidence of tumors (Martineau et al. 2002) and epizootic outbreaks (Hall et al. 1992, [1]Aguilar and Borrell 1994).

1.1.1. Sources of POPs

POPs can originate from many sources, such as sewage outfalls and agricultural/industrial runoff (Friedlander et al. 2005). POPs are of concern because they can be transferred via ocean currents or the atmosphere to distances far from the original source. For example, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and organic pesticides have been found in the Arctic (MacDonald et al. 2000). However, highly chlorinated biphenyls, such as those in the mixture Aroclor 1268, are less mobile and remain close to sources of contamination (Hansen et al. 2004, Wania and Mackay 1996). Although many POPs, such as DDT and PCBs, have been banned in the United States for decades, they continue to be found in marine mammal tissues because of previous exposure, bioaccumulation, and use in other regions of the world (Fielder 2008, Van den Berg 2009).

The Hawaiian archipelago, like the Arctic, is very distant from continental sources of pollution; however, POPs have been found to be present in wildlife inhabiting the Hawaiian islands. Activities in the main Hawaiian Islands that may be sources of POPs include conversion of agricultural lands to residences and resorts, expansion of harbor facilities (Friedlander et al. 2005), and use of chlorinated insecticides to control agricultural pests (Bevenue et al. 1972, Tanita et al. 1976). One particular study assessed contaminant profiles in fish and sediments located in urban, agricultural, and forested sites of Oʻahu. Chlordane compounds were the most frequently observed compounds at urban sites, followed by dieldrin, polycyclic aromatic hydrocarbons (PAHs), and dichlorodiphenyltrichloroethane (DDT) compounds. PAHs, which will be discussed in detail later, had mixed (urban and agricultural) distributions. Furthermore, PAH

concentrations were highest in watersheds. In agricultural sites, DDT and its degradation products (DDD and DDE) were detected. Forested sites did not contain pesticides or polychlorinated biphenyls (PCBs) (Brasher and Wolff 2004).

PCBs have been banned from use in the United States since 1979, and they were previously used as heat transfer fluids, hydraulic lubricants, and dielectric fluids. PCBs may have leaked from transformers used in the past (Safe et al. 1994), leading to their current presence in Hawaiian wildlife. In particular, tilapia (*Oreochromis mossambicus)* inhabiting Manoa streams and the Ala Wai canal had PCB profiles resembling Aroclor 1254 (Yang et al. 2008), similar to that found in a study of the French Frigate Shoals (Miao et al. 2000). PCB 118 accounted for more than 55% of total PCBs in the study on Oʻahu. Overall, this study indicated that the average levels of PCBs found in these two sites on Oʻahu were in the middle range of PCB concentrations detected in fish worldwide (both freshwater and marine) (Yang et al. 2008).

POPs have also been detected in marine mammals from Hawaiʻi. In the Hawaiian monk seal (*Monachus schauinslandi*), PCBs were found to be highest in seals from Midway Atoll. This is likely related to past military occupation (Lopez 2012). PCBs and PBDEs were found to be significantly higher in animals from Oʻahu in comparison to animals from Molokaʻi and Kauaʻi. DDT was found to be ubiquitous among animals from all islands. In false killer whales (*Pseudorca crassidens*) from the main Hawaiian Islands, the most abundant POPs were DDTs and PCBs, ranging in concentrations from 1,000 to 83,000 ng/g lipid. Other POPs, including polybrominated diphenyl ethers (PBDEs), chlordanes, b-HCH, dieldrin, HCB and mirex were also measured, but these were found at much lower concentrations than DDTs and PCBs. POPs such as endosulfan

I and aldrin were below the limit of quantification (LOQ) for all animals included in this study. Mean ∑PCBs and ∑DDTs were lower than concentrations found in a variety of marine mammals from the west coast of the United States that feed primarily in urbanized areas (Ylitalo et al. 2009).

1.1.2. Classes of POPs

Halogenated Aromatic Hydrocarbons

The halogenated aromatic hydrocarbons (HAHs) are among the most widespread and persistent chemical contaminants in coastal environments (Varanasi et al. 1992). HAHs are trace contaminants created in industrial processes that involve chlorination in the presence of phenolic substrates (Schmidt and Bradfield 1996). They are lipophilic, contain many chlorine molecules (Figure 1.1) (Nguyen and Bradfield 2008), and are generally resistant to metabolism (Law and Whinnett 1992).

The lipophilic nature of HAHs causes them to bioaccumulate and biomagnify throughout food chains, and their potential carcinogenic threat to wildlife has become a cause of concern. Planar HAHs (PHAHs), dioxin-like congeners with stereochemistry similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Figure 1.2), are of special interest because of their toxic potential, although found in the environment at low concentrations (Ahlborg et al. 1994. Safe et al. 1990). TCDD has been shown to cause a variety of physiological effects, including tumor promotion, immunosuppression, teratogenesis, and cardiac dysfunction (Schmidt and Bradfield 1996). Many other PHAHs are found in the environment, including halogenated dibenzodioxins and dibenzofurans (McKinney et al. 1985).

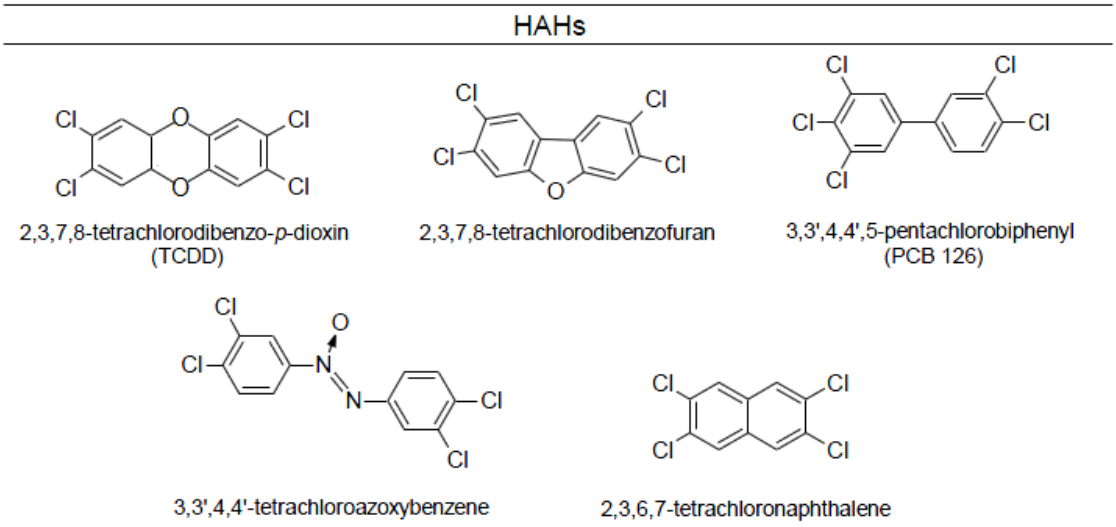

Figure 1.1. Representative Halogenated Aromatic Hydrocarbons. Figure adapted from Fertall 2010. These contaminants are by-products of industrial processes and are stable in the biotic environment (Schmidt and Bradfield 1996, Varanasi et al. 1992). The number, position, and nature of the halogen atoms, along with the structure of aromatic rings, influence the molecular activity of these contaminants (Birnbaum 1985).

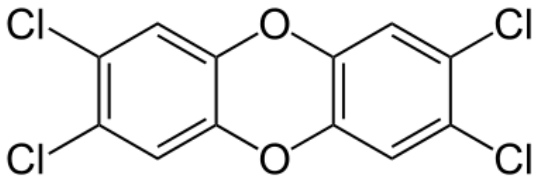

Figure 1.2. TCDD, a potent AHR ligand. It is chemically stable and highly resistant to metabolism in most biological systems (Okey 2007, Poland et al. 1976). Image from: http://chem.sis.nlm.nih.gov/chemidplus/.

Polycyclic Aromatic Hydrocarbons

PAHs are characterized by condensed benzene rings (Figure 1.3) (Cook et al. 1983), and they are introduced in the environment through natural and anthropogenic processes. PAHs are most often introduced into the environment through incomplete

combustion of fossil fuels and other organic matter. Natural sources of PAHs include volcanic activity and forest fires. Anthropogenic sources of PAHs include wood and coal burning, automobiles, heat and power plants, and refuse burning (Harvey 1991).

High molecular weight PAHs and low molecular weight PAHs differ in chemical activity. High molecular weight PAHs can be quickly adsorbed on the surface of organic and inorganic particles, and are therefore less bioavailable to organisms than low molecular weight PAHs (Neff 1979, NRCC 1983). However, high-molecular weight PAHs may be remobilized into the water column by biological activity, bioturbation, and currents. Organisms then are able to take up these compounds where they partition to lipid-rich tissues. Many of the high molecular weight PAHs are known to be carcinogenic, for example, dibenzo[*a*,*h*]anthracene, benzo[*b*]fluoranthene, and chrysene (Marsili et al. 2001).

Some sources indicate that lower molecular weight PAHs are more toxic to marine biota because of their greater bioavailability and higher solubility compared to higher molecular weight contaminants (Neff 1979, NRCC 1983). When taken up by organisms, lower molecular weight PAHs can be metabolized and excreted from the body (Law and Whinnett 1992)**.** Marsili and others (2001) found that in samples of striped dolphins (*Stenella coeruleoalba*) and fin whales (*Balaenoptera physalus*), the most abundant PAHs were of low molecular weight, making up ninety-percent of all the PAHs found in both species. These PAHs included naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, and fluoranthene.

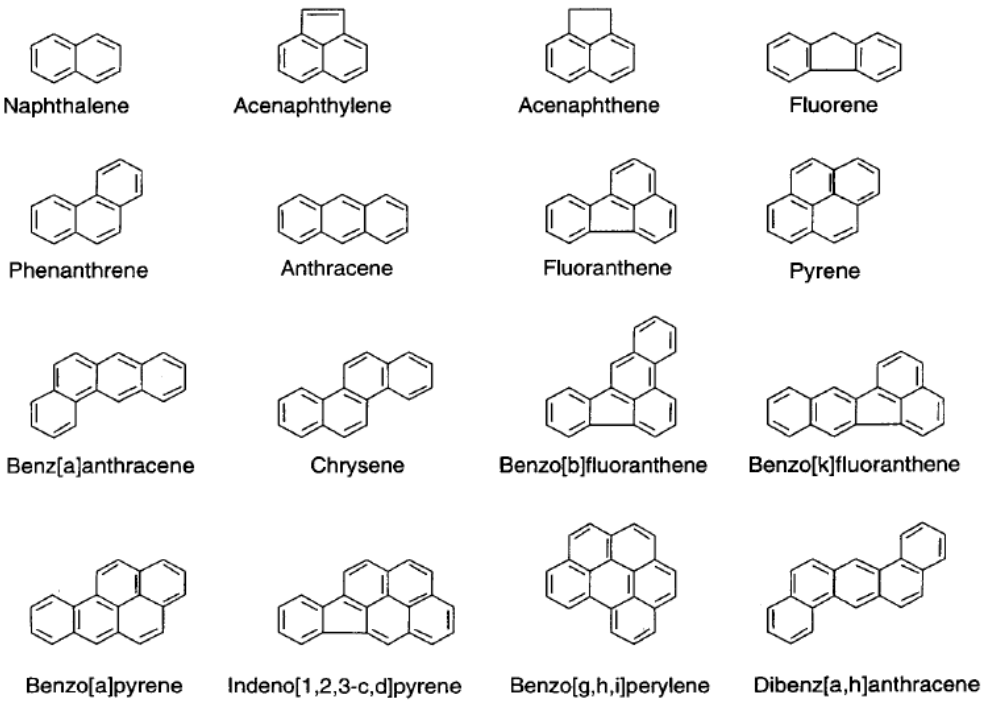

Figure 1.3. Representative polycyclic aromatic hydrocarbons. Figure adapted from Harvey et al. 2002. PAHs are composed of two or more aromatic (benzene) rings, which are fused together when a pair of carbon electrons is shared between them (Neff 1979). They are produced as by-products of fuel burning, either anthropogenic in nature or natural. Some compounds are known to be carcinogenic, mutagenic, and teratogenic (Harvey 1991).

Polybrominated Diphenyl Ethers

The toxic nature of polybrominated diphenyl ethers (PBDEs) has elicited recent concern. PBDEs (Figure 1.4) are known to cause thyroid disruption and neurobehavioral changes in laboratory animals. Mixtures of PBDEs are added as flame retardants to plastics, textiles, clothing, electronic circuit boards, and other materials in the manufacturing process (de Wit 2002). Similar to HAHs, these compounds are lipophilic and bioaccumulate in marine mammal tissues (de Wit et al. 2004, Ikonomou et al. 2002a, 2002b). There are no threshold levels established for toxicological effects of PBDEs in marine mammals.

Very high concentrations of PBDEs have been found in wildlife in North America (Ikonomou et al. 2002b, Hites 2004, LeBeuf et al. 2004), and the highest levels of PBDEs have been found in top marine predators (Fossi et al. 2006). Furthermore, they are known to travel long distances through the atmosphere and are present in remote regions of the world, such as Antarctica and the Arctic (de Wit et al. 2004, Corsolini et al. 2006). PBDEs have been found in wastewater effluent (de Boer et al. 2003, North 2004). This may be a PBDE point source in Hawai‘i because a number of plants have been known to discharge waste to the coastal ocean (Friedlander et al. 2005).

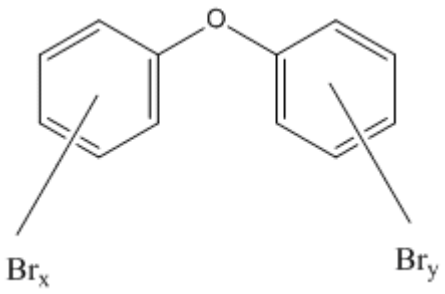

Figure 1.4. Structure of polybrominated diphenyl ethers. These emerging chemicals of concern are used as flame retardants for plastics and electrical devices, among other items (BSEF 2000, Darnerud et al. 2001, IPCS 1994). They are structurally similar to PCBs, DDT, and thyroid hormone 4 (Hooper and McDonald 2000). Their greatest toxicological impact seems to be thyroid hormone disruption, neurodevelopmental deficits, and cancer (reviewed in McDonald 2002).

Perfluorinated compounds (PFCs)

Perfluorinated compounds (PFCs) are used in industrial products for their surfactant properties ([1]U.S. EPA), and they are known to be carcinogenic chemicals. They have been shown to cause tumor formation in the liver, pancreas, and testis of rats (Kennedy et al. 2004). PFCs are ubiquitous in wildlife (Giesy and Kannan 2001), and there are a limited number of studies that have measured levels in cetacean populations.

A particular PFC, perfluorooctane sulfonate (PFOS) (Figure 1.5), is absorbed orally and binds to blood proteins in the serum and the liver (OECD 2002). This compound was the most abundant PFC in bottlenose dolphin (*Tursiops truncatus*) blubber samples (Fair et al. 2010). Overall, PFCs contributed less than 1% of total POPs in the blubber of bottlenose dolphins from Charleston, SC. However, these compounds may play a larger role than suggested by their relatively low percentage because of potential mobilization from the blubber (Fair et al. 2010).

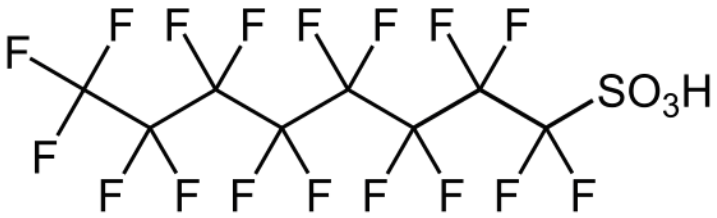

Figure 1.5. Perfluorooctane sulfonate (PFOS), an example perfluorinated compound. The carbon-fluorine bond (strongest single bond in organic chemistry) makes these pollutants incredibly stable in the environment. Long chain PFCs, such as PFOS, have a long half-life in humans ([2]U.S. EPA).

1.1.3. Factors Affecting the Toxicity of Contaminants in Marine Mammals

The lowest observed adverse effect level (LOAEL) for PCBs in marine mammal blubber was suggested to be 17,000 ng/g lipid weight (Kannan et al. 2000). These effects include disruption of vitamin A and thyroid hormone concentrations, suppression of natural killer cell activity, and reduced proliferative responses of lymphocytes to mitogens. As contaminant exposure increases, effects on innate and acquired immunity can result in premature mortality from infection (Hall et al. 2006). A table of measured

total PCB levels in the blubber of various species, four of which exceed the threshold value of 17,000 ng/g, is presented below in Table 1.1.

Table 1.1. Selected cetacean species analyzed for ∑PCBs throughout the world. Concentrations of ∑PCBs (ng/g lipid weight) in the blubber are reported.

Species	Location	∑PCBs	Author
beluga whale (*Delphinapterus leucas*)	St. Lawrence River estuary	Geometric mean for adult males (*n* =34): 11,800 ng/g	Hobbs et al. 2003
bottlenose dolphin (*Tursiops truncatus*)	Charleston, SC	Geometric mean for male dolphins (*n* =36): 93,980 ng/g	Fair et al. 2010
bowhead whale (*Balaena mysticetus*)	Northern Alaska	Mean (*n* =5): 296 +/- 23 ng/g	Hoekstra et al. 2005
harbour porpoise (*Phocoena phocoena*)	Baltic Sea	Mean for adult males (*n* =4): 46,000 +/- 29,000 ng/g	Berggrena et al. 1999
false killer whale (*Pseudorca crassidens*)	British Columbia	Mean for adult males (*n* =2): 45,000 +/- 2,500 ng/g	Jarman et al. 1996
false killer whale (*Pseudorca crassidens*)	Hawai‘i	Mean for adult males (*n* =2): 33,000 +/- 0 ng/g	Ylitalo et al. 2009
killer whale *(Orcinus orca)*	Offshore Alaska	Mean for adult males (*n* =4): 111,000 +/- 22,000 ng/g	Krahn et al. 2007
long-finned pilot whale *(Globicephala melas)*	Gulf of Maine	Mean (*n* =11): 1,677 +/- 694 ng/g	Weisbrod et al. 2001
sperm whale *(Physeter macrocephalus)*	Southern Australia	Mean for adult males *(n* =5): 1,300 +/- 1,200 ng/g	Evans et al. 2004
white-sided dolphin (*Lagenorhynchus acutus*)	Gulf of Maine	Mean (*n* =6): 5,682 +/- 1222 ng/g	Weisbrod et al. 2001

Depending on trophic level, food availability, migration, disease, gender, reproductive status, order of birth, and age, individuals may have higher or lower levels of contaminants in the blubber. The following paragraphs review trends in contaminant

levels related to these characteristics, along with other factors affecting susceptibility to contaminants.

Trophic Level

Trophic level influences contaminant burden because of processes of bioaccumulation and biomagnification of POPs that occurs in the food web. An example of greater contaminant burdens with higher trophic level has been shown in fishing-eating and mammal-eating killer whales in Prince William Sound, Alaska. Fish-eating killer whales predominately consume salmon, while mammal-eating whales mainly consume Dall's porpoise (*Phocoenoides dalli*) and harbor seals (*Phoca vitulina*). Higher levels of contaminants were found in the prey of mammal-eating killer whales that fed at the higher trophic levels, and mammal-eating killer whales had higher concentrations of persistent contaminants in their blubber compared to fish-eating killer whales (Ylitalo et al. 2001).

Food Availability

Seasonal variation in food availability may contribute to differences in lipid content in the blubber (Aguilar and Borrell 1990). The blubber is the site of the most lipid storage in cetaceans, and changes in nutrient availability affect blubber thickness and composition. Throughout the feeding season, progressive accumulation of fat reserves in the blubber has been noted, along with mobilization of lipid stores and decreased lipid content in correspondence to food scarcity. Active deposition and mobilization of lipids occurs primarily in the deep layers of the blubber (Aguilar and Borrell 1990). During

food scarcity, mobilization of fat reserves in this region may lead to mobilization of lipid-soluble POPs, which can induce physiological effects (Montie et al. 2008a).

Migration

Migration also plays a role in concentrations of contaminants within tissues, because it requires a high level of energy expenditure and draws heavily upon body lipid reserves (Burns et al. 1993). Fasting during migration may lead to mobilization of blubber lipid reserves, liberating contaminants into circulation (Tornero et al. 2004).

Disease

Several studies suggest a relationship between contaminant exposure and immunosuppression. A significant negative correlation was found between concentrations of specific PCBs and lymphocyte proliferative responses to mitogen stimulation in bottlenose dolphins (Lahvis et al. 1995). Beluga whales exposed to 5-25 μg/g wet weight mixtures of PCB and DDT congeners displayed significantly reduced splenocyte proliferative responses (De Guise et al. 1998). These cellular responses suggest that contaminant burdens can influence the onset of disease.

At the population level, Aguilar and Borrell ($_1$1994) found that striped dolphins that died during the 1990 morbillivirus epidemic in the Mediterranean sea had significantly higher PCBs in their blubber (median 778 mg/kg lipid weight) than animals sampled in years outside the epidemic (median 282 mg/kg lipid weight). Moreover, Jepson and others (2005) found that harbor porpoises that died from infectious disease had significantly higher total chlorobiphenyls (CBs) (mean, 27.6 mg/kg lipid) than

individuals that died from physical trauma (predominately bycatch) (mean, 13.6 mg/kg lipid).

Multiple stressors experienced by diseased animals must be taken into account when analyzing relationships between contaminant burdens and disease. Sick animals are often undernourished and may feed on different food sources than they would in a healthier state. Contaminant levels may be elevated in individuals that suffer lipid loss during infectious disease because of a concentrating effect in the blubber. Alterations in metabolic activities caused by disease may further elevate these contaminant levels (Aguilar et al. 1999). Although immunosuppression caused by contaminants has clearly been established (De Guise et al. 1998, Lahvis et al. 1995), disease presence is a result of many environmental and physiological stressors.

Gender, Reproductive Status, Order of Birth

Gender and reproductive status influence lipid dynamics and contaminant levels. Aguilar and Borrell (1990) found that lipid content of the blubber did not vary with reproductive state in male fin whales. However, reproductive state in females had a strong influence on average lipid content and stratification patterns in the blubber. Pregnant females had the highest average lipid content in the saturated internal layer, while lactating females had the lowest mean lipid content in this layer.

Lactating females have lower mean lipid content in the blubber because they transfer lipids and lipophilic contaminants to offspring. This essentially increases contaminant burdens in the young, and decreases contaminant levels in the mother ($_2$Aguilar and Borrell 1994, Beckmen et al. 1999, Krahn et al. 1999, Wageman and Muir

1994). Ylitalo and others (2001) found that reproductively active female killer whales had much lower concentrations of organochlorines than sexually mature males or immature males, while offspring had much higher organochlorine levels than their corresponding mothers. Hansen and others (2004) found the same trend in bottlenose dolphins, in which females of reproductive age showed the lowest concentrations of organochlorines in blubber, and adult males and juveniles were found to have the highest concentrations.

Birth order may also play a role in contaminant burden. The total amount of contaminants transferred from mother to offspring decreases with increasing number of births and lactation cycles (Ridgway & Reddy 1995). First recruits receive the most contaminants and may be at a greater risk than siblings born thereafter (Ylitalo et al. 2001). After the mother gives birth to her first calf, her contaminant levels tend to remain low for the rest of her reproductive lifespan (Hickie et al. 1999). They may, however, continue to accumulate between pregnancies (Krahn et al. 2009). Once a female reaches senescence, contaminant levels begin to increase with age (Ross et al. 2000, Tilbury et al. 1999). These trends may influence survivorship of individuals. Krahn and others (2002, 2004) found low survivorship in calves, adult male, and post-reproductive female southern resident killer whales, all of which had the highest levels of POPs. In another study, a contaminant risk model showed low survivorship of calves exposed to high levels of POPs from maternal transfer (Schwacke et al. 2002).

Physical and Chemical Structure of Contaminants

The molecular structure of POPs may affect their ability to be transferred from mother to offspring. Hansen and others (2004) found a higher proportion of ΣDDT in juveniles and adult male bottlenose dolphins, and a higher proportion of ΣPCBs in adult female bottlenose dolphins. A higher ratio of ΣDDT/ ΣPCBs has been found in adult males in comparison to adult females (reviewed in Aguilar et al. 1999). These trends may be because of selective partitioning during reproductive transfer, in which DDT and its degradation products are more efficiently transferred from blubber to milk compared to specific PCBs (Addison & Brodie 1987, Beckmen et al. 1999). For example, higher proportions of superhydrophobic octa-, nona-, and deca-biphenyls (for example, PCB 194, PCB 201) have been found in adult females compared to adult males and juveniles (Hansen et al. 2004). It is likely that these contaminants are not offloaded through lactation, possibly because of steric hindrance and membrane permeation resistance (Kannan et al. 1998, Maruya & Lee 1998). On the other hand, PCB 153, which is not easily metabolized by marine mammals, does not seem to follow this trend and has been found to be a common component of milk (Beckmen et al. 1999, Pomeroy et al. 1996). It was found to be lower in adult females compared to adult males and juveniles (Hansen et al. 2004).

Age and Growth

The relationship between age, content of total blubber lipids, and contaminant concentrations has been termed the "dilution effect" (Hickie et al. 1999). After weaning, juveniles eat a diet of fish composed of lower concentrations of lipophilic contaminants

compared to milk. Lower dietary intake of contaminants, along with rapid growth, results in lower contaminant burdens until growth rate plateaus (Hansen et al. 2004). PCB concentrations then increase with age in males depending on the contaminant levels of prey. Females may begin to accumulate PCBs again until reproduction begins (Hickie et al. 1999). The dilution effect does not take into account non-metabolizable POPs (Hansen et al. 2004), such as high molecular weight HAHs, that are not eliminated from the body. These HAHs may continue to be present in high amounts in subadults that are rapidly growing.

Montie and others (2008b) found that adult dolphins had lower content of total blubber lipids than subadults. This could be explained by decreases in surface-area-to-volume with growth, accompanied by decreased demand for insulation, and increased demand for energy to support growth (Dunkin et al. 2005, McLellan et al. 2002, Struntz et al. 2004, reviewed in Montie et al. 2008a). Lipid loss from the blubber in adults may be a causal agent for the redistribution of contaminants from the blubber into the plasma (Montie et al. 2008a).

Contaminant Affinity to Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AHR) is a soluble, ligand-activated transcription factor and member of the basic helix-loop-helix family of transcription factors (Gu et al. 2000). In its inactive form, it is located in the cytosol within a protein complex that includes two 90-kDa heat-shock proteins (HSP90), the co-chaperone p23, and a 43-kDa protein termed hepatitis B virus X-associated protein (Meyer et al. 1998). After an AHR agonist (such as an HAH or PAH) binds to the AHR, the AHR dissociates from HSP90

and the ligand-receptor complex translocates into the nucleus to form a heterodimer with ARNT (Figure 1.6). The AHR-ligand-ARNT complex then binds to promoter sequences termed dioxin-responsive enhancer elements (DRE) and transcriptional cofactors to activate transcription of several genes (Hankinson 1995, Schmidt and Bradfield 1996). These genes encode many metabolizing enzymes involved in the detoxification or bioactivation of contaminants (Nebert et al. 1991, Nebert & Gonzalez 1987). Examples of these enzymes include phase I metabolism enzymes: cytochrome P450 (CYP) 1A1, 1A2, 1B1, and 2S1; along with phase II metabolism enzymes: NAD(P)H: quinone oxidoreductase (NQO1), glutathione S-transferase A1 (GSTA1), cytosolic aldehyde dehyrdogenase-3 and UDP-glucuronosyltransferase 1A6 (Nebert and Duffy 1997, Rivera et al. 2002).

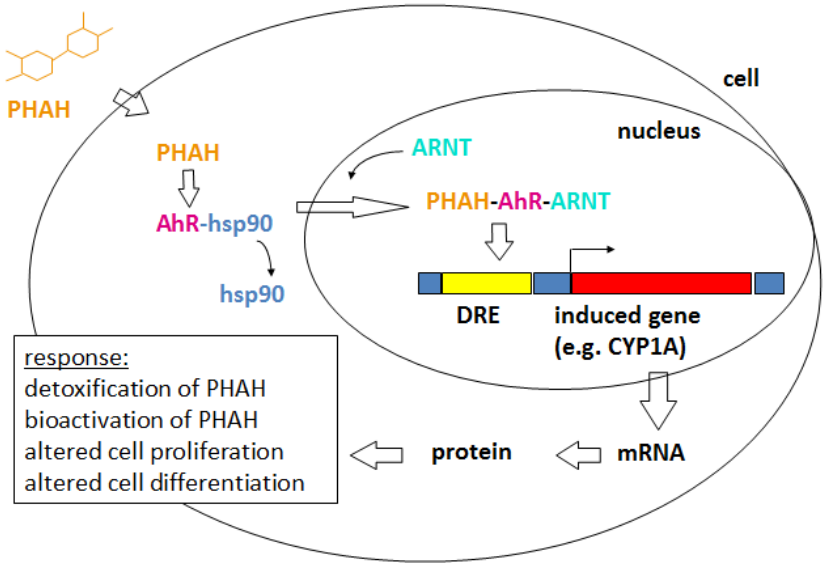

Figure 1.6. AHR response to a planar HAH (PHAH), resulting in CYP1A1 induction. CYP1A1, a phase I metabolizing enzyme, can metabolize the original ligand in such a way that it is excreted from the body, or it can bioactivate the original ligand by oxidative metabolism. Oxidative metabolism of an HAH can result in the creation of hydroxylated PCBs (OH-PCBs). Oxidized compounds as a result of CYP1A1 induction can have deleterious effects on DNA and proteins in the body (figure adapted from Jensen 2000).

The original function of the AHR in early metazoans and extinct invertebrate species may have involved the development of sensory structures or neurons. In mammals, it appears to be involved in development of the liver, ovary, cardiovascular system, and the immune system. Although the AHR has various functions, it evolved as part of an adaptive response system through its ability to regulate biotransformation enzymes (Hahn 2002). This adaptive response mechanism functions to minimize the body's burden of compounds with extended aromatic structures (Schmidt and Bradfield 1996), and it appears to have first evolved in early jawed vertebrates (gnathostomes) (Hahn 2002). Although the AHR evolved as a means to excrete and rid the body of xenobiotics, the enzymes associated with the AHR are also capable of metabolizing these compounds to electrophilic intermediates that alkylate cellular macromolecules. These metabolized xenobiotics can lead to changes in cellular function and genotoxicity (Schmidt and Bradfield 1996).

Correlations have been found between structure-AHR binding activity of HAHs and toxic responses (Goldstein and Safe 1989, Poland and Knutson 1982). Furthermore, affinity of particular contaminants to the AHR may differ among species, contributing to variable toxic responses exhibited between species. Jensen and others (2010) found that the beluga AHR bound [^{3}H]TCDD with an affinity that was two-fold higher than that of a dioxin-responsive mouse. Furthermore, the binding affinities of many compounds (similar in structure and function to [^{3}H]TCDD) to the AHR were highly correlated between the beluga and mouse species ($R^2 = 0.96$), and the beluga AHR showed higher affinity for most HAHs than the mouse AHR (Figure 1.7). These results may indicate that the beluga, and other cetacean species, are highly susceptible to the physiological effects

resulting from contaminant binding to the AHR. Other studies have shown that beluga exposed to low concentrations of PCBs show similar cellular responses to animals that are maximally induced (Wilson et al. 2005).

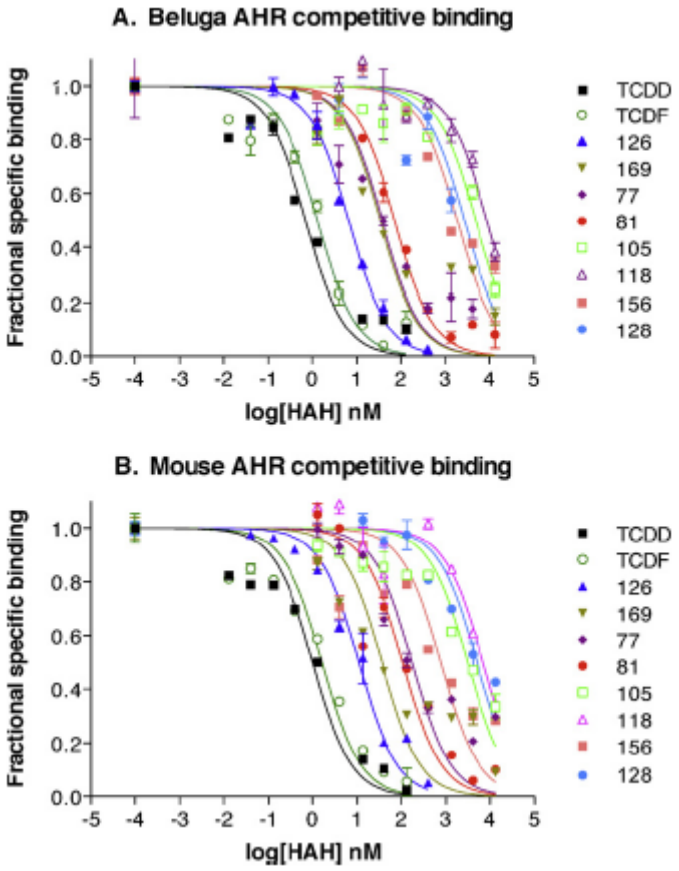

Figure 1.7. Competitive binding curves of ten HAHs to the AHR in both beluga (a) and mouse (b) species. Binding affinities (expressed as K_i) of ten representative HAHs relative to TCDD are expressed, and bars represent 1 standard deviation. Similar structure-binding relationships were found for beluga and mouse AHRs, and K_i values for the beluga AHR were consistently lower than those for the mouse AHR (Jensen et al. 2010).

1.2. CYP1A1 as a Biomarker of Contaminant Exposure

The effects of contaminants and stress in tissues can be assessed by analyzing molecular biomarkers that indicate a biological response, such as the presence of enzymatic activities or protein expression. An ideal biomarker is specific, sensitive, and responsive across a wide range of inducer concentrations. The following sections review the CYP proteins, in particular CYP1A1, a useful biomarker of contaminant exposure in cetaceans.

1.2.1. CYP Proteins

CYP proteins are a group of enzymes that are central to phase I metabolism of POPs (Boon et al. 2001). Phase I metabolism involves biotransformation of a variety of xenobiotic and endogenous compounds (Goksoyr & Forlin 1992, Lewis et al. 1998, Stegeman & Hahn 1994). It introduces functional groups into molecules (Caldwell et al. 1995, Parkinson 1996), which then can be conjugated into larger endogenous molecules via catalytic mediation by phase II enzymes (George 1994, Wolkers et al. 1998). Phase II metabolism increases hydrophilicity of the xenobiotic, facilitating excretion from the body (Parkinson 1996). These enzymes involved in phase I and phase II metabolism are most prevalent in hepatic tissues (McKinney et al. 2004).

1.2.2. CYP1A1

CYP1A1 induction via ligand-AHR interactions (Figure 1.6) is generally the best understood model for toxic responses mediated by the AHR (Safe 1998). CYP1A1 is induced by and involved in the metabolism of HAH and PAHs (Dennison and Heath-

Pagliuso 1998, Poland and Knutson 1982, Safe 1990). Metabolism may lead to detoxification or bioactivation of the original substrates, and bioactivation may lead to the generation of toxic metabolites (reviewed in Nebert and Russell 2002).

A specific role of CYP1A1 is its involvement in phase I oxidative metabolism of PCB congeners with chlorine substituents at one or both para positions, and with adjacent non-halogenated ortho and meta carbons on at least one ring (Kaminsky et al. 1981, Mills et al. 1985). In the case of lipophilic organohalogens lacking reactive groups, CYP1A1 catalyzes a reaction in which oxygen is introduced in the molecules (Nebert et al. 1991, Nebert and Gonzalez 1987), generating hydroxylated metabolites (OH-PCBs) (Kaminsky et al. 1981, Mills et al. 1985, White et al. 2000). OH-PCBs have been recognized as a group of contaminants that pose a threat marine mammal health (Brouwer et al. 1989, 1998, Zoeller et al. 2002) because they can interact with the thyroid hormone (TH) system through competitive binding with the TH transport protein (TTR) (Letcher et al. 2000). This may result in interference with thyroid hormone homeostasis, vital processes such as skeletal growth, development of the brain and inner ear, immune system function, maintenance of metabolic rate, fat metabolism, and sexual function (Brouwer et al. 1989, 1998, Zoeller et al. 2002). Furthermore, OH-PCBs have been shown to interfere with estrogen receptor. Loomis and Thomas (2000) found that the hydroxylated PCB 2,2',5'-trichloro-4-biphenylol acts through a membrane estrogen receptor to decrease androgen production in the testis of the Atlantic Croaker fish (*Micropogonias undulatus*).

Along with HAHs and PAHs, several non-polycyclic and non-planar compounds that have been classified as “nonclassical” CYP1A1 inducers have been shown to activate this gene despite inability to compete with TCDD for the AHR (Aix et al. 1994, Daujat et

al. 1992, Fontaine et al. 1998, 1999, Gradelet et al. 1997, Ledirac et al. 1997, Lee et al. 1996, Lesca et al. 1995). These compounds include omeprazole, thiabendazole, and primaquine among others (Figure 1.8). Many questions exist on the mechanisms in which they compounds induce CYP1A1 (Delescluse et al. 2000).

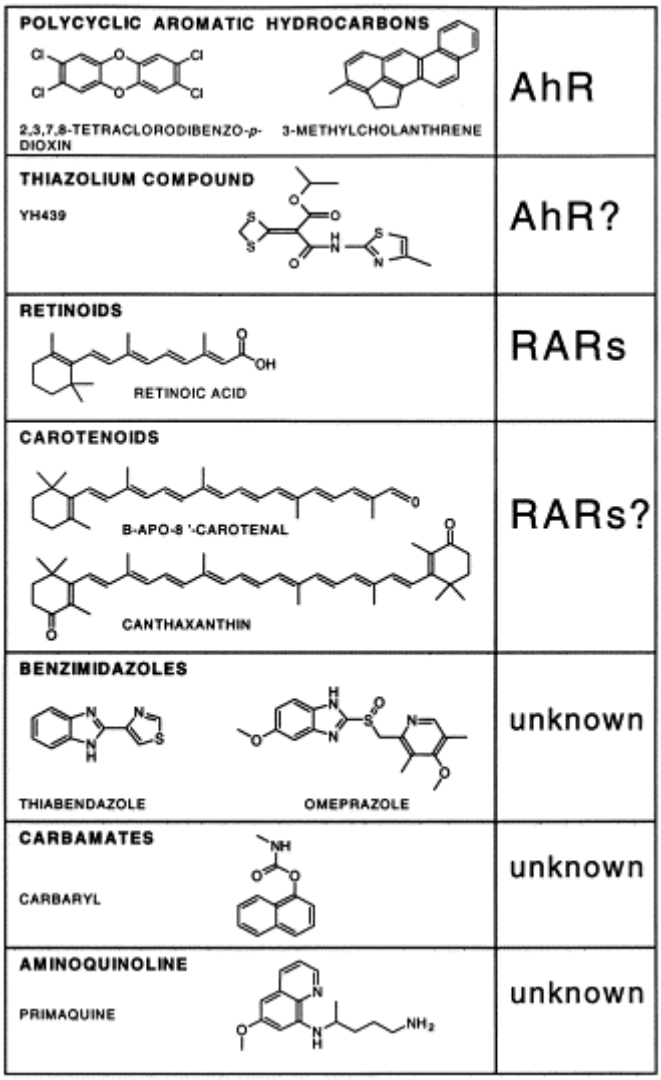

Figure 1.8. Principal families of compounds able to induce CYP1A1. Structures are shown on the left, and receptors involved are displayed on the right (Delescluse et al. 2000). RARs stands for retinoic acid receptors, which bind metabolites of vitamin A. Binding of retinoic acid to RARs affects the binding of other proteins that either induce or repress transcription of a nearby gene. Many of these genes control differentiation of a variety of cell types.

Natural aromatic substances with similar structure to anthropogenic PBDEs (flame retardants used in the manufacturing process) have been found in the blubber of True's beaked whale (*Mesoplodon mirus*). These compounds, MeO-PBDEs (Figure 1.9) were determined to be natural in origin by analysis of their radiocarbon (^{14}C) content (Teuten et al. 2005). It is unknown whether or not these compounds induce CYP1A1, however, their industrial counterpart PBDEs are known to induce CYP1A1 (Montie et al. 2008a, Von Meyerinck et al. 1990).

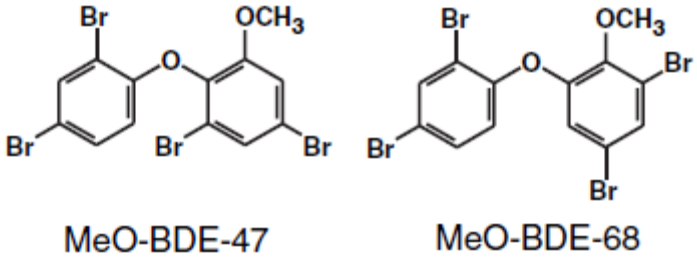

Figure 1.9. MeO-PBDEs, analogues to PBDEs, isolated from True's beaked whale (*M. Mirus*). These were determined to be natural in origin through radiocarbon (^{14}C) content (Teuten et al. 2005). Other studies have indicated that these compounds are produced by marine sponges (*Dysidea* sp.) (Anjaneyulu et al. 1996), red algae, and cyanobacteria (Malmvärn et al. 2008).

1.2.3. Detecting CYP1A1 expression

CYP1A1 can be analyzed by enzymatic activities, immunohistochemistry, western blot (Fossi et al. 2008), immunofluoresence, and real-time PCR (Fossi et al. 2006). Catalytic characterization studies have identified CYP1A-mediated EROD, PROD, MROD (7-ethoxy-, pentoxy- and methoxyresorufin- O-deethylase, respectively) enzymatic activities (Addison et al. 1998, White et al. 1994, 2000), which also may serve as indicators of CYP1A1 activity.

Immunohistochemistry is a very sensitive method for studying CYP1A1 expression in integument (blubber and skin) samples from cetacean species (Angell et al. 2004). Through use of immunohistochemistry, level of CYP1A1 expression can be determined as a product of intensity (measured qualitatively as a 0-5 score by the observer after antibody-antigen coupling) and occurrence (measured as number of cells expressing CYP1A1 per unit area) (Montie et al. 2008a). Expression can be compared to contaminant concentrations in selected tissues to determine if CYP1A1 is an effective biomarker of non-metabolizable contaminant (HAH) exposure.

1.2.4. Cellular and Tissue-specific Expression of CYP1A1

Previous studies on terrestrial animals have indicated that the endothelium is an excellent location to study CYP1A1 expression because of its abundant AHR expression and biological responses of edema and inflammation upon exposure to agonists (Bayou-Denizot et al. 2000, Hennig et al. 2002). The endothelial cells are in direct contact with blood borne xenobiotics and may play an important role in their transfer and metabolism (Godard et al. 2004). Montie and others (2008a) found that CYP1A1 was strongest and most often seen in vascular endothelial cells of arterioles and capillaries in the blubber of bottlenose dolphins (Montie et al. 2008a). CYP1A1 was also expressed in fibroblasts, arterial smooth muscles cells, arterial endothelial cells, and venule endothelial cells in these animals. In another study, CYP1A1 was quantified in cetacean skin biopsies, and it was found to be highest in endothelial cells between the epidermis and dermis junction in spinner dolphins (*Stenella longirostris*), pantropical spotted dolphins (*Stenella attenuata*), and humpback whales (*Megaptera novaeangliae*) from the Indian Ocean (Jauniaux et al.

2011). In a study of stranded cetaceans from Hawai‘i, CYP1A1 expression was high in the endothelial cells of the blubber (Fertall 2010).

In the previously mentioned study on stranded Hawaiian cetaceans, CYP1A1 was also found to be high in the lung and liver. Inhalation of PAHs may explain the high level of CYP1A1 expression in the lung of these animals, and the lung may be an important gateway for contaminant exposure (Fertall 2010). Other studies have demonstrated that CYP1A1 activity is relatively high in the lung of cetaceans (Garrick et al. 2006). On the other hand, high levels of CYP1A1 in the liver may be related to its noteworthy role in Phase I metabolism. Induction of CYP1A1 in the hepatocytes of the liver may lead to metabolism of PCBs and production of OH-PCBs (Kaminsky et al. 1981, Mills et al. 1985, White et al. 2000). Furthermore, liver CYP1A1 expression has been shown to be highly correlated to mono-*ortho* and non-*ortho* PCBs in the blubber of Arctic beluga whale (White et al. 1994). High CYP1A1 expression found in the lung, blubber, and liver may represent pathways of contaminant exposure, storage in lipids and mobilization to circulation, and resulting effects on target organ systems respectively.

1.2.5. Assessing CYP1A1 expression in the blubber

Examining CYP1A1 expression in the blubber must take into account the fact that the blubber does not operate as a discrete unit, but is characterized by layers of distinct function. Montie and others (2008b) established that the blubber of live bottlenose dolphins (*Tursiops truncatus*) from the southeast coast of the United States showed three distinct layers. The superficial blubber layer contained few, small adipocytes and a large amount of structural fibers. The middle blubber layer was characterized by few structural

fibers, and the greatest quantity of adipocytes that were largest in size. The deep blubber layer exhibited an intermediate amount and size of adipocytes in comparison to the superficial and middle blubber layers. The deep blubber layer also displayed an intermediate amount of structural fibers compared to the superficial and middle blubber layers (Figure 1.10)

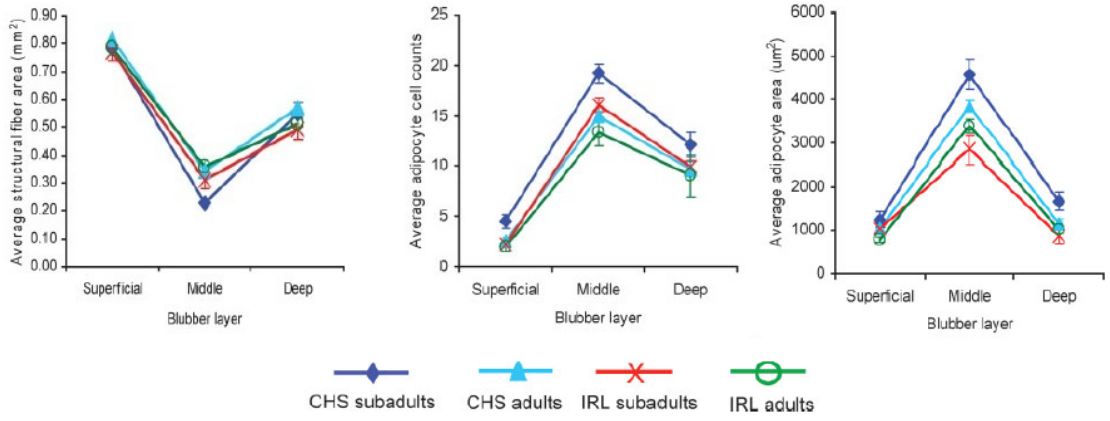

Figure 1.10. Average structural fiber areas (mm^2), adipocyte cell counts, and adipocyte areas (μm^2) across the blubber layers of bottlenose dolphins from the southeast coast of United States. Figure adapted from Montie et al. 2008b. CHS represents animals from Charleston Harbor, SC, and IRL represents animals from Indian River Lagoon, FL. CHS animals experience consistently colder water temperatures than IRL dolphins throughout the year.

Along with key cell types, fatty acids are stratified throughout the blubber layers. Studies on biochemical stratification in bottlenose and common dolphins (Delphinus sp.) distinguished layers based on analysis of saturated, monounsaturated, and polyunsaturated fatty acids. The superficial blubber layer contained higher amounts of monounsaturated fatty acids, while the deep blubber layer had higher amounts of saturated and polyunsaturated fatty acids (Samuel and Worthy 2004, Smith and Worthy 2006) (Figure 1.11). Long-chain polyunsaturated fatty acids are usually of dietary origin (Fraser et al. 1989, Iverson et al. 1997), and other studies have also indicated that these dietary fatty

acids may be deposited first in the deep blubber layer (Ackman et al. 1975a, 1975b, Koopman et al. 1996, 2003). Deposition of these fatty acids of dietary origin primarily in the deep blubber is important for understanding the role of this layer as an active source of energy supply (Koopman et al. 1996), involved in lipid deposition and mobilization (Ackman et al. 1975b, Aguilar and Borrell 1990, Lockyer et al. 1984).

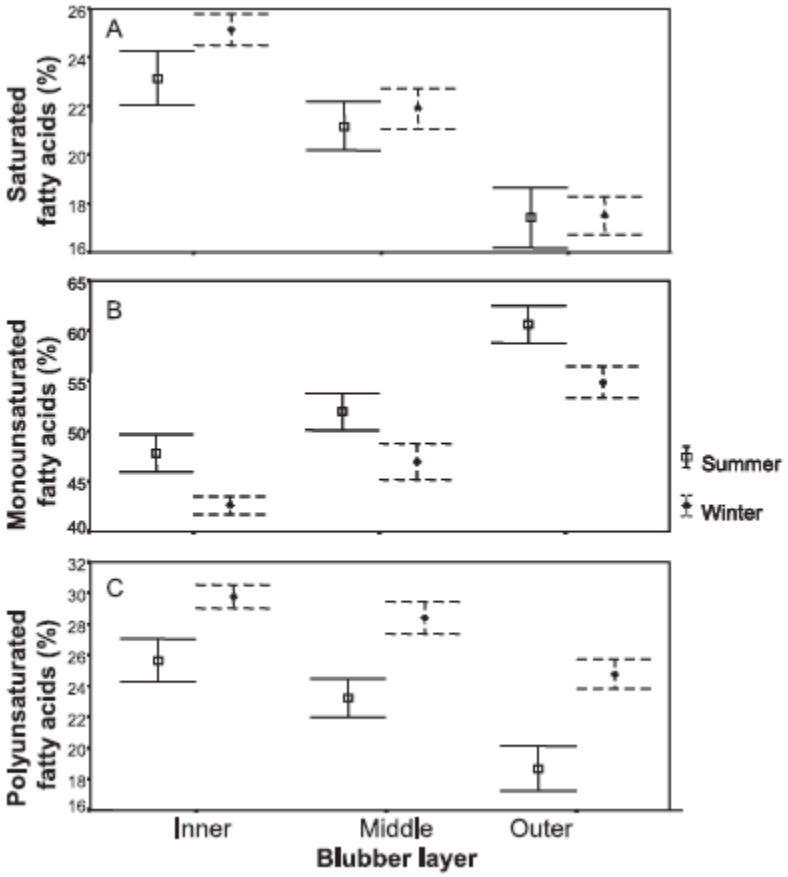

Figure 1.11. Average percentage of saturated, monounsaturated, and polyunsaturated fatty acids found in the inner, middle, and outer blubber layer of bottlenose dolphins from the southeast coast of the United States. Figure adapted from Samuel and Worthy 2004. Error bars represent standard error.

In a later study by Montie and others (2008a), CYP1A1 expression was highest in the endothelial cells of the deep blubber layer compared to the middle and superficial

blubber layers of bottlenose dolphins from Charleston Harbor, SC. This result was explained by lipid mobilization in the deep blubber layer, redistribution of lipophilic contaminants into circulation, leading to induction of CYP1A1. Lipid mobilization is triggered by seasonal changes in temperature, lactation in reproductive female dolphins, or physiological stress. Because stratification of CYP1A1 was not significant in dolphins from Indian River Lagoon, FL (in which seasonal water temperatures are consistently higher than in Charleston Harbor), it was concluded that more drastic seasonal changes in water temperature attributed to stratification patterns found (Montie et al. 2008a).

Further studies should indicate whether or not seasonal changes in temperature play a major role in distribution of CYP1A1 throughout the blubber. These studies are useful for determining whether or not dart biopsy methods of sampling are effective for analyzing CYP1A1 in the blubber. For example, animals in Hawai‘i experience small seasonal changes in temperature. Seasonal surface water temperatures in Hawai‘i vary by approximately 7°F, from a low of 73°F between late February/March, to a high of 80°F in late September/early October (NOAA 2007). This small-scale seasonality has been observed in the upper 128m of waters, yet no seasonality has been observed below this depth in intermediate and deep waters (Bingham and Lukas 1996, Lukas and Santiago 1996). Bottlenose dolphins from the southeast coast of the United States that showed stratification of CYP1A1 throughout the blubber, however, experienced seasonal surface temperature changes of 29°F (from 52°F in the winter to 81°F in the summer) (Montie et al. 2008b). This study did not indicate trends in deep water temperatures at sampling sites throughout the year.

1.2.6. Trends expected in CYP1A1 expression

As mentioned previously, some studies have shown that contaminant concentrations correlate significantly with CYP1A1 expression. CYP1A1 expression in the liver correlated with mono-*ortho* PCBs in the blubber (White et al. 1994). When looking solely at integument (blubber and skin), Wilson and others (2007) found that total PCB and toxic equivalent quotient concentrations in blubber correlated weakly with dermal endothelial CYP1A1 expression. However, Σmono-*ortho* PCBs concentrations did not show this relationship. Furthermore, an in vitro study found that CYP1A1 expression in skin-biopsy samples correlated significantly with exposure to varying concentrations of β-Naphthoflavone (Godard et al. 2004).

Moreover, CYP1A1 is a valuable biomarker of contaminant exposure because it is able to be present when contaminants, such as PAHs, are rapidly metabolized and eliminated from the body (Law & Whinnett 1992). Therefore, it is not expected that CYP1A1 should always correlate significantly with contaminant concentrations if inducers are able to be metabolized and eliminated. To take into account the metabolic nature of the PAHs, some studies have inferred their presence by the existence of aromatic DNA adducts in tissues (Ray et al. 1991). If all potential inducers are resistant to metabolism, such as the HAHs, the relationship between CYP1A1 expression and contaminant exposure would likely be more direct.

1.2.7. CYP1A1 expression with age, gender, reproductive state, and trophic level

Although CYP1A1 has been found to correlate with POP concentrations in many studies, CYP1A1 inter-group variability may not follow the same trends as seen with

POPs based on age and gender. CYP1A1 expression was not dependent on gender for humpback whales, spinner dolphins, and pantropical spotted dolphins from the Indian Ocean (Jauniaux et al. 2011). CYP1A1 expression was not correlated with gender and age in bottlenose dolphins from Sarasota Bay, FL (Wilson et al. 2007). Although it is expected that adult males and first-born calves should have the highest contaminant levels, CYP1A1 expression may not necessarily be higher in these gender and age groups. This would be a result of the fact that CYP1A1 is often induced when lipids and contaminants are mobilized into circulation, as occurs in lactating females or nutritionally stressed animals. For example, CYP1A1 varied with reproductive status in bottlenose dolphins from Charleston, SC, with pregnant-lactating females having the highest CYP1A1 expression (Montie et al. 2008a). Therefore, it is important to take into account the fact that CYP1A1 is a biomarker of contaminant exposure, yet individual physiology may have a large influence on expression patterns.

Although CYP1A1 expression may not exactly follow contaminant trends for age and gender because of individual variability in nutritional status, there appears to be an influence of trophic position on CYP1A1 expression. CYP1A1 was highest in spinner dolphins in comparison to pantropical spotted dolphins and humpback whales in skin biopsies collected from the Indian Ocean (Jauniaux et al. 2011). However, mysticetes (that feed at a lower trophic level) had comparable CYP1A1 expression scores to odontocetes (that feed at a higher trophic level) in a study by Angell et al. (2004). A table of semi-quantitative CYP1A1 expression determined for a variety of species is listed in Table 1.2.

Table 1.2. Reported semi-quantitative CYP1A1 expression (0-15) in blubber of various species from multiple studies. Scores from Angell et al. (2004) are reported as means, scores from Jauniaux et al. (2011) are reported as highest sample score, and the score from Godard-Codding et al. (2011) is reported as median. Sample sizes (*n*) are included in parentheses.

Species	CYP1A1 expression	Location	Author
beluga whale (*Delphinapterus leucas)*	5.2 +/- 1.6 (13)	Mackenzie Delta, Canada	Angell et al. 2004
bottlenose dolphin (*Tursiops truncatus*)	4.7 +/- 2.7 (6)	Gulf of Mexico	Angell et al. 2004
harbour porpoise (*Phocoena phocoena*)	0.1 +/- 0.17 (9)	Grand Manan, NB, Canada	Angell et al. 2004
humpback whale (*Megaptera novaeangliae*)	2.3 +/-2.6 (13)	Gulf of St. Lawrence	Angell et al. 2004
humpback whale (*Megaptera novaeangliae*)	1 (15)	Mayotte, Mozambique	Jauniaux et al. 2011
killer whale (*Orcinus orca*)	0.0+/-0.0 (2)	British Columbia, Canada	Angell et al. 2004
northern right whale (*Eubalaena glacialis*)	3.8 +/- 2.5 (83)	Bay of Fundy	Angell et al. 2004
pantropical spotted dolphin (*Stenella attenuata*)	2 (7)	Mayotte, Mozambique	Jauniaux et al. 2011
Risso's dolphin (*Grampus griseus*)	8.1 +/- 3.0 (5)	Cape Cod, MA	Angell et al. 2004
Sowerby's beaked whale (*Mesoplodon bidens*)	1.8 +/- 1.8 (3)	Northwest Atlantic	Angell et al. 2004
sperm whale *(Physeter macrocephalus)*	0.6 +/- 1.1 (9)	Galapagos	Angell et al. 2004
sperm whale *(Physeter macrocephalus)*	1.7 (25)	Galapagos	Godard-Codding et al. 2011
spinner dolphin *(Stenella longirostris)*	5 (24)	Mayotte, Mozambique	Jauniaux et al. 2011
white-sided dolphin *(Lagenorhynchus obliquidens)*	8.0 (1)	Cape Cod, MA	Angell et al. 2004

1.2.8. Geographic influence on CYP1A1 expression

Proximity to continents has been shown to affect overall CYP1A1 expression in cetacean populations. CYP1A1 was found to be highest in sperm whales from the Galapagos Islands, followed by those from Gulf of California. Sperm whales from these regions had significantly higher CYP1A1 expression compared to sperm whales from the Kiribati region and the region between Galapagos and Kiribati (Pacific Crossing 1). Significantly lower expression of CYP1A1 was seen in regions furthest from continents in this study (Godard-Codding et al. 2011). Although the authors could not correlate CYP1A1 expression with contaminant concentrations in the blubber in this study, they indicated that expression may have been related to other contaminants not analyzed in the study: dioxin-like PCBs, dioxins, and natural products.

Another study looked at CYP1A1 expression in beluga whales from two different geographic regions, the Gulf of St. Lawrence estuary and the Arctic. These two habitats differ in their levels of environmental contaminants. The Gulf of St. Lawrence estuary is much more highly contaminanted than the Arctic sampling location, and belugas from this region show an elevated incidence of tumors. Contrary to what would be expected based on the high levels of contamination, CYP1A1 in the liver of St. Lawrence Estuary beluga whales was very low (averages ranged 0.3-3.3), whereas Arctic beluga whales showed high levels of CYP1A1 expression (averages ranged 10-12.4). In all other tissues (adrenal gland, bladder, lung, skin, testis), CYP1A1 expression was high for both sites and not significantly different. Explanations for these patterns of CYP1A1 expression include downregulation of CYP1A1 in the liver when individuals are exposed to extremely high levels of contaminants (Wilson et al. 2005), as shown with fish exposed

to high levels of non-*ortho* PCB congeners. It is unknown exactly how CYP1A1 is down-regulated at high PCB exposure, but it may be blocked in its post-transcriptional stage (Schlezinger and Stegeman 2001). Moreover, the high levels of CYP1A1 in the Arctic belugas that are exposed to much lower levels of contaminants may be explained by the high sensitivity of the beluga whale AHR to HAHs (Jensen and Hahn 2001). Highly contaminated regions or food sources may be an exception to the rule of higher CYP1A1 expression with higher contaminant exposure. The potential for downregulation of CYP1A1 must be taken into account when analyzing samples from species that inhabit heavily contaminated waters or consume food at the highest trophic levels.

1.2.9. Conclusions: Threatened Species and Future Work

Toxicological studies on bioaccumulation, biomagnification, and adverse health effects at the individual, population, and ecosystem levels are incredibly important in the context of increasing anthropogenic threats to marine systems worldwide. Correlations have been found between PCB toxicity and epizootic outbreaks of morbillivirus and phocine distemper virus in striped dolphins of the Mediterranean Sea ([1]Aguilar & Borrell 1994) and harbor seals in the Baltic Sea (Hall et al. 1992). In addition, models have predicted depressed population growth of bottlenose dolphins because of exposure to high levels of PCBs (Hall et al. 2006). The St. Lawrence beluga (Muir et al. 1996), striped dolphins from the western Mediterranean (Kannan et al. 1993), killer whales from British Columbia (Ross et al. 2000), and bottlenose dolphins from the Southeast United States (Hansen et al. 2004) are all known to be exposed to high contaminant levels. Future research on the effects of increasing contaminant exposure on local populations is

important for understanding the future status of these species overall.

Little research has been conducted on the molecular responses to contaminants in Hawaiian cetaceans. The purpose of this thesis project is to examine the health status of stranded and free-ranging Hawaiian odontocetes for a biomarker of contaminant exposure, CYP1A1. This work will determine whether CYP1A1 is a useful biomarker of contaminant exposure in Hawaiian odontocetes. Lastly, this thesis project should indicate which species inhabiting the waters of Hawai‘i are most affected at the molecular level to contaminant exposure, providing a baseline for future monitoring of CYP1A1 expression in Hawaiian odontocetes.

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Chapter 2: Characterization of the Blubber of Hawaiian Odontocetes: Cellular and CYP1A1 Stratification

Abstract

The blubber of cetaceans is characterized by distinct layering of fatty acids, adipocytes, structural fibers, and connective tissue. This layering gives blubber its multifunctional nature, allowing it to exhibit changes based on the nutritional demands of an individual and provide insulation against the thermodynamically challenging aquatic environment. A key enzyme influenced by lipid dynamics in the blubber is cytochrome P4501A1 (CYP1A1), an established biomarker of contaminant exposure. This enzyme has been found to be prevalent in endothelial cells of the deep blubber layer. Stratification of CYP1A1 with highest presence in the deep blubber layer occurs because of lipid and contaminant mobilization within this layer during times of nutritional stress or seasonal changes in water temperature.

We assessed stratification of key cell types and CYP1A1 in Hawaiian odontocetes to determine the utility of dart biopsy samples that often obtain only the superficial to middle blubber layers. Significant differences in adipocyte quantities, adipocyte areas, and endothelial cell quantities occurred in the blubber ($p < 0.0001$). The middle blubber layer contained significantly smaller quantities of endothelial cells compared to the deep and superficial blubber layers. The middle blubber layer also displayed the greatest amount of adipocytes, along with the largest adipocytes. Furthermore, a significant difference existed for adipocyte area between thin and robust condition groups within the deep blubber layer ($p = 0.002$). These results contributed to an understanding of lipid mobilization in the deep blubber layer, a valid explanation for CYP1A1 stratification with highest presence in the deep blubber layer ($p = 0.013$). Stratification of key cell types and CYP1A1 in the present study was most likely a result of physiological stress and lipid mobilization that individuals experienced prior to stranding.

Significant differences in adipocyte area ($p = 0.041$) and adipocyte quantities ($p <0.0001$) existed among age classes within the middle blubber layer. These results, along with similar results found in the literature, suggest that the thermal properties of the middle blubber layer change with growth. No significant differences in CYP1A1 expression occurred for age class ($p = 0.182$) or gender ($p = 0.310$), a result also reported in the literature. A significant difference in CYP1A1 expression was found for species groups, with striped dolphins expressing lower levels of CYP1A1 in comparison to bottlenose dolphins and spinner dolphins ($p = 0.017$). More information is needed on the trophic structure of Hawaiian odontocetes for further conclusions, however, we hypothesize that the offshore feeding habits of striped dolphins may expose these individuals to a different set of contaminants than encountered by island-associated populations.

Lastly, CYP1A1 expression in the deep blubber layer correlated significantly with expression in the middle blubber layer ($p = 0.0004$), along with the superficial blubber layer ($p = 0.001$). Therefore, we suggest that dart biopsy samples can be used for future analysis of CYP1A1; however, these samples must consider area of the biopsy tissue because of the influence of higher CYP1A1 expression in the deep blubber layer.

2.1. Introduction

Blubber is the layer of fatty tissue between the epidermis and muscle that provides support for movement, buoyancy (Parry 1949), and insulation from heat loss in the aquatic habitat (Dunkin et al. 2005, Worthy and Edwards 1990). Because of its high lipid content, it is a dynamic layer of tissue that is used for energy storage and supply depending on the nutritional needs of an animal (Aguilar and Borrell 1991, Koopman et al. 1996, 2002, Struntz et al. 2004). Because blubber is a site for lipid storage, it is an interesting area to study the molecular effects of lipophilic persistent organic pollutants (POPs) that accumulate within the blubber. POPs are a significant threat to odontocete species because of the processes of bioaccumulation in the blubber and biomagnification up the food web. Odontocetes are exceptionally susceptible because of their large fatty tissue reserves, high position in food webs, and long life spans (Boon et al. 1992). Exposure to POPs has been linked to reproductive impairment (Subramanian et al. 1987), reduced reproductive success (Wells et al. 2005), decreased immune function (Lahvis et al. 1995), higher incidence of tumors (Martineau et al. 2002), and epizootic outbreaks (Aguilar and Borrell 1994) in odontocete species.

The blubber does not solely operate as one unit, but is multifunctional with distinct layers of different cell types and functions. Analysis of fatty acids has revealed that the deep blubber layer is an active source of energy supply (Koopman et al. 1996), involved in lipid deposition and mobilization. The outermost layer, however, is a stable, structural layer with only a small role in energy storage. The middle blubber layer represents the transitional zone between the superficial and deep blubber layers (Ackman et al. 1975, Aguilar and Borrell 1990, Lockyer et al. 1984). Montie and others (2008b)

established that the blubber of live bottlenose dolphins from the southeast coast of the United States shows three distinct layers. In their study, the superficial blubber layer contained few, small adipocytes, and a large amount of structural fibers. The middle blubber layer was characterized by few structural fibers, and the greatest quantity and size of adipocytes. The deep blubber layer exhibited an intermediate amount and size of adipocytes, and amount of structural fibers, in comparison to the superficial and middle blubber layers. Understanding the structure of blubber is critical for many cetacean studies that depend on dart biopsy sampling. In some cases, dart biopsy sampling may only obtain a small sample of tissue that is not representative of the full blubber depth. Depending on the analysis, these samples must be interpreted with caution because of the importance of the metabolically active inner blubber layer ($_2$Krahn et al. 2004).

Odontocetes draw upon storage lipids in the deep blubber layer during times of stress, illness, migration, reproduction/lactation, and low food availability. Along with lipid mobilization, POPs are also mobilized from blubber storage into circulation where they are able to be metabolically altered. It is suggested that POPs may be metabolized in the endothelial cells as soon as they reach circulation; however, the predominant site of phase I metabolism is the liver (Garrick et al. 2006, Montie et al. 2008a, White et al. 2000). As they travel through circulation, POPs may have large effects on organ systems in the body, including endocrine, neurological, reproductive, developmental, and immunological systems (Robertson and Hansen 2001).

Cytochrome P4501A1 (CYP1A1) expression is a well-known biomarker of POP exposure that has been observed in the blubber of a variety of cetacean species (Angell et al. 2004, Fertall 2010). CYP1A1 is induced when lipophilic polycyclic aromatic

hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons (PHAHs) are mobilized from lipid storage, diffuse through cellular membranes, and bind to the cytosolic aryl hydrocarbon receptor (AHR). CYP1A1 is then involved in phase I oxidative metabolism in which it may bioactivate the original PAH or PHAH ligands. PAHs may be converted to epoxide intermediates, that can further be converted to highly reactive diol-epoxide intermediates. These diol-epoxides are known to cause DNA and protein adducts, initiating carcinogenesis and cell transformation (Conney 1982, Shimada and Fujii-Kuriyama 2004). CYP1A1 may also metabolize PHAHs (such as PCB congeners with chlorine substituents at one or both para positions, with adjacent non-halogenated ortho and meta carbons on at least one ring) to hydroxylated metabolites (OH-PCBs) (Letcher et al. 2000). OH-PCBs may cause changes to thyroid hormone (TH) homeostasis, interfering with vital processes such as skeletal growth, brain and ear development, immune system function, metabolism, and reproduction (Brouwer et al. 1989, 1998, Zoeller et al. 2002).

CYP1A1 expression is stratified within the blubber of bottlenose dolphins from Charleston Harbor, SC (CHS) (Montie et al. 2008a), with highest expression found in capillary endothelial cells of the deep blubber layer. CYP1A1 expression in the deep blubber was positively correlated to concentrations of OH-PCBs in the plasma. This significant relationship was explained by lipid mobilization in the deep blubber layer, leading to contaminant mobilization into plasma and induction of CYP1A1 within this layer. Lipids mobilization in the deep blubber layer may occur for a variety of reasons: starvation, fasting, lactation, or adaptation to warmer water temperatures (Figure 2.1).

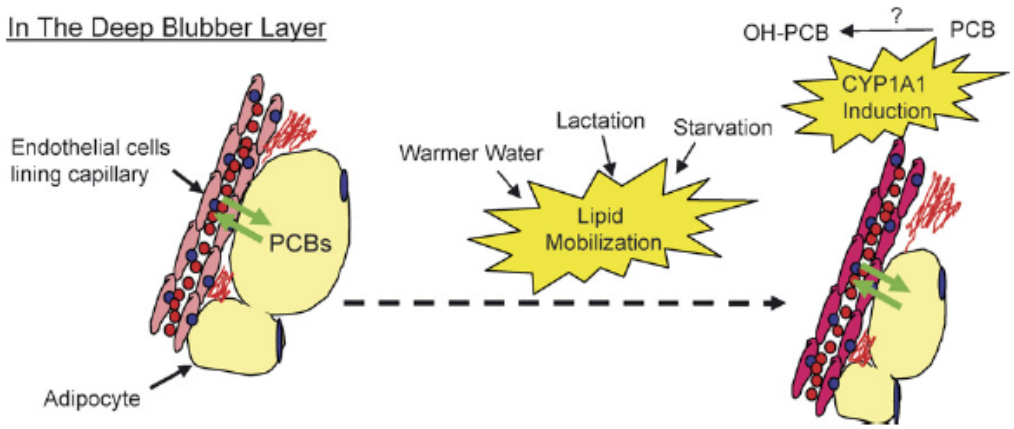

Figure 2.1. Graphic representation of how lipids are mobilized from the deep blubber layer under certain conditions, leading to mobilization of PCBs and induction of CYP1A1. As PCBs induce CYP1A1 in the endothelial cells, they may be metabolized by the enzyme to OH-PCBs and further carried throughout circulation. However, because the liver is the predominant source of phase I metabolism, PCBs may be carried throughout circulation and then metabolized in the liver to OH-PCBs (from Montie et al. 2008a).

Because stratification of CYP1A1 is significant in CHS dolphins, and not bottlenose dolphins from Indian River Lagoon, FL (IRL), more drastic seasonal fluctuations in sea surface temperature (adaptation to much warmer waters in the summer) was suggested to be the driving force for lipid mobilization and CYP1A1 stratification in CHS dolphins (Montie et al. 2008a). CHS dolphins experience colder temperatures from January through June each year compared to IRL dolphins (Montie et al. 2008b). Therefore, it is possible that the CHS dolphins experience larger fluctuations in adipocyte size throughout the year, with highest lipid content seen in colder months to provide insulation, and drastic thinning and mobilization of lipids and contaminants in the warmer months.

The purpose of this study is to extend analysis of cellular and CYP1A1 stratification to cetaceans that inhabit the waters of Hawai‘i, where the seasonal changes

in surface water temperature an animal experiences are small, but where the changes in water temperature from the thermocline to feeding depths are significant. Surface temperatures vary by approximately 7°F (low of 73°F between late February/March to a high of 80°F in late September/early October), and night to day changes are one or two degrees ($_1$NOAA). This small variability in daily and seasonal temperature of the waters surrounding Hawai‘i may be enough to cause cellular and CYP1A1 stratification. However, it is possible that Hawaiian odontocetes will exhibit less distinctive cellular and CYP1A1 stratification among the blubber layers (deep, middle, and superficial) as displayed in the IRL dolphins that experienced warmer annual sea-surface temperatures (Montie et al. 2008a, 2008b). If consistent sea surface temperatures throughout the year prevents seasonal mobilization of lipids and residing contaminants, then blubber of Hawaiian cetaceans may not be stratified with respect to cellular distribution and CYP1A1 expression.

Intermediate, deep, and bottom waters surrounding Hawai‘i do not show changes in temperature that resemble a seasonal cycle (Bingham and Lukas 1996, Lukas and Santiago 1996). However, species that feed as deep as 1000m, such as beaked whales (family *Ziphiidae*) (Baird et al. 2006), may experience temperature changes of 40°F from surface waters to depth in Hawai‘i ($_1$NOAA, Noda et al. 1981). Therefore, on a daily basis, deep-diving animals experience large fluctuations in temperature. Differences in cellular and CYP1A1 stratification between deep-diving and shallow-diving species is yet to be analyzed.

In this study, adipocyte quantities, adipocyte areas, endothelial cell quantities, and CYP1A1 expression throughout the blubber of eleven species of stranded Hawaiian

odontocetes was examined. Importantly, this stratification analysis enables us to assess the potential application of CYP1A1 as a biomarker in dart biopsy samples from free-ranging animals in the circumstance that the biopsy does not penetrate all blubber layers. For these reasons, this study contributes critical information for future toxicological and health assessment work in Hawaiian odontocetes.

2.2. Methods

2.2.1. Sample collection

Blubber samples from stranded animals were received from the Hawai‘i Pacific University Marine Mammal Stranding Program. All animals stranded in the main Hawaiian Islands from 1997-2011. Stranded animals were reported by the public or government officials, and necropsied as quickly as possible in the field or at facilities on O‘ahu. Necropsies took place usually within 24 hours of the initial report. Blubber samples collected for this study were fixed in 10% neutral buffered formalin (NBF).

Blubber samples were retrieved from formalin storage and placed in ethanol for transport to the Department of Pathobiology, College of Veterinary Medicine at University of Tennessee or the histology core facility at John A. Burns School of Medicine, University of Hawai‘i. Blubber samples were trimmed and embedded in paraffin blocks. The embedded tissues were sectioned at 5 μm and placed on slides. The slides were then returned to Hawai‘i Pacific University for staining and analysis. The following cetacean blubber samples were used for the cellular stratification analysis and CYP1A1 stratification analysis (Table 2.1).

Table 2.1. Stranded odontocetes studied in cellular stratification analysis. This list includes the field number, accession or slide number, necropsy date, location of stranding, species, age class, gender, and animal body condition. A total of n = 31 animals were analyzed for the cellular stratification analysis. For the CYP1A1 stratification analysis, n = 29 of the same animals from the cellular stratification analysis were analyzed for expression.

Field #	Accession #	Necropsy Date	Stranding Location	Species	Age Class	Gender	Condition
12470	12470	1/2/1997	Mokule'ia, Oʻahu	*S. coeruleoalba*	Adult	Male	Robust
NMFS-SA-03-05-SD	08-021	1/31/2003	Kailua, Oʻahu	*S. attenuata*	Adult	Female	Thin
MMRP *S. attenuata*	06-177	7/12/2004	Oʻahu	*S. attenuata*	Adult	Female	Thin
NMFS-SC-05-24-SD	06-174	3/14/2005	Kaneohe, Oʻahu	*S. coeruleoalba*	Adult	Female	Thin
SLP2006001	06-217 M11-54	7/23/2006	Makapu'u, Oʻahu	*F. attenuata*	Adult	Female	Not determined
KW-2007-004	07-296 M11-53	7/1/2007	Punalu'u, Oʻahu	*S. longirostris*	Subadult	Male	Thin
KW-2007-005	07-294 M11-52	9/30/2007	Wai'anae, Oʻahu	*S. longirostris*	Adult	Female	Thin
KW-2008-002	08-018	1/16/2008	Anahola, Kauaʻi	*S. longirosris*	Adult	Female	Robust
KW-2008-004	08-206	3/15/2008	Lahaina, Maui	*S. longirostris*	Adult	Male	Robust
KW-2008-005	08-205	6/12/2008	Honolulu, Oʻahu	*S. attenuata*	Subadult	Male	Robust
KW-2008-006	08-207	6/21/2008	Hilo, Hawaiʻi	*S. coeruleoalba*	Calf	Female	Thin
KW-2008-008	08-204	7/29/2008	Kaunakakai, Molokaʻi	*Z. cavirostris*	Subadult	Male	Not determined
KW-2008-010	08-283 M11-51	10/22/2008	Po'ipu, Kauaʻi	*O. orca*	Subadult	Male	Not determined
KW-2008-012	10-35-16	9/23/2010	Polihale, Kauaʻi	*S. longirostris*	Adult	Male	Thin
KW-2009-001	09-054	3/13/2009	Kahalu'u, Oʻahu	*S. coeruleoalba*	Subadult	Female	Thin
KW-2009-004	09-053	3/13/2009	Wai'anae, Oʻahu	*S. longirostris*	Calf	Female	Robust
KW-2009-006	09-132	5/25/2009	Ma'alaea, Maui	*F. attenuata*	Adult	Male	Not determined
KW-2009-007	09-133	6/04/2009	Kaho'olawe	*T. truncatus*	Adult	Male	Robust
KW-2009-008	09-134	6/14/2009	Kailua, Oʻahu	*S. coeruleoalba*	Subadult	Male	Robust
KW-2009-009	09-153	7/06/2009	Ma'alaea, Maui	*S. coeruleoalba*	Adult	Female	Thin
KW-2009-011	09-154	7/06/2009	Kihei, Maui	*S. coeruleoalba*	Calf	Female	Thin
KW-2009-014	10-36-14	9/22/2010	Wai'anae, Oʻahu	*P. electra*	Calf	Male	Not determined
KW-2009-015	10-107	11/26/2009	Kailua-Kona, Hawaiʻi	*S. attenuata*	Adult	Male	Thin
KW-2010-006	KW-2010-006	4/16/2010	Waimanalo, Oʻahu	*S. longirostris*	Subadult	Male	Robust
KW-2010-008	KW-2010-008	6/16/2010	Miloli'i, Hawaiʻi	*S. coeruleoalba*	Adult	Male	Robust
KW-2010-012	KW-2010-012	8/29/2010	Ma'alaea, Maui	*M. densirostris*	Subadult	Male	Not determined
KW-2010-019	10-38-01	11/27/2010	Kawela, Molokaʻi	*P. crassidens*	Adult	Female	Not determined
KW-2011-001	11-20-23	2/14/2011	Lydgate Park, Kauaʻi	*T. truncatus*	Subadult	Female	Robust
KW-2011-003	KW-2011-003	4/13/2011	Waikoloa, Hawaiʻi	*T. truncatus*	Calf	Male	Thin
KW-2011-006	M11-46-16	4/24/2011	Waikoloa, Hawaiʻi	*T. truncatus*	Calf	Female	Thin
KW-2011-007	M11-50-31	5/22/2011	Lydgate Park, Kauaʻi	*T. truncatus*	Adult	Male	Robust
KW-2011-008	11-089	5/31/2011	Laʻie, Oʻahu	*P. macrocephalus*	Neonate	Female	Not determined

2.2.2. Cellular Stratification Analysis

Hematoxylin and Eosin

Slides were placed in a routine set of xylene, ethanol, distilled water, hematoxylin, and eosin dips according to modified Sigma Aldrich Co. (St. Louis, MO, USA) protocols (Fertall 2010). First, sections were deparaffinized by placement in two xylene trays for ten minutes each. They were then rehydrated by two changes of absolute ethanol for five minutes each. Next, they were placed in 95% ethanol for two minutes, and then in 70% ethanol for two minutes. They were then set in distilled water, stained with Mayer's hematoxylin for eight minutes, and washed in warm running tap water for ten minutes. Afterwards, they were held in distilled H_2O, rinsed in 95% ethanol for ten dips, and counterstained in eosin phloxine B solution (or eosin Y solution) for one minute. Last, they were dehydrated with 95% ethanol, two changes in absolute ethanol for five minutes each, and cleared with two changes of xylene for five minutes each. The slides were then mounted with Permount® (Fisher Scientific) (Fertall 2010, modified from Sigma Aldrich protocols).

Analysis of cellular distribution

Hematoxylin and eosin stained slides were viewed to determine if a gradient in adipocyte area, adipocyte quantity, and endothelial cell quantity existed among the superficial, middle, and deep blubber layers of Hawaiian odontocetes. First, each slide was viewed without magnification to determine if a color gradient existed on each tissue, indicating stratification of cell types. The eosin stain has been found to bind to more fibrous tissue, giving it a pink-red appearance, while it does not stain adipocytes. If the

tissue on the slide contained three representative layers (a pink-red toned superficial layer, white-toned middle layer, and pink-red toned deep layer) as indicated by previous studies (Montie et al. 2008b), the layer gradient was marked with a Sharpie® pen on the back of the slide. Next, the slides were viewed with an Olympus BX43 microscope at a total magnification of 400x (40x objective, 10x eyepiece). A picture of the blubber was taken every 0.48 mm throughout the length of the blubber with an Infinity 2-3 digital microscope camera. Pictures were sorted by layer according to the boundaries initially marked off on the back of the slides.

A sum of endothelial cells was determined for each picture, and an average of endothelial counts per picture was taken for each blubber layer of an individual. To count adipocytes, two diagonals were drawn across each picture, and the number of adipocytes that intersected each diagonal was counted and recorded (Figure 2.2). In cases for which the same adipocyte crossed both diagonals, the adipocyte was only counted once. An average of adipocyte counts per picture was taken for each blubber layer of an individual. For adipocyte area, a 100 µm scale bar at 400x total magnification was used to calibrate measured pixels to distance in Image J software 1.45M, (National Institutes of Health). Adipocyte area (μm^2) was measured for ten adipocytes on each picture. The average adipocyte area was recorded for each picture (Figure 2.3). An average was then taken of this value for all pictures within the blubber layer of each individual.

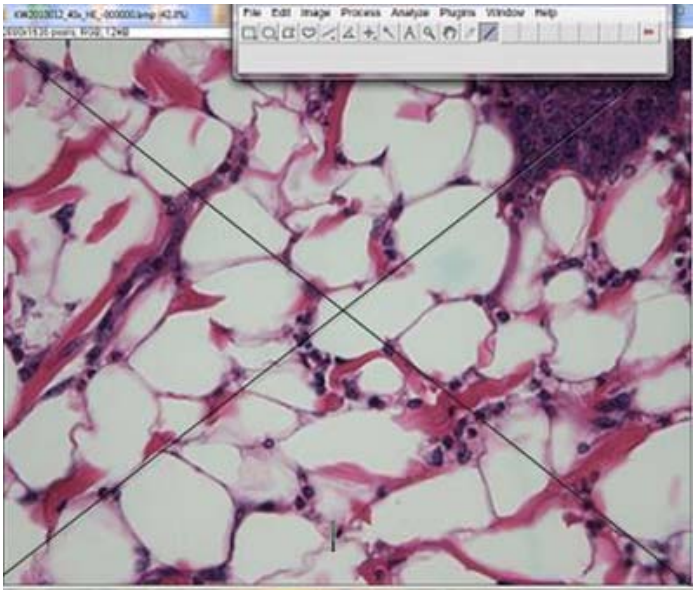

Figure 2.2. Method used to count adipocyte quantities throughout the blubber. Adipocytes that intersected each diagonal were counted for each picture/segment throughout the blubber, and then averaged within layers for a layer-specific mean quantity for each individual. Image J Software, 1.45 M (National Institutes of Health) was used for drawing diagonals across the tissue.

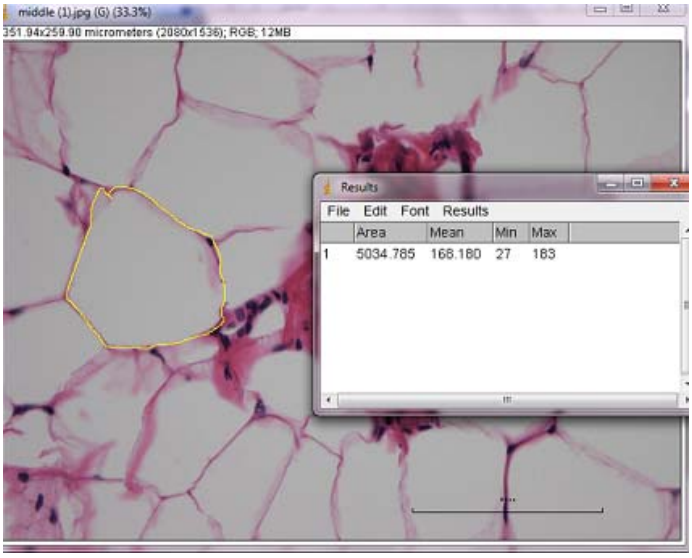

Figure 2.3. Method in which adipocyte area was measured throughout the blubber. Pixels were measured for a 100 µm scale bar and calibrated for measuring adipocyte area in μm^2. Ten adipocytes areas were measured for each picture, and then averaged for mean adipocyte area per picture. An average of these values was then taken for all pictures within the blubber layer of each individual. Measurements were taken with Image J software, 1.45 M (National Institutes of Health).

Animal condition

Body condition is a subjective assessment made at necropsy that can be problematic because of the large number of species that are common in Hawaiian waters and the infrequency of strandings. Because of the low sample size of most species in the sample archive, coupled with the variation of blubber thickness caused by size, a less subjective assessment of body condition was required. Gross necropsy reports and the accompanying photo catalog was re-examined for all individuals representing species for which $n > 3$ (bottlenose dolphins, pantropical spotted dolphins, spinner dolphins, and striped dolphins). After careful examination, the animal condition was categorized by the following formula:

Dorsal blubber thickness (cm)/total length (cm)

Based on analysis of animal body condition in necropsy reports, an animal was considered "thin" if its condition equaled less than 0.006, whereas it was considered "robust" if it scored 0.006 and higher. This method was chosen after taking the range of animal condition scores (0.0006-0.0127), and dividing by two (0.006). After comparing scores to photos and data from necropsy reports, it was decided that 0.006 was an appropriate division between thin and robust animals. Furthermore, previous analysis has used this method of quantifying animal condition, in which a negative correlation was found between CYP1A1 protein expression and blubber condition ($p = 0.004$; $R^2 = 0.52068$) (Urekew 2011). A distribution of animal condition by species in the present study is shown below (Figure 2.4).

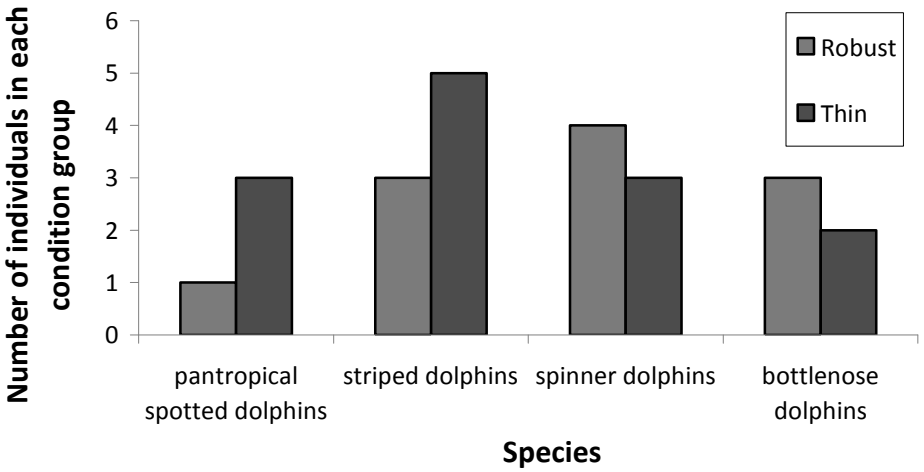

Figure 2.4. Number of individuals in each condition group (thin or robust) within species.

Statistics for cellular stratification analysis

All within-layer averages for each individual (endothelial cells quantities, adipocyte cell quantities, and adipocyte area) were transformed with the fourth root to achieve normal distributions based on analysis of data distribution within columns, along with data distribution and homogeneity of variances by each factor. In all cases, the multivariate F tests were used, and univariate tests were not used as sphericity was found to be violated in some cases. The multivariate F-tests are powerful and robust to violations in repeated-measures MANOVA assumptions (JMP® support online).

First, two-factor repeated measures MANOVA were performed for age and gender, age and condition, gender and condition, species and gender, and species and condition. The species and age factors were not considered together since there were no pantropical spotted dolphin calves in this study. The species and gender trial excluded all individuals with $n = 1$, since only one gender was represented within these species. Furthermore, only *Stenella* sp. and *Tursiops* sp. were included in analyses with condition

as a factor, because these were the only species categorized by the condition formula described previously. Because of these exclusions, the two-factor analyses were only useful to make sure no interactions existed among the factors that would confound the repeated measures analysis. Because no interactions were found among any of the factors in this study, repeated-measures analysis was then performed against each factor individually to determine if a significant effect occurred for age, gender, species, or condition alone. Furthermore, these one-factor repeated measures indicated whether a layer and factor interaction was present. In all cases, groups with $n < 5$ were excluded from the ANOVAs.

In the case of an interaction between layer and any factor (e.g. layer and condition), repeated-measures was run within groups of a factor (e.g. thin and robust animals), and one-way ANOVAs were performed for each individual layer by the factor of interest. In the case of no interaction between layer and factor, repeated-measures analysis was performed on the entire dataset, and not within groups of a factor. After all repeated-measures analyses, multivariate ANOVA contrasts were also performed for each blubber layer against the other two to determine which layers differed significantly from one another.

One-way ANOVAs were performed within each layer for adipocyte area and quantity by age and condition. Previous studies have shown that the composition of the middle blubber layer varies based on age, and the deep blubber layer varies based on animal condition (Montie et al. 2008b). Data were analyzed with JMP 9.0.2 Statistical software (©2010 SAS Institute Inc), with significance set at $\alpha = 0.05$.

2.2.3. CYP1A1 stratification analysis

Immunohistochemistry

Tissues were processed on slides according to the methods described above. For CYP1A1 stratification analysis, two slides were received for each tissue. One slide was used for analysis of CYP1A1 stratification with a primary polyclonal antibody against rat hepatic CYP1A1, with a rabbit host (Chemicon International, Cat. No. AB1247; diluted 1:4500 in 0.9% phosphate buffered saline (PBS)). The other slide was used as a negative control slide without use of antibody to account for non-specific staining not indicative of CYP1A1-antibody interactions. For each run of slides, an animal that was previously shown to have high expression of CYP1A1 in the integument was used as a positive control (Fertall 2010). This control was treated like all other animals examined in the study with a positive antibody slide used for analysis of CYP1A1 expression, and a negative control slide to account for background staining.

Slides were prepared for analysis by incubations in xylene and ethanol. They were placed in xylene for ten minutes, followed by two additional five-minute incubations in xylene. They were then incubated in 100% ethanol twice, for one minute each time. Next, they were placed in 70% ethanol twice, for one minute each time, followed by placement in 30% ethanol for two minutes (Fertall 2010, modified from Chemicon Immunophosphatase Secondary Detection System 2005). Slides were then placed in MilliQ® water for five minutes, followed by a rinse procedure in PBS. Lastly, a PAP barrier was drawn around each tissue to contain immunohistochemical reagents on the tissues.

Primary antibody slides

Blocking serum (Normal Horse Serum, Vector laboratories, Cat. No. S-2000) was placed on the tissues for twenty minutes, and excess serum was blotted from the sections. The primary antibody AB1247 was then placed on the appropriate slides for one hour. After incubation for one hour, the slides underwent two, two-minute rinse procedures with PBS. Slides were then incubated for another hour with the primary antibody, followed by the rinse procedure again. Tissues were then incubated with biotinylated secondary antibody (Vectastain® ABC-AP kit, rabbit IgG, Vector Laboratories, Cat. No. AK-5001) for thirty minutes, followed by the rinse procedure. Afterwards, tissues were incubated with alkaline phosphatase reagent labeled with avidin (Vectastain® ABC-AP kit, rabbit IgG, Vector Laboratories, Cat. No. AK-5001) for thirty minutes. Following the rinse procedure, tissues were incubated with substrate solution **(**Vector® Red in 200 mM tris-HCl, pH 8.5, Vector Laboratories, Cat. No. SK-5100**)** for thirty minutes. Slides were rinsed and counterstained with hematoxylin for one minute. After a final rinse, they were coverslipped with non-aqueous Permount® (Fisher Scientific, Cat. No. SP15-500).

Negative control slides

Negative control slides underwent the same procedure as above for the primary antibody slides, except they did not receive the primary antibody for any of the hour-long incubations. Instead, they were allowed to incubate in rinse procedure until the appropriate time for blocking serum to be placed on the slides, so that both primary antibody slides and negative control slides received the biotinylated secondary antibody

(and following steps of the procedure) at the same time. This occurred during the last half of the second hour-long incubation for primary antibody slides.

Determining CYP1A1 expression

Since previous studies showed that the endothelial cells of the blubber have the highest expression of CYP1A1 compared to other cells (Montie et al. 2008a), this study only examined endothelial cells for CYP1A1 expression. Intensity and occurrence of CYP1A1 in endothelial cells within the blubber layers was determined through use of the Olympus BX43 microscope at a total magnification of 200x (20x objective, 10x eyepiece). Intensity was measured on a 0-5 scale, with 0 indicating no staining, and 5 indicating the brightest amount of staining as seen in previous studies (Angell et al 2004, Montie et al. 2008a, Wilson et al. 2007).

CYP1A1 was quantified for the entire blubber layer of each individual by three different methods: semi-quantitative CYP1A1 expression, CYP1A1 expression per area (mm^2), and CYP1A1 expression/total number of endothelial cells. There are advantages to all three of these methods. First of all, the semi-quantitative method has been used extensively throughout the literature (Angell et al. 2004, Hooker et al. 2007, Wilson et al. 2005, 2007). Semi-quantitative CYP1A1 expression in this study is determined as:

(Intensity (0-5) *occurrence of endothelial cells (0-3) within blubber layer for primary antibody slide) - (Intensity (0-5)*occurrence of endothelial cells (0-3) within blubber layer for negative control slide).

The lowest level of intensity or occurrence was 0 (no staining present), five was the highest level of intensity, and 3 was the highest level of occurrence (all cells stained). Therefore, the range of semi-quantitative expression possible is 0-15. This semi-quantitative method is useful for tissues in which CYP1A1 is expressed in a variety of cell types, and quantifying cells and intensity collectively is a difficult task. Montie et al. (2008a) established that semi-quantitative CYP1A1 expression correlated significantly with CYP1A1 expression/area (mm^2). They also proposed the latter as a more accurate method of determining expression levels in endothelial cells of the blubber. CYP1A1 expression/area in this study is determined as:

[(Intensity (0-5)*occurrence (number of stained cells within blubber layer of primary antibody slide))- (Intensity (0-5)*occurrence (number of cells stained cells within blubber layer of negative antibody slide))]/total area (mm^2) of blubber layer

Instead of determining occurrence on a 0-3 scale, this method quantifies total expression within the blubber, and then accounts for area of tissue that would influence amount of CYP1A1 quantified. CYP1A1 scores for each layer were also determined through another quantitative method, CYP1A1 expression/total number of endothelial cells:

[(Intensity (0-5)*occurrence (number of stained cells within blubber layer of primary antibody slide))- (Intensity (0-5)*occurrence (number of cells stained cells within blubber layer of negative antibody slide))]/total number of endothelial cells within blubber layer

This quantification method has not yet been reported in the literature for analysis of CYP1A1 expression in cetacean blubber. Total number of endothelial cells within the blubber were quantified in the hematoxylin & eosin slides. This method is a useful method of quantification because 1) only endothelial cell expression was quantified in this study, 2) it divides expression determined by the total amount of cells that could potentially express CYP1A1, and 3) it provides a 0-5 scale of CYP1A1 expression (5 meaning all cells stained at an intensity of 5, the highest possible expression).

Statistical analysis: comparison of scoring methods

The three scoring methods were statistically compared through multivariate pairwise correlations to see if relationships existed among the scoring methods. All significance values were set at $\alpha = 0.05$.

Statistical analysis: CYP1A1 stratification

After testing a suite of transformations, CYP1A1 expression per area and per total number of endothelial cells were transformed by the fourth root ($x^{\wedge 1/4}$), and semi-quantitative CYP1A1 expression was transformed by the square root ($x^{\wedge 1/2}$) to meet ANOVA assumptions of normality and homogeneity of variance. CYP1A1 (all methods) was statistically analyzed as mentioned previously for the cellular stratification analysis with factorial repeated measures MANOVA, and statistical significance was set at $\alpha = 0.05$. Linear regression was used for determining relationships among CYP1A1 expression within blubber layers, and data were transformed with the fourth root for these

analyses so that residuals were normally distributed. Data were analyzed in JMP 9.0.2 Statistical software (©2010 SAS Institute Inc).

2.3. Results

2.3.1. Is cellular stratification present in Hawaiian odontocetes?

This study first examined whether there is apparent layering, or stratification, of endothelial cell quantities, adipocyte area, and adipocyte quantities, across blubber cross-sections from Hawaiian odontocetes. Stratification with respect to these cell parameters occurred in the sample set of this study.

Stratification of endothelial cell quantities existed in the blubber of Hawaiian odontocetes ($p < 0.0001$). The deep blubber layer had a significantly higher average endothelial cell quantity than the middle blubber layer ($p < 0.0001$). The superficial blubber layer had a significantly higher average endothelial cell quantity than the middle blubber layer ($p = 0.002$) (Figure 2.5).

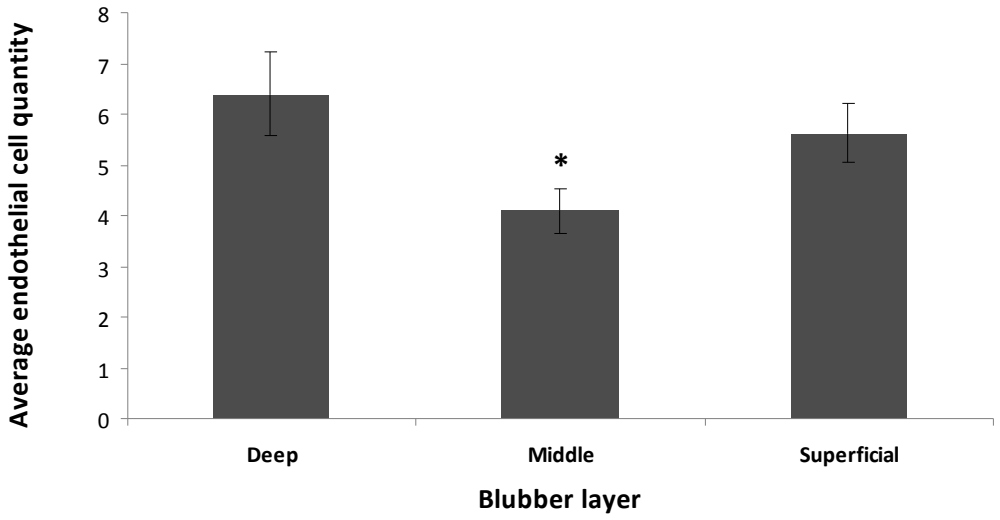

Figure 2.5. Average endothelial cell quantity throughout the blubber layers. The sample set included $n = 32$ individuals. Error bars represent standard error. The asterisk indicates that the middle blubber layer differed significantly from the other layers, $p < 0.05$.

Average adipocyte area (μm^2) was stratified throughout the blubber layers ($p < 0.0001$) of Hawaiian odontocetes as well, and all blubber layers differed significantly from one another (deep and middle: $p = 0.002$, deep and superficial: $p < 0.0001$, middle and superficial: $p < 0.0001$) (Figure 2.6). Furthermore, stratification of adipocyte quantities occurred throughout the blubber layers ($p < 0.0001$). All blubber layers differed significantly from one another (deep and middle: $p = 0.006$, deep and superficial: $p = 0.0002$, middle and superficial: $p < 0.0001$) (Figure 2.7). These patterns of stratification are similar to that found in bottlenose dolphins from the southeast coast of the United States (Montie et al. 2008b).

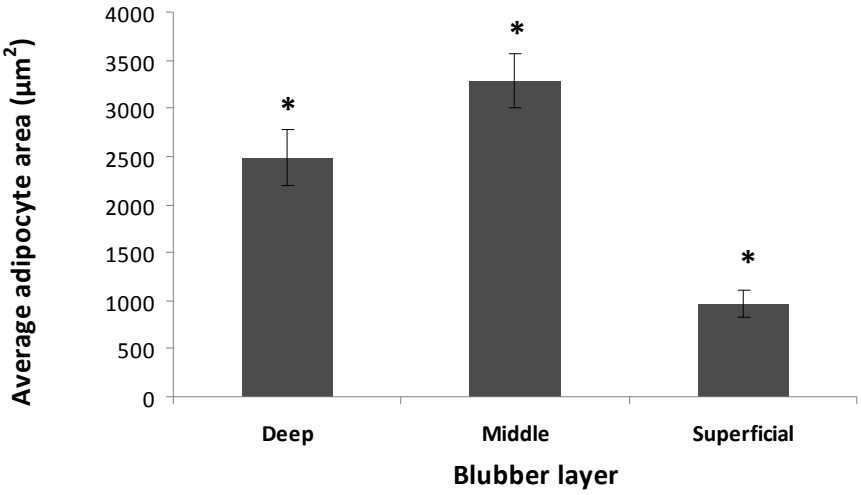

Figure 2.6. Average adipocyte area (μm^2) throughout the blubber layers. The sample set included $n = 32$ individuals. Error bars represent standard error. The asterisk on all layers indicates that all layers differed significantly from one another, $p < 0.05$.

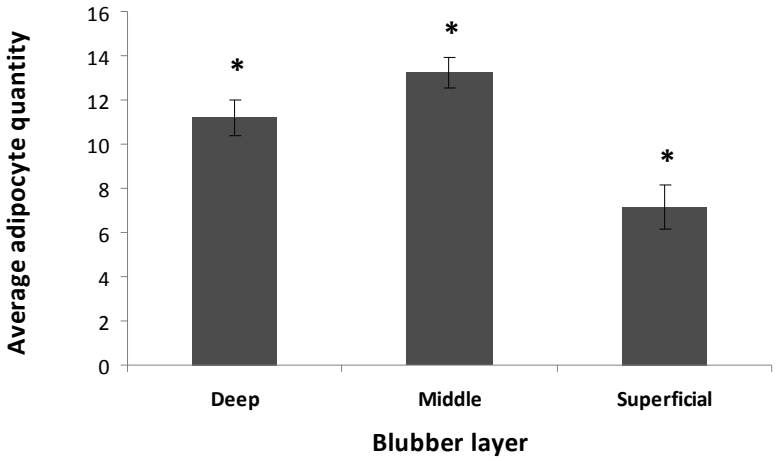

Figure 2.7. Average adipocyte quantity throughout the blubber layers. The sample set included $n = 32$ individuals. Error bars represent standard error. The asterisk on each layer indicates that all layers differed significantly from one another, $p < 0.05$.

2.3.2. Is CYP1A1 stratified throughout the blubber layers?

After establishing that there was clear cellular structure in the blubber of Hawaiian odontocetes, we next examined whether CYP1A1 expression in endothelial cells followed a similar pattern. However, cellular heterogeneity made it a challenge to describe CYP1A1 expression in a quantitative manner, so the first step was to determine the most appropriate way to generate the data. Upon analysis of the three different CYP1A1 scoring methods throughout the blubber layers, CYP1A1 expression/total number of endothelial cells most accurately represented CYP1A1 expression in stranded animals with variable blubber condition. This method quantifies CYP1A1 by normalizing expression independent of the influence of adipocyte area, that varies with animal condition and potentially influences ability to see expression.

CYP1A1 expression/total number of endothelial cells was stratified significantly throughout the blubber layers ($p = 0.013$). The deep blubber had significantly higher CYP1A1 expression than the middle and superficial blubber layers ($p = 0.020$ and

p = 0.004 respectively). The middle blubber layer did not differ significantly from the superficial blubber layer (p = 0.357) (Figure 2.8). Taken together with stratification of adipocyte cell area throughout the blubber (Figure 2.6), these results indicate lipid and contaminant mobilization, resulting in higher CYP1A1 expression in the deep blubber layer.

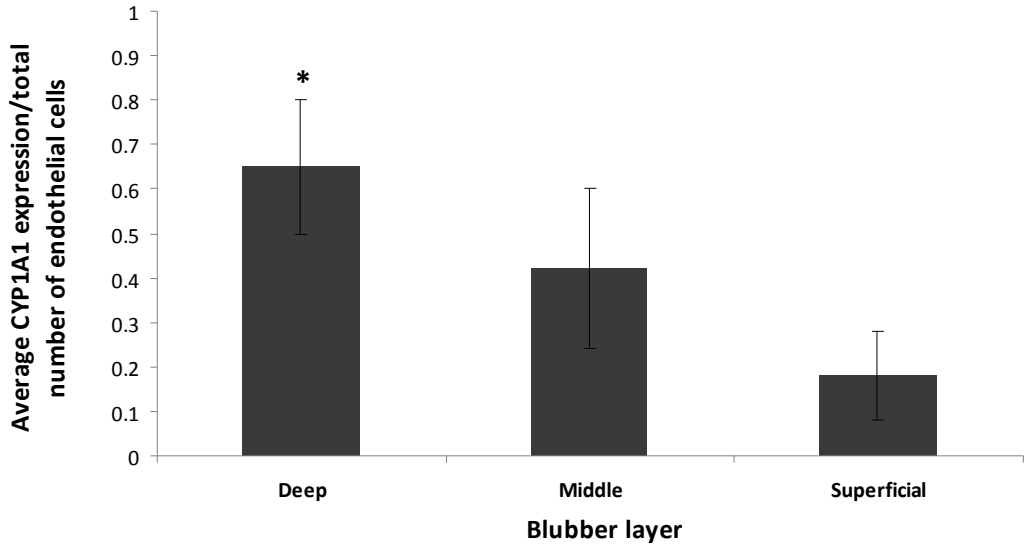

Figure 2.8. Average CYP1A1 expression throughout the blubber layers. The sample set included n = 30 individuals. Error bars represent standard error. The asterisk shows that the deep blubber layer had significantly higher expression than the middle and superficial blubber layers, $p < 0.05$.

2.3.3. Are CYP1A1 expression levels correlated across layers?

A key reason to assess the relationship between CYP1A1 expression in the blubber layers was to determine whether or not dart biopsies, which are more likely to sample the superficial to middle blubber layers, are appropriate for future CYP1A1 expression studies. In this study, CYP1A1 expression in the middle and superficial blubber layers correlated significantly with expression in the deep blubber layer (R^2=0.34, p = 0.0004, Figure 2.9 and R^2= 0.20, p = 0.001, Figure 2.10 respectively).

Since cellular heterogeneity makes layers easy to identify, this is a promising result for the prospects of using dart biopsies for future analysis of CYP1A1.

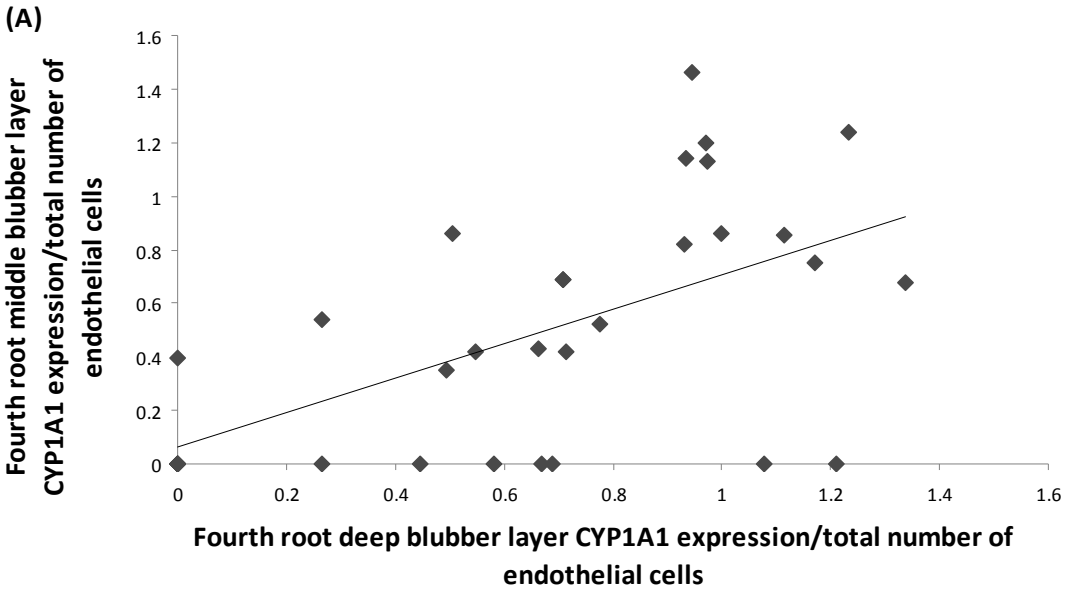

Figure 2.9. Correlation between deep and middle blubber layer CYP1A1 expression. A significant relationship was found among the blubber layers ($R^2 = 0.34$, $p = 0.0004$).

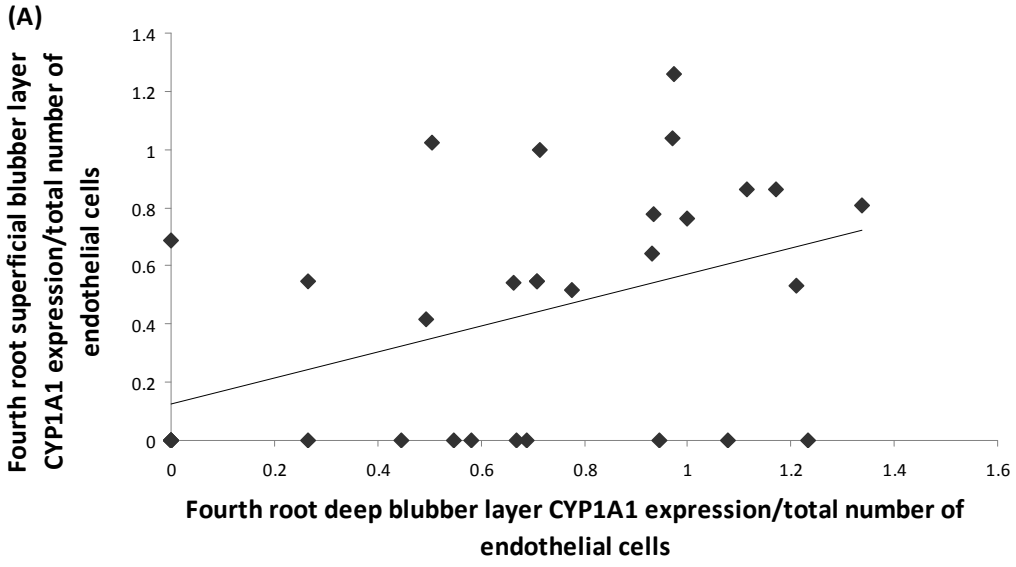

Figure 2.10. Correlation between deep and superficial blubber layer CYP1A1 expression. A significant relationship was found among the blubber layers ($R^2 = 0.20$, $p = 0.001$).

2.3.4. Are there significant trends in cellular distribution and CYP1A1 expression for different condition, species, age, or gender classes?

Although cellular and CYP1A1 stratification trends in a sample of pooled species were the main focus of this study, we also investigated whether or not cellular characteristics and CYP1A1 expression varied for individuals of different body condition, species, age class, and gender. Many studies have previously assessed CYP1A1 expression by species, gender, and age (Angell et al. 2004, White et al. 1994, Wilson et al. 2007), however, animal condition is a factor that has not yet been quantified and analyzed in the literature. Montie and others (2008a) showed that male dolphins and simultaneously pregnant-lactating females dolphins with smaller adipocytes had higher CYP1A1 expression in the deep blubber. These results show that nutritionally stressed animals may express higher levels of CYP1A1, and emphasize the importance of metabolism in the deep blubber layer.

Analysis of cellular characteristics and CYP1A1 expression by animal condition

In the within-subjects response for the repeated-measures MANOVA, there was a layer and condition interaction for adipocyte area across blubber layers ($p = 0.036$). Because of this significant interaction, condition groups were analyzed within each layer to determine whether or not there were significant differences in adipocyte area based on animal condition. Only adipocyte area in the deep blubber layer differed significantly based on animal condition (Figure 2.11) (deep: $p = 0.0015$, middle: $p = 0.164$, superficial: $p = 0.103$).

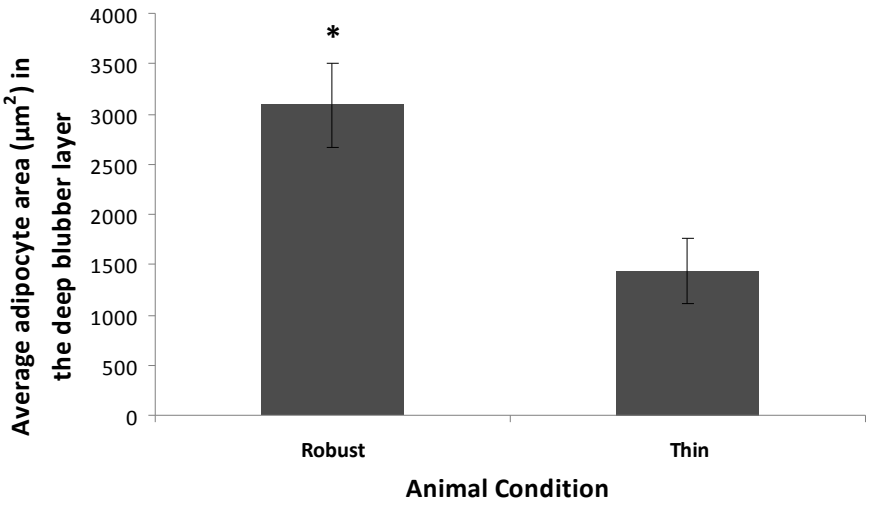

Figure 2.11. Average adipocyte area (µm^2) for condition groups in the deep blubber layer. Thin (n = 13) and robust (n = 11) animals were analyzed. Error bars represent standard error. The asterisk indicates that robust and thin animal condition groups differed significantly from one another, $p < 0.05$.

Adipocyte quantities (p = 0.940), endothelial cell quantities (p = 0.085), and CYP1A1 expression (p = 0.559) (Figure 2.12) did not differ significantly among condition groups.

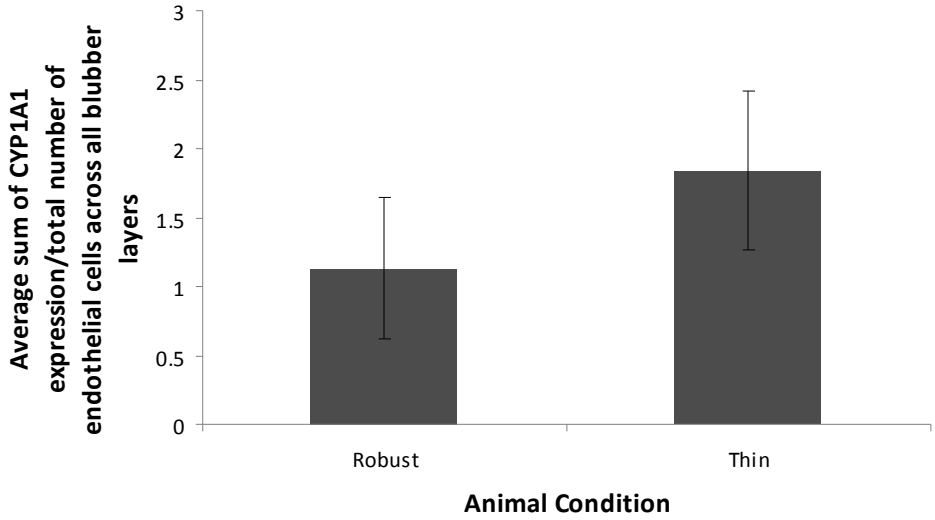

Figure 2.12. Average sum of CYP1A1 expression for condition groups. Thin (n = 11) and robust (n = 11) animals were analyzed, $p > 0.05$. Error bars represent standard error.

Analysis of differences by species

No significant differences in average sum of endothelial cells (p = 0.436), adipocyte quantities (p = 0.119), and adipocyte area (p = 0.643) existed for different species across all blubber layers. However, the average sum of CYP1A1 expression across all blubber layers differed significantly among species, with lower CYP1A1 present in striped dolphins in comparison to bottlenose dolphins and spinner dolphins (p = 0.017) (Figure 2.13).

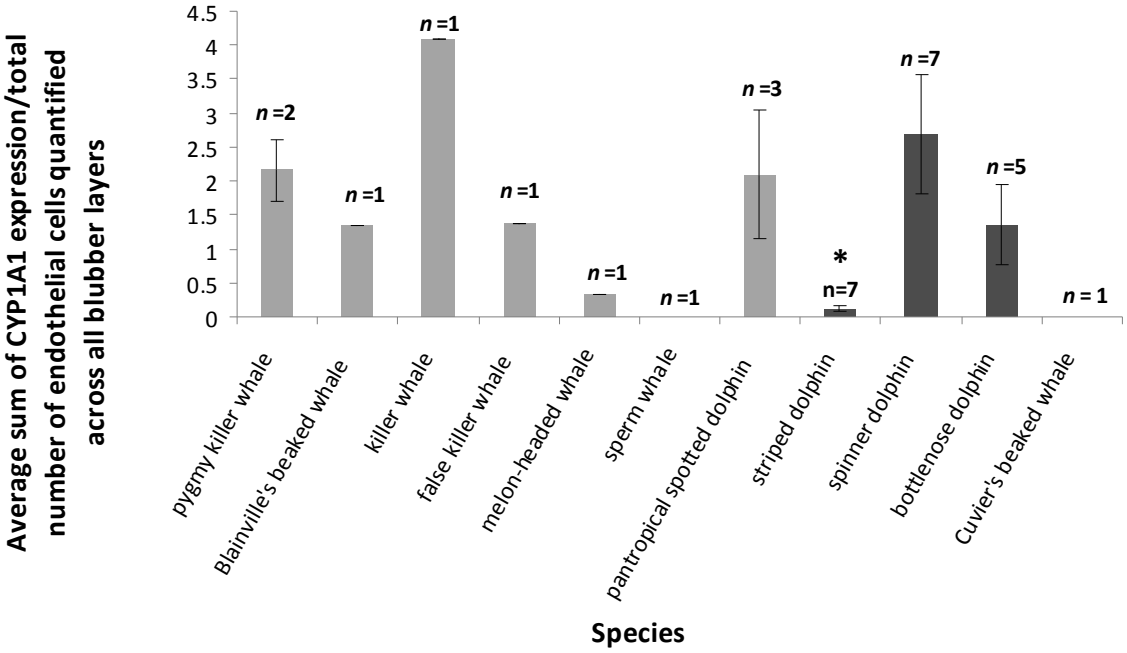

Figure 2.13. Average sum of CYP1A1 expression across all blubber layers for different odontocetes. Species included: pygmy killer whales, *Feresa attenuata*, Blainville's beaked whale, *Mesoplodon densirostris,* killer whale, *Orcinus orca*, false killer whale, *Pseudorca crassidens*, melon-headed whale, *Peponocephala electra*, sperm whale, *Physeter macrocephalus*, pantropical spotted dolphins, *Stenella attenuata,* striped dolphins, *Stenella coeruleoalba*, spinner dolphins, *Stenella longirostris*, bottlenose dolphins, *Tursiops truncatus*, and Cuvier's beaked whale, *Ziphius cavirostris*. Error bars represent standard error. The asterisk indicates that striped dolphins had significantly lower average CYP1A1 expression/total number of endothelial cells in comparison to the other species highlighted in dark gray with $n > 4$ (spinner dolphins and bottlenose dolphins), $p < 0.05$.

Analysis of differences in cellular characterization and CYP1A1 based on age class

Average sum of endothelial cells across all layers did not differ significantly among age classes ($p = 0.146$). However, in the middle blubber layer, adipocyte quantity in calves differed significantly from the other age classes ($p < 0.0001$) (Figure 2.14). P-values for the deep and superficial blubber layer adipocyte quantity for age were $p = 0.147$ and $p = 0.204$ respectively.

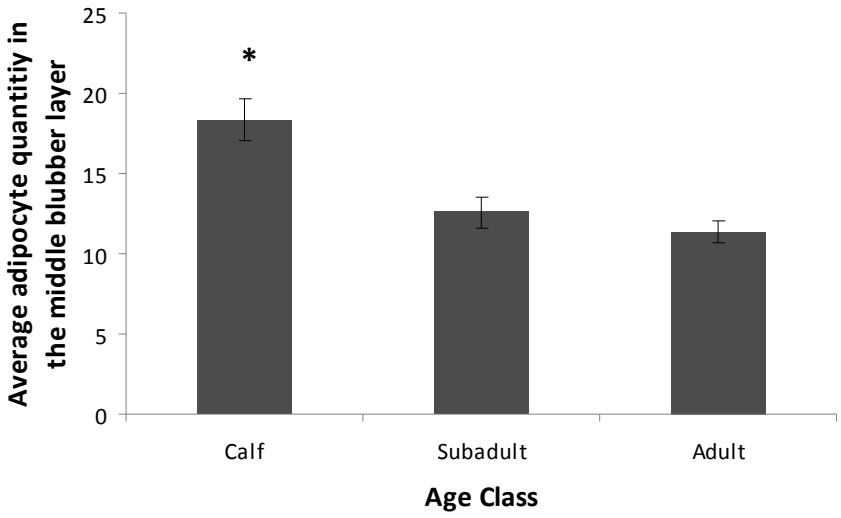

Figure 2.14. Average adipocyte quantity in the middle blubber layer by age class. The sample set included $n = 7$ calves, $n = 9$ subadults, and $n = 16$ adults. Error bars represent standard error. The asterisk indicates that calves differed significantly from the other age classes, $p < 0.05$.

One-way ANOVA was performed for adipocyte area (μm^2) by age within layer. Only the middle blubber layer displayed significant differences among age groups for adipocyte area ($p = 0.041$), with calves having significantly smaller adipocytes than subadults (Figure 2.15). The deep blubber layer almost displayed a significant difference in adipocyte area based on age ($p = 0.050$). The superficial layer did not show a significant difference in adipocyte area based on age ($p = 0.186$).

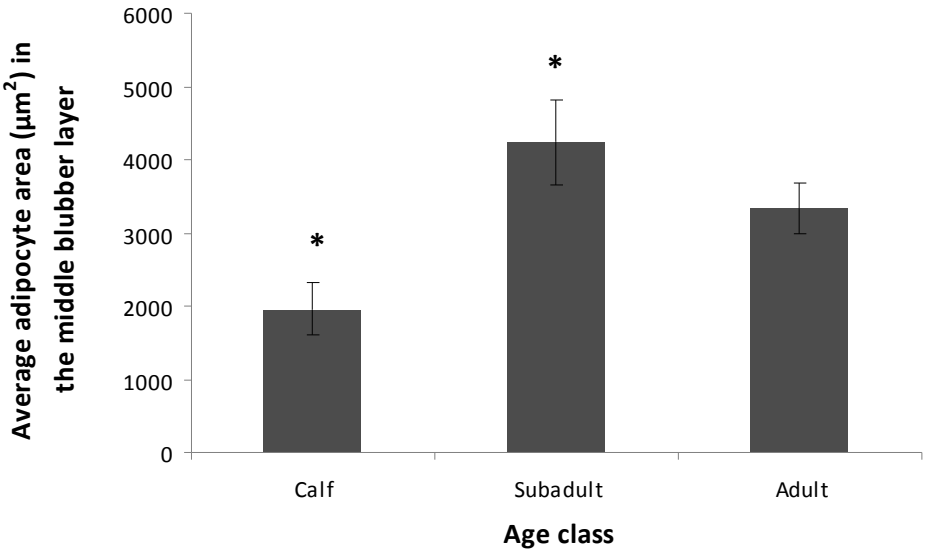

Figure 2.15. Average adipocyte area (µm^2) in the middle blubber layer by age class. The sample set included n = 7 calves, n = 9 subadults, and n = 16 adults. Error bars represent standard error. The asterisks above the calf and subadult age class indicate that these two age classes differed significantly from one another, yet do not differ significantly from the adult age class, $p < 0.05$.

CYP1A1 expression/total number of endothelial cells did not differ significantly across age class (p = 0.182) (Figure 2.16).

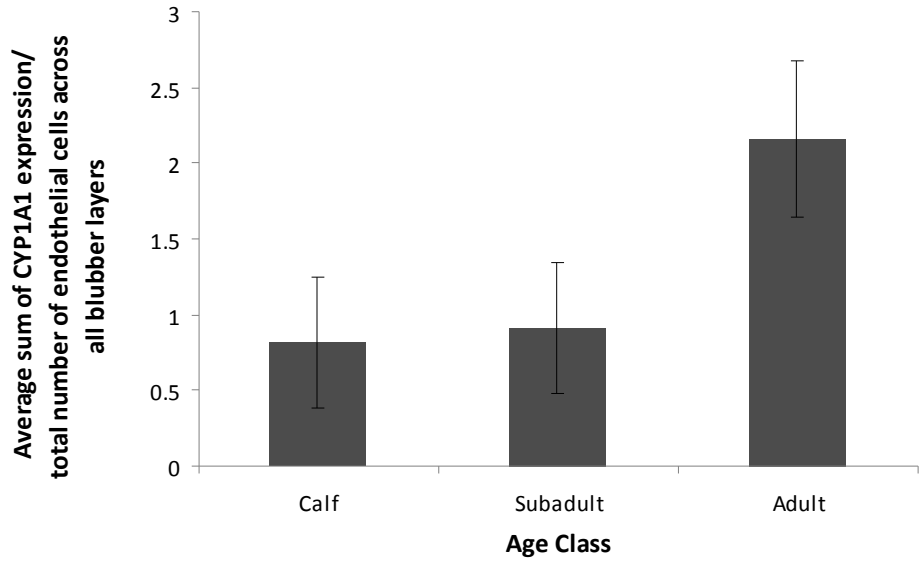

Figure 2.16. Average sum of CYP1A1 expression across all blubber layers by age class. The sample set included n = 7 calves, n = 9 subadults, and n = 14 adults, $p > 0.05$. Error bars represent standard error.

Analysis of differences in cellular characterization and CYP1A1 based on gender

Many studies have not found significant differences in cellular characteristics (Montie et al. 2008b) and CYP1A1 expression (Godard-Codding et al. 2011, Jauniaux et al. 2011, Wilson et al. 2007) for males and females. Significant differences in cellular characteristics of the blubber in this study may be the result of the sample set with more thin females than thin males (n = 9 vs. n = 4 respectively). Females showed significantly higher average sum of endothelial cells across all blubber layers (p = 0.036) compared to males (Figure 2.17).

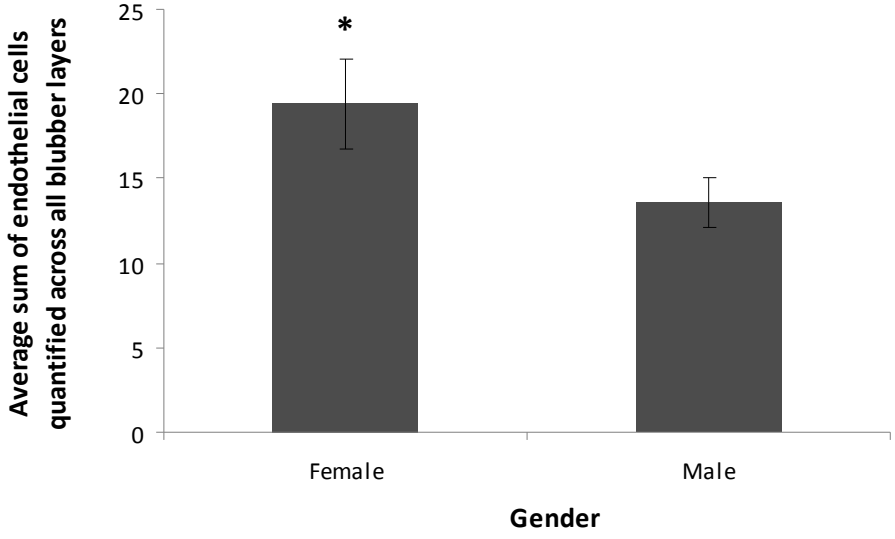

Figure 2.17. **Average sum of endothelial cells across all blubber layers for individuals of different gender.** The sample set included n = 15 females and n = 17 males. Error bars represent standard error. The asterisk represents a significant difference among females and males, $p < 0.05$.

No significant differences in average sum of adipocytes across all blubber layers existed for different gender (p = 0.920), yet, a significant difference was present for average sum of adipocyte area (μm^2) across all blubber layers based on gender (p = 0.036). Males displayed greater average adipocyte area throughout all blubber layers compared to females (Figure 2.18).

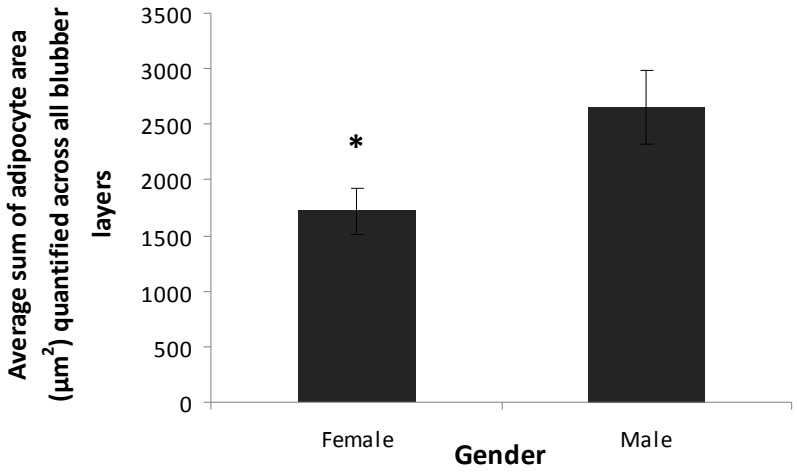

Figure 2.18. Average adipocyte area (μm^2) across all blubber layers for individuals of different gender. The sample set included n = 17 males and n = 15 females. Error bars represent standard error. The asterisk indicates a significant difference among females and males, $p < 0.05$.

CYP1A1 expression/total number of endothelial cells did not differ significantly among gender (p = 0.329) (Figure 2.19).

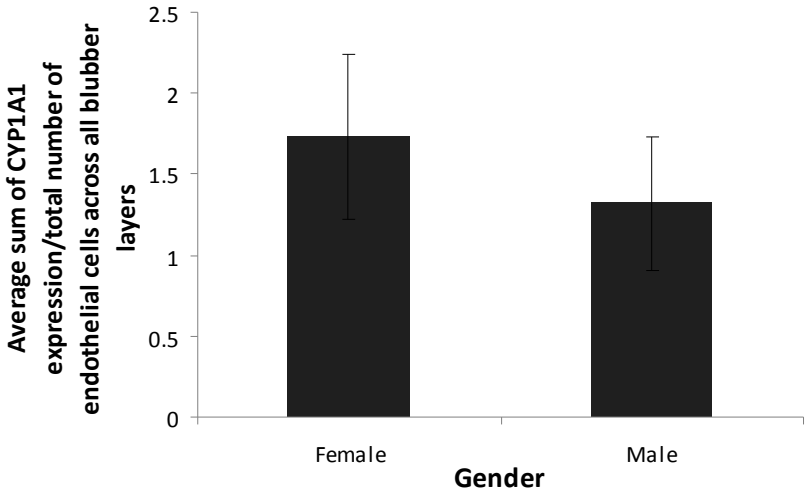

Figure 2.19. Average sum of CYP1A1 expression across all blubber layers for individuals of different gender. The sample set included n = 13 females and n = 17 males, $p > 0.05$. Error bars represent standard error.

2.4. Discussion

Endothelial cell distribution, adipocyte cell size and distribution, and CYP1A1 expression were characterized in the blubber of Hawaiian odontocetes that experience relatively consistent sea-surface temperature annually. Cellular stratification and CYP1A1 stratification illustrate the multifunctional nature of the blubber and its distinct layering. Stratification of endothelial cells throughout the blubber layers confirms the dynamic nature of the blubber, in which blood flow is carefully controlled to retain or release heat in the thermodynamically challenging aquatic environment. Distinct stratification of adipocyte quantity, adipocyte area, and CYP1A1 expression can be related to food supply and nutrition, potentially deep-diving patterns to colder waters on a daily basis, and the fact that all animals in this study were stranded animals that experienced health abnormalities or stress.

This study also investigated CYP1A1 stratification to determine whether or not dart biopsies should be appropriate for future CYP1A1 analysis. Significant correlations of CYP1A1 expression among the blubber layers helped establish the utility of dart biopsies for further analysis of free-ranging odontocetes.

2.4.1. Cellular and CYP1A1 stratification

Endothelial cells

The middle blubber layer had significantly fewer endothelial cells than the superficial ($p = 0.002$) and deep blubber layers ($p < 0.0001$). The middle blubber layer is termed the "transitional zone" between the structural, inert superficial blubber layer, and the dynamic deep blubber layer (Aguilar and Borrell 1990, Struntz et al. 2004). With the

largest and most adipocytes in comparison to the other layers, this layer provides insulation to the body core in which circulation is prevalent. Furthermore, this layer protects against a water medium that conducts heat away from the body twenty-five times faster than air (Dunkin et al. 2005, Parry 1949, Scholander et al. 1950). The greater amounts of endothelial cells seen in the superficial and deep blubber layers are important for releasing or retaining heat to or from the environment. Odontocetes do not have the means to release heat as terrestrial mammals do: evaporation, convection, and radiation controlled by surface temperatures. They instead have to employ a "forced convection" of heat to water flowing past the body, controlled by circulation within the blubber (Parry 1949). Previous studies have characterized circulation within the blubber of whales, showing arterioles that are present from the base of the epidermis all the way to the surface of the skin, with many small arterioles feeding capillaries within the dermal papillae. Furthermore, venules connect venules in the dermis and travel to the hypodermis. "Accompanying venules" alongside arterioles are known to travel to the base of the epidermis, providing the countercurrent exchange required to retain heat in the body (Parry 1949, Norris 1966). In the present study, it is likely that many small connecting blood vessels in the superficial and deep blubber layer contributed to the greater number of endothelial cells quantified within these layers, since often the middle blubber layer contained few, but very large blood vessels.

Stratification of adipocyte quantity, areas, and CYP1A1 expression

Stratification of adipocyte quantity, area, and CYP1A1 expression was assessed to determine if Hawaiian odontocetes that experience relatively consistent sea-surface

temperatures (73-80°F) ($_1$NOAA) and deep-water temperatures throughout the year (variable by depth) (Lukas and Santiago 1996) show less distinct stratification of the blubber. Less stratification of the blubber was seen in IRL dolphins that experience less seasonality and warmer sea surface temperatures throughout the year (Montie et al. 2008a, b) compared to CHS dolphins. Although Hawaiian odontocetes experience warmer, and more consistent sea-surface temperatures annually than IRL dolphins, stratification of adipocyte quantities ($p < 0.0001$), adipocyte area ($p < 0.0001$), and CYP1A1 expression ($p = 0.013$) was distinct for the sample set as seen in CHS dolphins.

Also similar to the findings of Montie et al. (2008b) for CHS dolphins, the greatest amount and largest adipocytes were present in the middle blubber layer, followed by the deep blubber layer, with smallest amounts and areas of adipocytes found in the superficial blubber layer. Average adipocyte area (μm^2) within the middle layer in the present study (3331 μm^2 for adults and 4236 μm^2 for subadults) was more similar to averages in the middle blubber layer of CHS dolphins (3700 μm^2 for adults and 4600 μm^2 for subadults) than averages in the middle blubber layer for IRL dolphins (3200 μm^2 for adults and 2700 $\mu m^2$for subadults). These results are surprising because CHS bottlenose dolphins experienced cold sea-surface water temperatures (SST) in winter months (51-55°F in January and February). If cold SST drove stratification, it would be assumed that Hawaiian odontocetes would have average adipocyte areas similar to IRL dolphins that experience warmer seasonal temperatures (60-66°F in January and February) (Montie et al. 2008b).

The more similar cellular characteristics of the blubber among Hawaiian odontocetes and CHS dolphins may not be related to annual SST, but rather to the

declines in temperatures experienced during foraging at depth. It is important to note that IRL dolphins live in a habitat that averages four feet in depth (Indian River Lagoon National Estuary Program), so these animals may experience very limited changes in temperature on a daily basis when foraging. These animals most likely do not require as much insulation because of less drastic changes in annual SST and temperature with depth in their relatively shallow habitat. Many of the species analyzed in the present study are known to feed at mesopelagic and bathypelagic depths in Hawai‘i (Baird et al. 2006, Benoit-Bird and Au 2003, Clarke and Young 1998, McSweeney et al. 2007, Norris et al. 1994, Shomura and Hida 1965), along with other regions of the world (Blanco et al. 1995, Clarke and Young 1998, Dolar et al. 2003, $_2$NOAA, $_3$NOAA). Hawaiian cetaceans that forage at depth may experience steep declines in temperature, as temperature decreases from 75°F to 60°F from the thermocline (> 60m) to 200m below, and then gradually decreases to 40°F at 900m (Noda et al. 1981). Therefore, the similarity in adipocyte areas in the middle blubber layer for CHS dolphins and Hawaiian odontocetes may be explained by daily foraging to colder waters at depth.

Adipocyte morphology and distribution observed in this study suggest that lipid mobilization was a driving force for CYP1A1 stratification found in these stranded animals. The significant difference in adipocyte area for animal condition groups in the deep blubber layer indicated the importance of this layer for nutritive condition and lipid storage/retrieval. Thin animals displayed significantly smaller average adipocyte area compared to robust animals (1434 +/- 327 SE compared to 3088 +/- 416 SE μm^2) in the deep blubber layer (p = 0.006). This trend in adipocyte area based on condition was not significant for the middle (p = 0.164) and superficial blubber layers (p = 0.103). Blubber

thickness and lipid content have been shown to be indicative of nutritive condition in other studies on cetaceans (Aguilar and Borrell 1990), and the effect of nutrition has been shown to be most dramatic in the deep blubber layer (Koopman et al. 2002, Montie et al. 2008b, Struntz et al. 2004). Moreover, thin animals had higher average CYP1A1 expression/total number of endothelial cells, although this result was not significant (p = 0.559). Altogether, these results demonstrate the importance of animal condition on blubber composition and CYP1A1 expression patterns. More specifically, thin animals in this study mobilized lipids and associated contaminants to a greater extent than robust animals, inducing higher average levels of CYP1A1.

2.4.2. Utility of biopsy samples for analysis of CYP1A1

CYP1A1 expression in the deep blubber layer correlated significantly with expression in the middle (p = 0.0004) and superficial blubber layers (p = 0.001). Therefore, dart biopsies that obtain only the superficial to middle blubber layers can be useful for analysis. Interpretation of CYP1A1 in dart biopsies should take into account the area of biopsy, since larger biopsies may obtain the deep blubber layer which would contribute to higher expression found within individuals. Dividing expression of CYP1A1 by area should reduce the influence of greater-depth biopsies on expression levels. Dividing CYP1A1 expression per total number of endothelial cells in biopsy samples does not seem as critical, since blubber composition does not appear to be as variable in presumably healthy, free-ranging animals.

2.4.3. Analysis of key cell types and CYP1A1 expression based on age, gender, and species

Age

Average adipocyte area and quantity was useful for elucidating trends specific to age. There was a pattern of decreasing average adipocyte quantity with age throughout all blubber layers, with calves and adults differing significantly in average sum of adipocyte quantity ($p = 0.005$). The middle blubber layer drove the trends. Montie et al. 2008b observed similar results: CHS subadults had more adipocytes than adults, and this difference was found within the middle blubber layer.

In the present study, calves and subadults differed significantly in average sum adipocyte area across all blubber layers ($p = 0.009$). There was a trend of increasing adipocyte area with age until adulthood, in which average adipocyte area declined. When looking only within layers, significant differences based on age were restricted to the middle blubber layer ($p = 0.041$). Montie and others (2008b) found that significance in adipocyte area based on age was confined to the middle blubber layer. This led them to predict that the middle blubber layer is ontogenetic in function, showing variation with growth.

The trend of decreasing adipocyte quantity with age and increasing adipocyte area with age may be explained by decreases in surface-area to volume with growth, with less demand for insulation and greater need for energy to support growth (Dunkin et al. 2005, McLellan et al. 2002, Struntz et al. 2004). Many, smaller adipocytes may be important for greater surface area and insulation in calves, while fewer, larger adipocytes may be more important for energy storage as the individual grows and actively forages in subadulthood. The observance of increasing adipocyte area with growth ended with

adults in the present study, as a decline in average adipocyte area was seen from subadults to adults. It is predicted that once growth-rate maxes out in adulthood, the trend of increasing adipocyte area with age diminishes. The smaller average adipocyte areas for adults in this study may reflect lipid loss in the blubber that occurs in adulthood as seen in Montie and others (2008a).

There were no age-related differences in CYP1A1 expression. This is consistent with results reported elsewhere in the literature (Jauniaux et al. 2011, Wilson et al. 2003, 2007, 2010); yet, it is interesting based on the fact that contaminant levels are known to change with age class (Hansen et al. 2004, Hickie et al. 1999, Krahn et al. 2002, $_1$2004, Ylitalo et al. 2001).

Gender

More endothelial cells were quantified in females compared to males (p = 0.036). Moreover, larger adipocytes were measured in males compared to females (p = 0.036). It is important to note that there were more thin females than males in this study (n = 9 vs. n = 4 respectively), which may have influenced the higher number of endothelial cells and smaller adipocytes seen in females. Although female odontocetes are smaller than male odontocetes (Perrin et al. 2008), it is hypothesized that animal condition was more influential for the results obtained because of the variability in blubber composition that exists among stranded animals of different condition categories.

No significance existed for CYP1A1 expression across gender (p = 0.746), a result reported in previous studies (Godard-Codding et al. 2011, Jauniaux et al. 2011, Wilson et al. 2003, 2007, 2010). Similarly to the results obtained for age, these results are

interesting because studies have found significant differences in contaminant levels for different genders (Hansen et al. 2004, Ylitalo et al. 2001).

Species

A notable finding of this study was that striped dolphins had significantly lower CYP1A1 expression than spinner dolphins and bottlenose dolphins ($p = 0.017$). Spinner and bottlenose dolphins are known to have island-associated populations (Baird et al. 2003, Martien et al. 2011), while striped dolphins in Hawai‘i are mostly sighted in deep offshore waters (R.W. Baird, pers. comm.). Therefore, striped dolphins may be exposed to a different types and levels of contaminants in offshore waters than species inhabiting waters closer to shore. Sperm whales from regions furthest from continents have significantly lower CYP1A1 expression compared to sperm whales inhabiting waters closer to continents (Godard-Codding et al. 2011). This suggests that despite the ubiquitous presence of contaminants in the marine environment, there may be differences in contaminant exposure in the near-shore and off-shore environment.

2.4.4. Conclusions

Analysis of stranded animals for cellular stratification provided the framework to better understand the physiological process of lipid mobilization and CYP1A1 induction in several species of Hawaiian odontocetes, and how the multifunctional nature of the blubber affects these processes. The deep blubber is vital for energy stores and supply, and composition of this layer is most influenced by nutritive condition. Thin animals draw upon lipids within this layer to a greater extent than robust animals, inducing higher

average levels of CYP1A1 as contaminants travel into circulation. Stratification of CYP1A1 was prevalent in Hawaiian odontocetes, most likely because of physiological stress prior to stranding that caused the animals to draw upon lipid reserves. However, stratification of CYP1A1 may have been influenced by reproduction and lactation in female dolphins, low food availability, or potentially diving at depth for extended periods of time. Small-scale seasonal changes in temperature probably have only a small effect on cellular and CYP1A1 stratification in Hawaiian odontocetes.

Correlations of CYP1A1 expression across blubber layers suggest that dart biopsies that sample the superficial to middle blubber layers may be useful for future CYP1A1 analysis. Caution must be taken for variable depth samples, however, and CYP1A1 expression should be normalized by area of biopsy. Biopsies should always be taken from visibly healthy, free-ranging animals, because blubber condition in sick, distressed animals may influence quantification of CYP1A1 in samples.

Future work should assess the trophic position of Hawaiian odontocetes to see if CYP1A1 expression follows trends in structure. Future research could also examine relationships between CYP1A1 and disease state if possible in stranded animals. CYP1A1 is related to decreased immune function in cetaceans (Lahvis et al. 1995), and an analysis of CYP1A1 expression levels in conjunction with histopathology may reveal how induction of this enzyme in certain tissues affects whole organ systems. Much is to be learned from CYP1A1 as a biomarker of contaminant exposure and its relationship to the health status of an individual.

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Chapter 3: Cytochrome P4501A1 expression in the blubber of free-ranging Hawaiian odontocetes

Abstract

Odontocetes are sentinel species for examining the molecular effects of contaminants because of their high trophic position, long life spans, and blubber that accumulates lipophilic contaminants. Cytochrome P4501A1 (CYP1A1) is a biomarker of contaminant exposure and molecular effects in many species, including odontocetes. Established immunohistochemical methods were used to assess CYP1A1 expression in blubber biopsies from a variety of free-ranging Hawaiian odontocetes ($N = 10$ species: Blainville's beaked whale (*Mesoplodon densirostris*), bottlenose dolphins (*Tursiops truncatus*), Cuvier's beaked whales (*Ziphius cavirostris*), false killer whales (*Pseudorca crassidens*), melon-headed whales (*Peponocephala electra*), pantropical spotted dolphins (*Stenella attenuata*), pygmy killer whales (*Feresa attenuata*), rough-toothed dolphins (*Steno bredanensis*), short-finned pilot whales (*Globicephala macrorhynchus*), and a sperm whale (*Physeter macrocephalus*). False killer whales displayed significantly higher expression than melon-headed whales ($p = 0.010$). Differences in expression may be a result of different trophic levels of these two species; false killer whales in Hawai‘i are observed to prey on large game species, and stomach contents from stranded melon-headed whales in Hawai‘i consist of a combination of fish and squid species from mesopelagic to bathypelagic depths. No significant differences were found based on age class ($p = 0.308$) or sex ($p = 0.545$) across all samples in this study. However, within male false killer whales, juveniles displayed significantly higher CYP1A1 expression than adults ($p = 0.016$).

Total PCBs were measured in the false killer whale biopsies used in this study. Significant relationships were found for ∑PCBs (ng/g wet weight and lipid weight) and CYP1A1 expression ($p = 0.001$ and $p = 0.015$) respectively. No significance was found for the relationship between TEQ (ng/g wet weight and lipid weight) and CYP1A1 expression ($p = 0.226$ and $p = 0.430$ respectively). It is important to note that out of all PCBs analyzed, the only measured dioxin-like congeners were PCB 105, 118, and 156. No significant differences were found for CYP1A1 expression among social clusters of false killer whales ($p = 0.406$), but average expression was highest in cluster 3 animals that primarily spend time north of Maui and Moloka‘i. Altogether, this study is the first study to assess CYP1A1 expression in free-ranging Hawaiian odontocetes. It may provide a foundation for future health analyses and will be valuable for long-term monitoring of the molecular effects of AHR agonists in Hawaiian odontocetes.

3.1. Introduction

Persistent Organic Pollutants (POPs) are ubiquitous throughout the marine environment, and because of their lipophilic nature, they bioaccumulate and biomagnify throughout the food web. POPs are known to be transported long distances to remote regions via atmospheric deposition and evaporation events (Wania and Mackay 1996). Although Hawaiʻi is far from continental sources of POPs, it is not invulnerable to high levels of contaminant exposure. A variety of POPs have been found in Hawaiʻi, in various habitats and organisms (Brasher and Wolff 2004, Hunter et al. 1995, Lopez et al. 2012, Yang et al. 2008, Ylitalo et al. 2008, 2009).

Particular classes of POPs can exhibit their toxic effects via the aryl hydrocarbon receptor (AHR), a soluble, ligand-activated transcription factor and member of the basic helix-loop-helix family of transcription factors (Gu et al. 2000). The most established, high-affinity AHR agonists include the halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) (reviewed in Denison et al. 2002). HAHs are trace contaminants created in industrial processes that involve chlorination in the presence of phenolic substrates (Schmidt and Bradfield 1996). Examples of HAHs include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic HAH, and the more environmentally abundant polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs). In odontocetes, high levels of PCBs have been linked to decreased immune function (De Guise et al. 1998, Lahvis et al. 1995), death from infectious disease (Jepson et al. 2005), and epizootic outbreaks (Aguilar and Borrell 1994). PAHs are created by combustion of organic matter, and have natural and anthropogenic sources. Principal sources of PAHs include

combustion of fossil fuels for heat and power generation, and refuse burning (Harvey 1991). Evidence suggests that high levels of PAHs in the St. Lawrence estuary are involved in etiology of cancer in beluga whales from this region (Martineau et al. 2002).

PAHs and HAHs have been quantified in Hawaiʻi. PAHs have been quantified in sediments from watersheds of mixed (urban and agricultural) land use, and levels were within the highest twenty-five percentile of the United States (Brasher and Wolff 2004). Polychlorinated biphenyl (PCB) profiles resembling Aroclor 1254 have been found in tilapia (*Oreochromis mossambicus*) inhabiting the Manoa stream and the Ala Wai canal located on the island of Oʻahu (Yang et al. 2008). ∑PCBs have been found to be as high as 6,100 ng/g lipid weight in the Hawaiian monk seal (*Monachus schauinslandi*) from the main Hawaiian Islands (Lopez et al. 2012), and 33,000 ng/g lipid weight in free-ranging false killer whales (*Pseudorca crassidens*) surrounding the main Hawaiian Islands (Ylitalo et al. 2009). This level of exposure quantified within free-ranging false killer whales exceeds the threshold concentration of 17,000 ng/g lipid weight, suggested for marine mammals based on various toxicological endpoints such as thyroid hormone concentrations and natural killer cell activity (Kannan et al. 2000). ∑PCBs exceeding this threshold concentration have also been quantified within stranded Hawaiian cetaceans (Bachman et al. in preparation).

Because AHR agonists are known to exist at measurable levels in Hawaiʻi, a molecular biomarker of contaminant exposure, cytochrome P4501A1 (CYP1A1) was investigated. CYP1A1 is an enzyme induced by PAH or HAH binding to the AHR, and it is subsequently involved in the metabolism of these agonists (Parkinson 1996). CYP1A1 is involved in detoxification of the original ligand. However, bioactivation of the original

ligand may occur and lead to toxicity or neoplasia. Bioactivation occurs when detoxification pathways are saturated at high substrate concentrations, resulting in the production of reactive metabolites beyond the capability of cellular defenses (Conney 1982, Miller et al. 1981, Phillips 1983, reviewed in Whitlock 1999). Examples of more toxic metabolites created by CYP1A1 include hydroxylated-PCBs (OH-PCBs) (Kaminsky et al. 1981, Mills et al. 1985, White et al. 2000) that are known to interfere with thyroid hormone homeostasis, immune function, development, metabolism, and sexual function (Brouwer et al. 1989, 1998, Zoeller et al. 2002). PAHs can be metabolized to epoxide intermediates by CYP1A1, and further metabolized to highly reactive diol-epoxide intermediates capable of binding to DNA and initiating carcinogenesis (Conney 1982, Shimada and Fujii-Kuriyama 2004). Therefore, CYP1A1 presence indicates that the individual is responding at the molecular level to contaminant exposure, potentially resulting in negative physiological consequences. It is a useful biomarker of contaminant exposure because it may be present even when inducers capable of being rapidly metabolized, such as PAHs (Varanasi 1989), have been removed from the body.

This study is the first to assess CYP1A1 expression in the blubber of ten species of free-ranging Hawaiian odontocetes. Previous analysis of CYP1A1 in stranded Hawaiian cetaceans determined that the blubber is an indicator tissue for expression (Fertall 2010). Furthermore, levels of CYP1A1 expression are correlated in the deep and middle, and deep and superficial blubber layers (Chapter 2), confirming the utility of dart biopsies for analysis of CYP1A1. The specific aims of this study are to (1) determine whether or not different species groups exhibit variability in CYP1A1 expression levels,

(2) investigate patterns of expression among age and sex, (3) compare levels of expression within false killer whales to measured contaminant concentrations (wet weight (ng/g), lipid weight (ng/g), and TEQ), and (4) examine differences in expression among false killer whale social clusters (Baird et al. 2012).

3.2. Materials and methods

3.2.1. Sample collection

Blubber biopsies were collected from 113 Hawaiian odontocetes in October and December of 2009, and April through December of 2010, as part of ongoing studies of odontocetes in the main Hawaiian Islands by Cascadia Research Collective (Baird et al. 2008a). Samples were collected from individuals on the northwest to southwest sides of the islands of Hawaiʻi and Oʻahu (Figure 3.1 A). Samples were collected using a 45 kg pull Barnett RX-150 crossbow and Larsen biopsy tips that measured 25 mm long and 8 mm wide. Biopsy darts were fit with a high-density foam collar to prevent tissue retrieval greater than 18 mm. Biopsy samples were stored in a cooler with ice packs while in the field. Biopsy samples were subsampled into two equal size cross-sections of blubber so that contaminant analysis and immunohistochemistry could be performed from the same biopsy of each individual. Biopsy tissue depths are shown in slides processed by hematoxylin and eosin staining (Figure 3.1B).

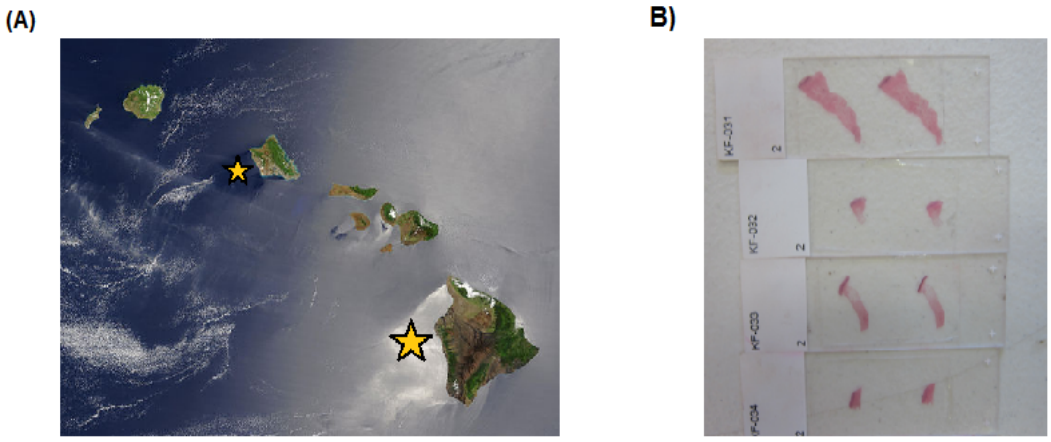

Figure 3.1. Location of sample collection and variability of biopsy depth within this study. (A) Samples were collected from individuals on the northwest to southwest sides of the island of Hawaiʻi and Oʻahu. (B) Tissues stained with hematoxylin and eosin, showing variability of biopsy depths, photo (c) Kerry Foltz.

Cross-sections used for contaminant analysis were transferred to a -20°C freezer at the end of the day, and then transferred within days to a -80°C freezer for long-term storage. Immunohistochemistry samples were transferred and stored in individual sealed containers of 10% neutral buffered formalin (NBF). Cascadia Research Collective photo-identified samples from each individual, and compared photographs to each other and the catalog to prevent replicates and to determine population identity. A list of sample sizes for each species group analyzed in this study is given in Table 3.1.

Table 3.1. Sample sizes of Hawaiian odontocetes.

Species	Number of samples
Blainville's beaked whale (*Mesoplodon densirostris*)	3
Bottlenose dolphin (*Tursiops truncatus*)	3
Cuvier's beaked whale (*Ziphius cavirostris*)	2
False killer whale (*Pseudorca crassidens*)	36
Pygmy killer whale (*Feresa attenuata*)	5
Melon-headed whale (*Peponocephala electra*)	15
Pantropical spotted dolphin (*Stenella attenuata*)	23
Rough-toothed dolphin (*Steno bredanensis*)	15
Short-finned pilot whale (*Globicephala macrorhynchus*)	10
Sperm whale (*Physeter macrocephalus*)	1
Total	**113**

3.2.2. Age determination

Age class (juvenile, subadult, adult) of biopsied individuals were noted in the field by Cascadia Research Collective based on body size relative to other individuals, presence of calves in close proximity (indicating adulthood), pigmentation patterns (e.g. changes throughout time of pantropical spotted dolphins), scarring patterns over time and erupted teeth (for beaked whales). In some cases, age was only a probable estimate. These probable estimates were excluded in the ANOVA analyses of CYP1A1 expression by age.

3.2.3. Sex determination

Sex was determined genetically by colleagues at Southwest Fisheries Science Center in subsamples of the biopsies from false killer whales, bottlenose dolphins, pygmy killer whales, melon-headed whales, and rough-toothed dolphins. Sex of Blainville's beaked whale, Cuvier's beaked whale, short-finned pilot whales, and the sperm whale in

this study were determined by morphology in the field and photo-identification; in a few cases, sex was not available (n = 5 short-finned pilot whales, n = 23 pantropical spotted dolphins, n = 2 rough-toothed dolphins, and a Blainville's beaked whale in this study).

3.2.4. Social cluster determination

False killer whale social groups or cluster (1, 2, or 3) were determined by Baird et al. 2012 through analysis of association patterns using photo-identification data. Six individuals were not assigned to any of the three clusters, but were in closely related clusters. For the purpose of this analysis, these individuals were assigned to the closest cluster. Five individuals of the false killer whale dataset had unidentified clusters (altogether, n = 31 of n = 36 false killer whales were assigned clusters).

3.2.5. Analysis of CYP1A1 by immunohistochemistry

Samples were transferred to a histologist, who embedded tissues in paraffin and cut them at 5 μm using a Microm 330 rotary microtome. Blubber biopsies were mounted on Leica Biosystems 1mm X-tra® Slides for hematoxylin and eosin (H&E) staining and CYP1A1 analysis respectively. After slides were deparaffinized and rehydrated with a series of xylene, decreasing concentrations of ethanol, and Milli-Q® purified water incubations, blocking serum (Normal Horse Serum, Vector laboratories, Cat. No. S-2000) was placed on the slides for twenty minutes. After incubation with blocking serum, slides were treated with the polyclonal anti-CYP1A1 antibody (Chemicon International, Cat. No. AB1247; diluted 1:4500 in 0.9% phosphate buffered saline). Comparable levels of CYP1A1 expression were quantified in blubber biopsies stained with AB1247 and

additional cuts of the same blubber biopsies previously stained with the anti-CYP1A1 monoclonal antibody (MAb) 1-12-3 (0.3 µg/ml). Slides were treated with the anti-CYP1A1 antibody for two hour-long incubations, separated by a five minute rinse procedure with 0.9% phosphate buffered saline (PBS). Slides were then treated with biotinylated secondary antibody, followed by alkaline phosphatase reagent labeled with avidin (Vectastain® ABC-AP kit, rabbit IgG, Vector Laboratories, Cat. No. AK-5001). Five minute rinse procedures with PBS followed each incubation. Lastly, slides were incubated with alkaline phosphatase substrate (Vector® Red in 200 mM tris-HCL, pH 8.5, Vector Laboratories, Cat. No. SK-5100), rinsed, and incubated with hematoxylin for one minute prior to being coverslipped with mounting medium (Permount®, Fisher Scientific, Cat. No. SP15-500). Negative control slides were used to account for background staining not indicative of CYP1A1 expression, and were processed without use anti-CYP1A1 antibody. Positive controls of tissues known to display high CYP1A1 expression (Fertall 2010) were used to verify appropriate staining as well.

Because vascular endothelial cells of the arterial system and capillaries are known to express the highest levels of CYP1A1 in the blubber (Angell et al. 2004, Montie et al. 2008, Wilson et al. 2007), CYP1A1 expression was quantified in these cells, below the dermal papillae to the lowest layer of each biopsy dart. CYP1A1 expression was quantitatively determined as a product of staining intensity (0-5 scale, 5 being the highest intensity) and occurrence (total number of stained endothelial cells). This product was normalized per area of blubber biopsy (mm^2) as described by Montie et al. (2008). Area of tissue was determined through use of ImageJ 1.45m software (National Institutes of

Health, USA). Background staining of negative controls was subtracted from CYP1A1 expression determined for slides stained with primary antibody.

3.2.6. Contaminant analysis of blubber biopsies

Individual and total PCB concentrations (ng/g wet weight) were determined for $n = 31$ false killer whale biopsies by the Northwest Fisheries Science Center via the gas chromatography-mass spectrometry methods of Sloan et al. (2005) and Ylitalo et al. (2009). Measured PCBs are listed in Table 3.2.

Table 3.2. PCBs quantified in false killer whales of this study for comparison to CYP1A1 expression levels.

PCBs analyzed in this study (mono-*ortho* PCBs highlighted in **bold**)		
PCB17	PCB99	PCB177
PCB18	PCB101	PCB180
PCB28	**PCB105**	PCB183
PCB31	PCB110	PCB187
PCB33	**PCB118**	PCB191
PCB44	PCB128	PCB194
PCB49	PCB138	PCB195
PCB52	PCB149	PCB199
PCB66	PCB151	PCB205
PCB70	PCB153	PCB206
PCB74	**PCB156**	PCB208
PCB82	PCB158	PCB209
PCB87	PCB170	
PCB95	PCB171	

Percent lipid was determined in biopsies by thin-layer chromatography with flame ionization detection through the methods of Ylitalo et al. (2005). Lipid weight ∑PCB concentrations (ng/g lipid) were determined by dividing ∑PCB concentrations (ng/g wet weight) by (percent lipid * 0.01). Toxic Equivalency Quotients (TEQ) were determined for each individual biopsy sample based on the three AHR agonists measured in this

study: the mono-*ortho* PCBs 105, 118, and 156. The 2006 international mammalian toxic equivalency factors (TEFs) for PCB 105, 118, and 156 (0.00003) were multiplied by individual concentrations of these AHR agonists (using both wet weight ng/g and lipid weight ng/g), and then summed to determine TEQ (wet weight ng/g and lipid weight ng/g) for each individual biopsy sample (Van den Berg et al. 2006). For PCB 156, $n = 4$ were below the limit of detection (LOD). Therefore, we divided the LOD by two for an estimate of the contaminant concentration for these individuals.

3.2.7. Statistical analysis

Statistical analysis of CYP1A1 expression by species, age, and sex

CYP1A1 expression/area (mm^2) was transformed by the fourth root to improve data normality and homogeneity of variances. One-way ANOVA was performed for species, and a Tukey-Kramer post-hoc test was used to determine pairwise differences among the groups. Factorial ANOVA was conducted for age and sex, excluding individuals with undetermined sex or probable age estimates. Because there was no interaction between age and sex, one-way ANOVAs were conducted for age and sex individually. In the case of the one-way ANOVA for age, only probable age estimates were excluded. For the one-way ANOVA by sex, only undetermined sexes were excluded.

Statistical analysis of CYP1A1 expression in false killer whales

Within false killer whales, differences in CYP1A1 expression and contaminant levels among social clusters were analyzed using one-way ANOVA. Differences in

CYP1A1 expression and contaminant levels for age and sex within false killer whales were analyzed using factorial and one-way ANOVA. For the above statistical tests, data were transformed with the fourth root to improve normality and homogeneity of variances. Linear regression was used for analysis of the relationship between CYP1A1 expression (dependent variable) and contaminant levels (∑PCBs (ng/g wet weight), ∑PCBs (ng/g lipid), TEQ (ng/g wet weight), and TEQ (ng/g lipid) (independent variables). CYP1A1 was log-transformed when analyzing the relationship between expression and TEQ, so that distribution of residuals did not violate normality.

For the above statistical tests on false killer whales, analyses were conducted with and without the exclusion of three individual outliers that expressed unusual patterns of CYP1A1 expression and contaminant levels. These individuals included HIPc220, a probable adult female with extremely low contaminant levels: 310 ng/g wet weight, 2,164 ng/g lipid weight (concentrations 9 and 10 times less than the lower 95% mean of 2,735 ng/g wet weight and 21,414 ng/g lipid weight respectively). HIPc220 had the highest CYP1A1 expression score (3 times greater than the upper 95% mean of 16.47/mm^2). It is hypothesized that HIPc220 may have been a post-partum female who previously offloaded contaminants to her offspring through lactation as seen in other studies (Addison and Brodie 1987, Borrell et al. 1995). The other two excluded individuals were HIPc184, an adult male, and HIPc266, a probable subadult female. Both individuals had the highest ∑PCBs (respectively, 3 and 4 times greater ∑PCBs (ng/g wet weight) as the upper 95% mean of 6,434 ng/g, 2 times greater ∑PCBs (ng/g lipid) as the upper 95% mean of 38,819 ng/g). HIPc184 and HIPc266 had low CYP1A1 expression scores, 2 times less than the lower 95% mean of 8.21/mm^2. It is possible that these two individuals

down-regulated CYP1A1 expression at very high contaminant exposure, as seen in previous studies in liver (Schlezinger and Stegeman 2001,Wilson et al. 2005).

Design considerations and post-hoc analyses

For all ANOVA and linear regression analyses in this study, $\alpha = 0.05$. Groups with $n < 5$ were excluded from ANOVA analyses, except for the analyses of age and sex within species groups (melon-headed whales, rough toothed dolphins, and short-finned pilot whales) in which it was not possible to exclude a group (see Table 3.3). Groups with $n < 5$ were also not excluded in the analysis of CYP1A1 expression for age groups within female false killer whales, and sex within the juvenile age group (see Table 3.4). It was not possible to exclude a group with $n < 5$ from these analyses because there were only two groups in which to make comparisons.

Post-hoc power analyses were conducted following ANOVA tests for which no significant differences were observed. A small effect size, $\delta = 0.10$, and a large effect size, $\delta = 0.50$, were used. Because data were transformed with the fourth root in this study for ANOVA analyses, effect sizes were also transformed with the fourth root to become $\delta = 0.56$ and $\delta = 0.84$. Calculated power was transformed back with the fourth power for reporting results. Data were analyzed using JMP 9.0.2 Statistical software (©2010 SAS Institute Inc).

3.3. Results

3.3.1. Does CYP1A1 differ significantly among species?

CYP1A1 was expressed specifically in key cell types and exhibited variability among individuals in this study (Figure 3.2 A-D).

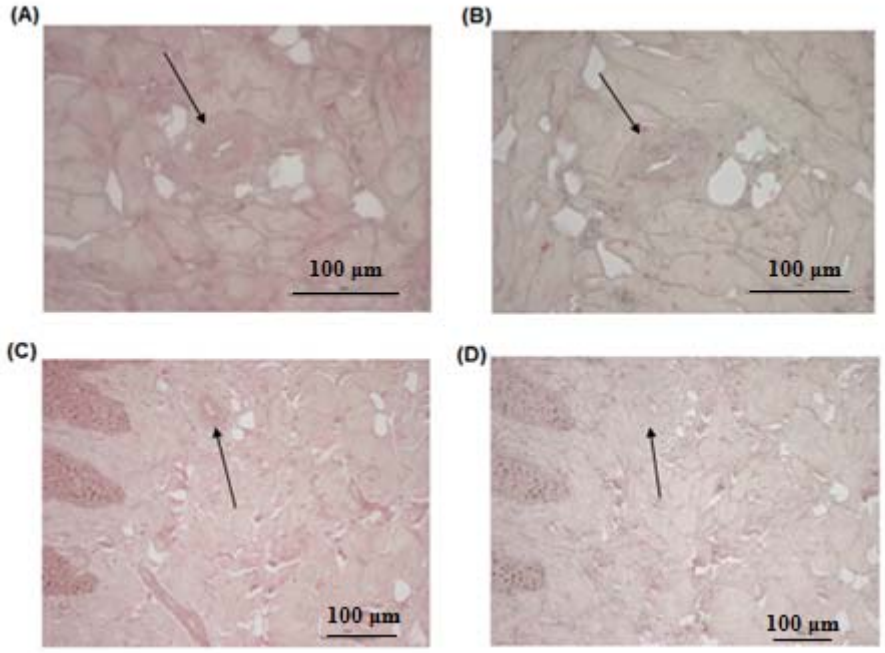

Figure 3.2. Representative CYP1A1 expression in biopsies. (A & B) Photos of blubber in the free ranging false killer whale HIPc210, an adult male from cluster 1; 40x objective. (C &D) Photos of rough-toothed dolphin with unidentified age and gender; 20x objective. Arrows point to vessels with endothelial cells expressing CYP1A1 (A & C) in tissues treated with anti-CYP1A1 antibody, and with no CYP1A1 detection (B & D) in negative control tissues. For scale, a 100 micrometer bar is placed on each photograph. Photos (c) Kerry Foltz.

For the analysis of CYP1A1 expression by species, six out of ten species had large enough sample sizes ($n > 4$) for comparisons among groups. CYP1A1 expression in false killer whales differed significantly from expression in melon-headed whales ($p = 0.010$) (Figure 3.3) Average CYP1A1 expression ranged from lowest expression found in melon-headed whales, to higher expression (in increasing order) found in pygmy killer

whales, short-finned pilot whales, pantropical spotted dolphins, rough-toothed dolphins, and false killer whales. For the analysis of differences among all species except false killer whales and melon-headed whales, the probability (power) to detect significant differences at $\delta = 0.10$ and $\delta = 0.50$ in the sample set of 53 individuals was 1.00 and 1.00 respectively.

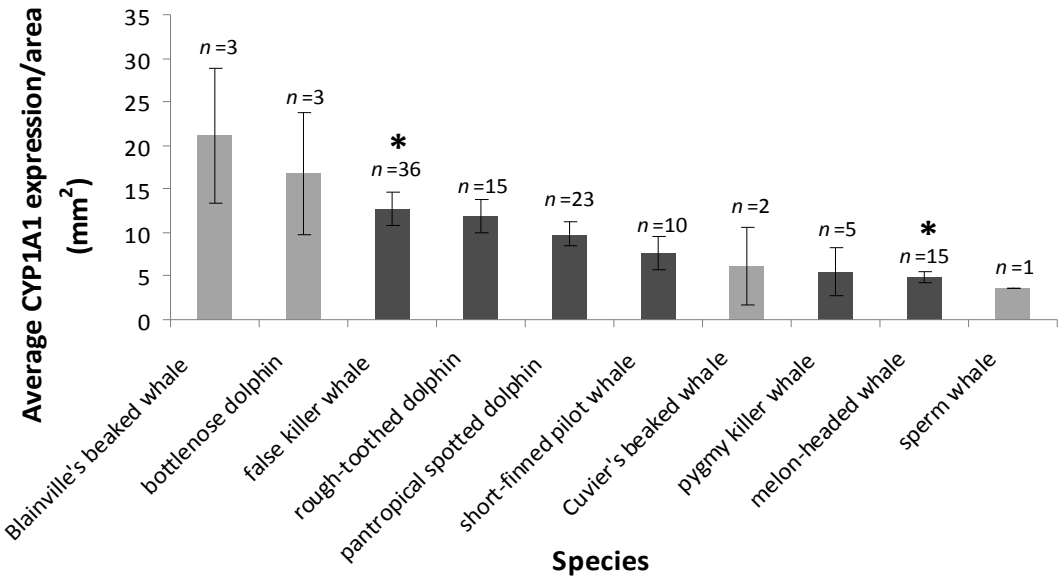

Figure 3.3. Average CYP1A1 expression for Hawaiian odontocetes. The sample set included $n = 113$ individuals. The asterisks show that false killer whales and melon-headed whales differed significantly from one another in the Tukey-Kramer test ($p = 0.010$). Species highlighted in light gray were excluded from the ANOVA and Tukey-Kramer test because of small sample size ($n < 5$). Error bars represent standard error.

3.3.2. Are there significant differences among age class and sex for all species pooled?

No interaction was found between age and sex in this study across all samples ($p = 0.170$). Thus, differences in CYP1A1 expression for age and sex were examined over the entire data set. No significant differences were found among different age classes ($p = 0.308$). The power to detect significant differences at $\delta = 0.10$ and $\delta = 0.50$ in

the sample set of 100 individuals was 1.00 and 1.00 respectively, confirming that these conditions would be adequate to see differences among age classes if they were present. Average CYP1A1 expression decreased with age group in this study, with juveniles displaying the highest average expression, and adults displaying the lowest average expression (Figure 3.4).

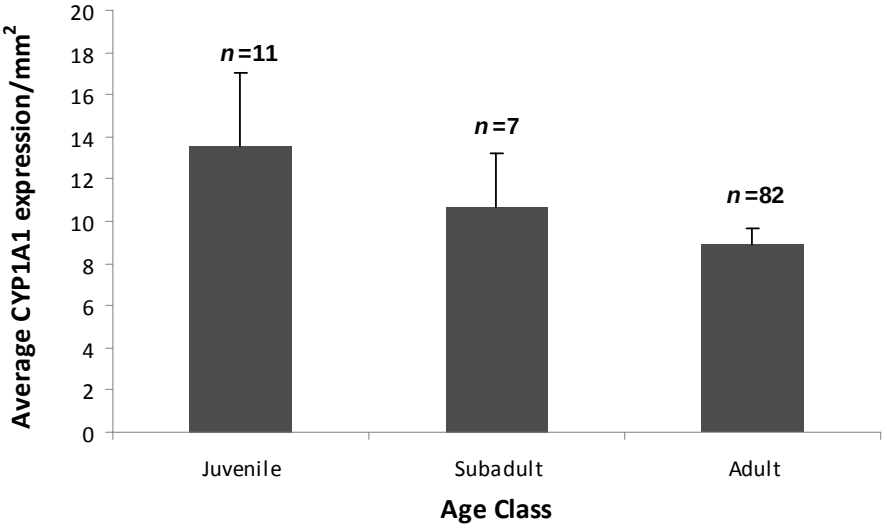

Figure 3.4. Average CYP1A1 expression for different age classes. No significant trends were found, $p > 0.05$. The sample set included $n = 100$ individuals. Individuals that had only probable estimates of age were excluded from the ANOVA analysis. Error bars represent standard error.

No significant differences for found for sex ($p = 0.552$). Power to detect significant differences at $\delta = 0.10$ and $\delta = 0.50$ in the sample set of 83 individuals was 1.00 and 1.00 respectively. On average, females displayed higher CYP1A1 expression (Figure 3.5).

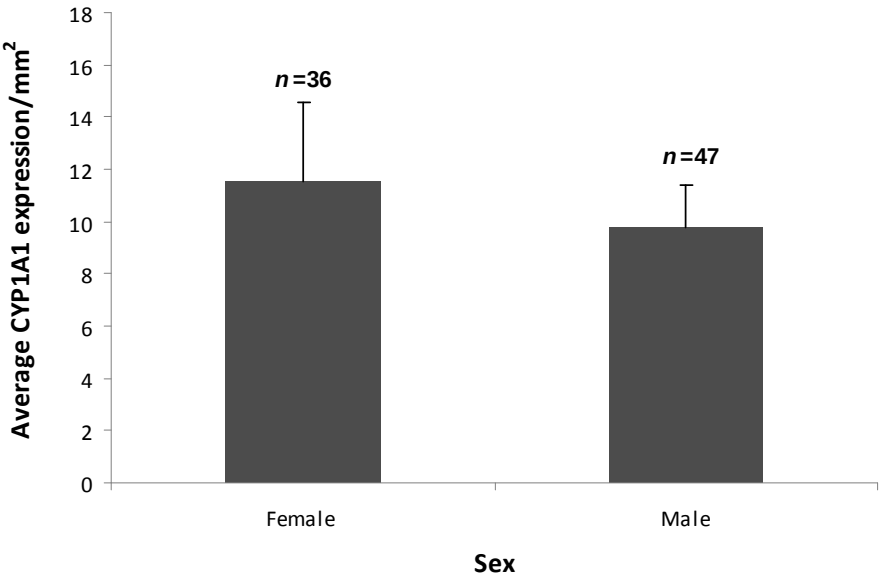

Figure 3.5. Average CYP1A1 expression by sex. No significant trends were found, $p > 0.05$. The sample set included $n = 82$ individuals. Error bars represent standard error.

3.3.3. Are there significant differences among age and sex within species groups?

Age and sex were examined within species groups with large enough total sample sizes ($n > 5$). For rough-toothed dolphins, no significant differences were found for sex ($p = 0.473$). No significant differences were found for age ($p = 0.319$). For melon-headed whales (all of which were adults), no significant differences were found for sex ($p = 0.490$). For short-finned pilot whales (all of which were males), no significant differences were found for age ($p = 0.263$). Although pantropical spotted dolphins had a large sample size ($n = 23$), all sexes were unknown and all individuals were adults except for one subadult. Therefore, this species group was not examined for age and sex differences.

The above results are summarized in Table 3.3 with corresponding sample sizes and power to detect significant differences. False killer whales were also examined for differences among age and sex, and the results are reported in the paragraphs below.

Table 3.3. Analysis of CYP1A1 by age and sex within species groups with large enough sample sizes ($n > 5$).

Species	P-value for age	power at δ = 0.1, 0.5	Sample size	P-value for sex	power at δ = 0.1, 0.5	Sample size
melon-headed whales	All samples were adults, n/a			0.490	1.00, 1.00	*N* = 15 *n* = 4 females *n* = 11 males
rough-toothed dolphins	0.319	1.00, 1.00	*N* = 10 *n* = 5 adults *n* = 3 subadults *n* = 2 juveniles	0.473	1.00, 1.00	*N* = 14 *n* = 7 females *n* = 7 males
short-finned pilot whales	0.263	0.96, 1.00	*N* = 14 *n* = 12 adults *n* = 2 subadults	All samples were males, n/a		

3.3.4. Analysis of false killer whale biopsy samples by age, gender, and social cluster

The following paragraphs review results obtained for CYP1A1 expression and contaminant levels within false killer whale blubber biopsies. The results reported in the paragraphs below are for statistical tests run with the exclusion of the three individual outliers mentioned previously (HIPc184, HIPc220, HIPc266), that displayed unusual levels and patterns in CYP1A1 expression and contaminant levels. However, all results, sample sets, and power for statistical tests with and without exclusion of outliers are reported Table 3.4 (CYP1A1 expression) and Table 3.5 (contaminant levels). Significant results did not vary when including or excluding these individuals in analyses by age, sex, or cluster. In some cases, the individual outliers were already excluded because of unknown cluster or probable estimates of age.

CYP1A1 by age and sex for false killer whales

An age and sex interaction ($p = 0.015$) for CYP1A1 expression occurred within the false killer whale species group. No significant differences in CYP1A1 expression were found for age class in false killer whales across all sexes ($p = 0.195$). No significant differences in CYP1A1 expression were found for sex in false killer whales across all age classes ($p = 0.812$). Within males, there was a significant difference based on age ($p = 0.016$). Juveniles had higher average CYP1A1 expression (Figure 3.6). Within females, no significant differences existed for age ($p = 0.324$). Within juveniles, there was a significant difference for sex ($p = 0.015$), with males having higher average CYP1A1 expression compared to females (although sample size was very low for females, $n = 3$). Within adults, no significant differences occurred for sex ($p = 0.381$).

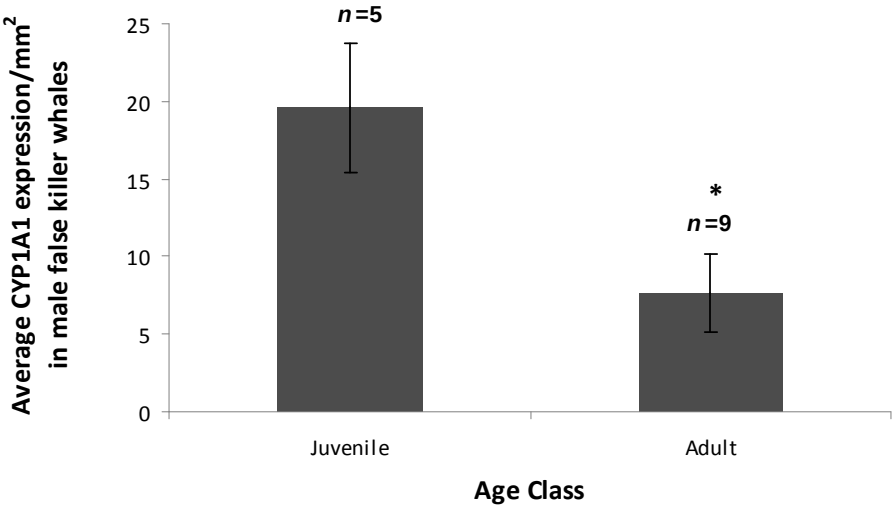

Figure 3.6. Average CYP1A1 expression by age class within male false killer whales. The asterisk represents significant differences found between the two age classes, $p = 0.016$. The sample set included $n = 14$ individuals. The individual outlier HIPc184 was excluded. Error bars represent standard error.

Contaminant levels by age and sex for false killer whales

No interactions were found for age and sex in factorial ANOVAs for ∑PCBs wet weight ($p = 0.485$), ∑PCBs lipid weight ($p = 0.589$), TEQ wet weight ($p = 0.492$), and TEQ lipid weight ($p = 0.501$). No significant differences were found based on age: ∑PCBs wet weight ($p = 0.417$), ∑PCBs lipid weight ($p = 0.921$), TEQ wet weight ($p = 0.093$), and TEQ lipid weight ($p = 0.197$). No significant differences were found based on sex: ∑PCBs wet weight ($p = 0.620$), ∑PCBs lipid weight ($p = 0.326$), TEQ wet weight ($p = 0.768$), and TEQ lipid weight ($p = 0.369$).

CYP1A1 and contaminant levels by cluster

No significant differences in CYP1A1 expression were found among social clusters 1, 2, and 3 ($p = 0.406$) (Figure 3.7). However, false killer whales from cluster 3 had higher average CYP1A1 expression overall. Contaminant levels did not differ significantly among social clusters 1 and 2: $p = 0.856$ (∑PCBs wet weight), $p = 0.823$ (∑PCBs lipid weight, Figure 3.8), $p = 0.975$ (TEQ wet weight), and $p = 0.907$ (TEQ lipid weight). No consistent trends were observed among Cluster 1 and Cluster 2 animals for contaminant levels. Although Cluster 3 was excluded from the ANOVA analysis because of small sample size ($n = 4$), this social group had higher average ∑PCBs (ng/g lipid weight) and TEQ (ng/g lipid and wet weight) compared to Cluster 1 and Cluster 2 animals.

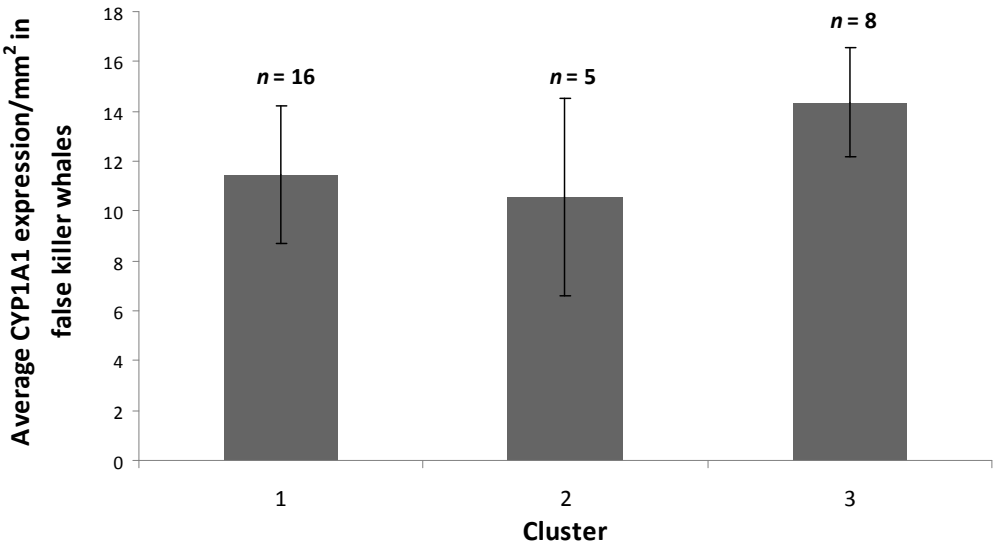

Figure 3.7. Average CYP1A1 expression among false killer whale social clusters. No significant trends were found, $p > 0.05$. The sample set included $n = 29$ individuals. Individual outliers ($n = 2$) were excluded from these analyses. Error bars represent standard error.

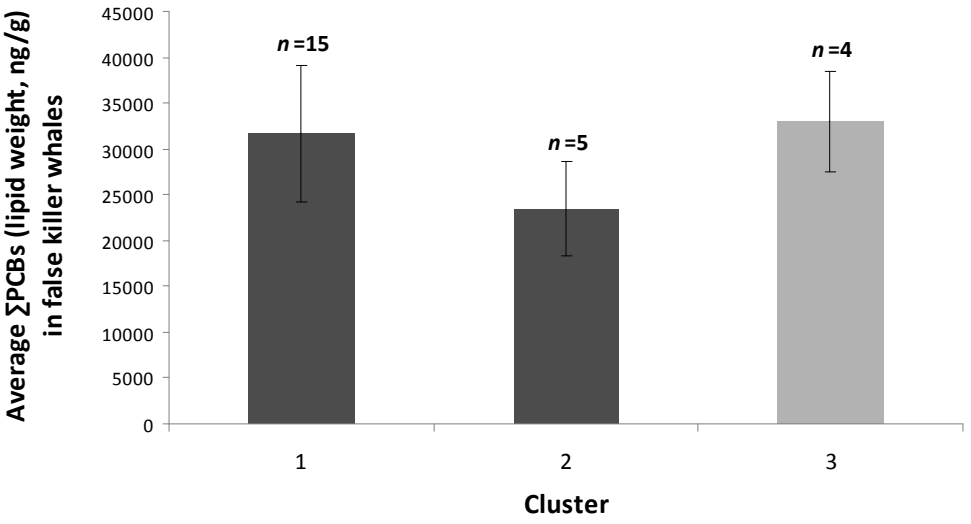

Figure 3.8. Average ∑PCBs (ng/g lipid weight) among false killer whale social clusters. No significant trends were found, $p > 0.05$. The sample set included $n = 20$ individuals. Cluster 3 (highlighted in light gray) omitted from ANOVA analysis because $n < 5$. Differences in sample size between Figure 3.7 and 3.8 reflect the fact that $n = 5$ false killer whales in this study did not have measured contaminant levels, yet had measured CYP1A1 expression. Individual outliers ($n = 2$) were excluded from these analyses. Error bars represent standard error.

Table 3.4. CYP1A1 expression trends in false killer whales. CYP1A1 was examined by factorial ANOVA without and with exclusion of individual outliers HIPc184, HIPc220, HIPc266. Significant p-values obtained are noted in bold print. Power was determined at δ = 0.10 and δ = 0.50, and it is reported in the respective order.

	Without exclusion of individual outliers				With exclusion of individual outliers			
	P-value	Power at δ= 0.1, 0.5	Sample size	Individual(s) excluded	P-value	Power at δ= 0.1, 0.5	Sample size	Individual(s) excluded
Cluster	0.400	1.00 1.00	*N* = 31; Cluster 1: *n* = 17 Cluster 2: *n* = 6 Cluster 3: *n* = 8	Undetermined clusters, *n* = 5	0.406	1.00 1.00	*N* = 29; Cluster 1: *n* = 16 Cluster 2: *n* = 5 Cluster 3: *n* = 8	Undetermined clusters, *n* = 5; HIPc220, HIPc266
Age	0.170	1.00 1.00	*N* = 24 Juveniles: *n* = 8 Adults: *n* = 16	Probable estimates of age, *n* = 12	0.195	1.00 1.00	*N* = 23 Juveniles: *n* = 8 Adults: *n* = 15	Probable estimates of age, *n* = 12; HIPc184
Gender	0.845	1.00 1.00	*N* = 36 Females: *n* = 17 Males: *n* = 19	None excluded	0.812	1.00 1.00	*N* = 33 Females: *n* = 15 Males: *n* = 18	HIPc184, HIPc220, HIPc266
Age within sex (females)	0.324	0.91 1.00	*N* = 9 Juveniles: *n* = 3 Adults: *n* = 6	Probable estimates of age, *n* = 8	Same result as without exclusion (HIPc184, only outlier with known age, is a male and not analyzed here)			
Age within sex (males)	**0.010**	1.00 1.00	*N* = 15 Juveniles: *n* = 5 Adults: *n* = 10	Probable estimates of age, *n* = 4	**0.016**	1.00 1.00	*N* = 14 Juveniles: *n* = 5 Adults: *n* = 9	Probable estimates of age, *n* = 4; HIPc184
Sex within age (juveniles)	**0.015**	1.00 1.00	*N* = 8 Females *n* = 3 Males: *n* = 5	Probable estimates of age, *n* = 4	Same result as without exclusion (HIPc184, only outlier with known age, is an adult and not analyzed here)			
Sex within age (adults)	0.338	1.00 1.00	*N* = 16 Females *n* = 6 Males: *n* = 10	Probable estimates of age, *n* = 4	0.381	1.00 1.00	*N* = 15 Females *n* = 6 Males: *n* = 9	Probable estimates of age, *n* = 4; HIPc184

Table 3.5. Contaminant level trends in false killer whales. Contaminant levels were examined by factorial ANOVA, without and with exclusion of individual outliers HIPc184, HIPc220, HIPc266. Results under significance and power are given in the order of ∑PCBs (ng/g wet weight), ∑PCBs (ng/g lipid weight), TEQ (ng/g wet weight), TEQ (ng/g lipid weight). Power was determined at $\delta = 0.10$ and $\delta = 0.50$, and it is reported in the respective order. Sample set varies with that of CYP1A1 expression because $n = 5$ false killer whales were not analyzed for contaminants.

	Without exclusion of individual outliers				With exclusion of individual outliers			
	P-value	Power at δ= 0.1, 0.5	Sample size	Individual(s) excluded	P-value	Power at δ= 0.1, 0.5	Sample size	Individual(s) excluded
Cluster			*N* =22 Cluster 1: *n* = 16 Cluster 2: *n* = 6	Undetermined clusters, *n* = 5. Cluster 3, *n* < 4			*N* = 20 Cluter 1: *n* = 15 Cluster 2: *n* = 5	Undetermined clusters, *n* = 5; Cluster 3, *n* < 4 HIPc220, HIPc266
∑PCBs (ng/g wet weight)	0.281	0.007 0.09			0.856	0.04 0.35		
∑PCBs (ng/g lipid weight)	0.303	0.0003 0.005			0.823	0.0005 0.005		
TEQ (ng/g wet weight)	0.336	1.00 1.00			0.975	1.00 1.00		
TEQ (ng/g lipid weight)	0.321	1.00 1.00			0.907	1.00 1.00		
Age			*N* = 20 Juveniles: *n* = 7 Adults: *n* = 13	Probable estimates of age, *n* = 11			*N* = 19 Juveniles: *n* = 7 Adults: *n* = 12	Probable estimates of age, *n* = 12; HIPc184
∑PCBs (ng/g wet weight)	0.859	0.03 0.28			0.417	0.10 0.63		
∑PCBs (ng/g lipid weight)	0.858	0.007 0.55			0.921	0.008 0.11		

TEQ (ng/g wet weight)	0.350	1.00 1.00			0.09	1.00 1.00		
TEQ (ng/g lipid weight)	0.325	1.00 1.00			0.197	1.00 1.00		
Gender								
			N = 31 Females: *n* = 15 Males: *n* = 16	None excluded			*N* = 28 Females: *n* = 13 Males: *n* = 15	HIPc184, HIPc220, HIPc266
∑PCBs (ng/g wet weight)	0.398	0.03 0.27			0.620	0.16 0.76		
∑PCBs (ng/g lipid weight)	0.223	0.002 0.04			0.326	0.004 0.05		
TEQ (ng/g wet weight)	0.623	1.00 1.00			0.768	1.00 1.00		
TEQ (ng/g lipid weight)	0.232	1.00 1.00			0.369	1.00 1.00		

3.3.5. Do contaminant levels and CYP1A1 expression correlate within false killer whale samples?

Without omission of the individual outliers HIPc184, HIPc220, and HIPc266, no statistical significance was present for all relationships between contaminant levels and CYP1A1 expression: ∑PCBs wet weight ($R^2 = 0.00$, $p = 0.883$), ∑PCBs lipid weight ($R^2 = 0.01$, $p = 0.564$), TEQ wet weight ($R^2 = 0.05$, $p = 0.698$), and TEQ lipid weight ($R^2 = 0.06$, $p = 0.895$). However, with exclusion of the three individual outliers, a significant relationship was found between CYP1A1 expression and ∑PCBs (ng/g wet weight) ($R^2 = 0.36$, $p = 0.001$), along with ∑PCBs lipid weight (ng/g lipid weight) ($R^2 = 0.20$, $p = 0.015$). However, there was no significant relationship between CYP1A1 expression and

TEQ wet weight ($R^2 = 0.09$, $p = 0.226$), and CYP1A1 expression and TEQ lipid weight ($R^2 = 0.04$, $p = 0.430$) (Figure 3.9 A-D).

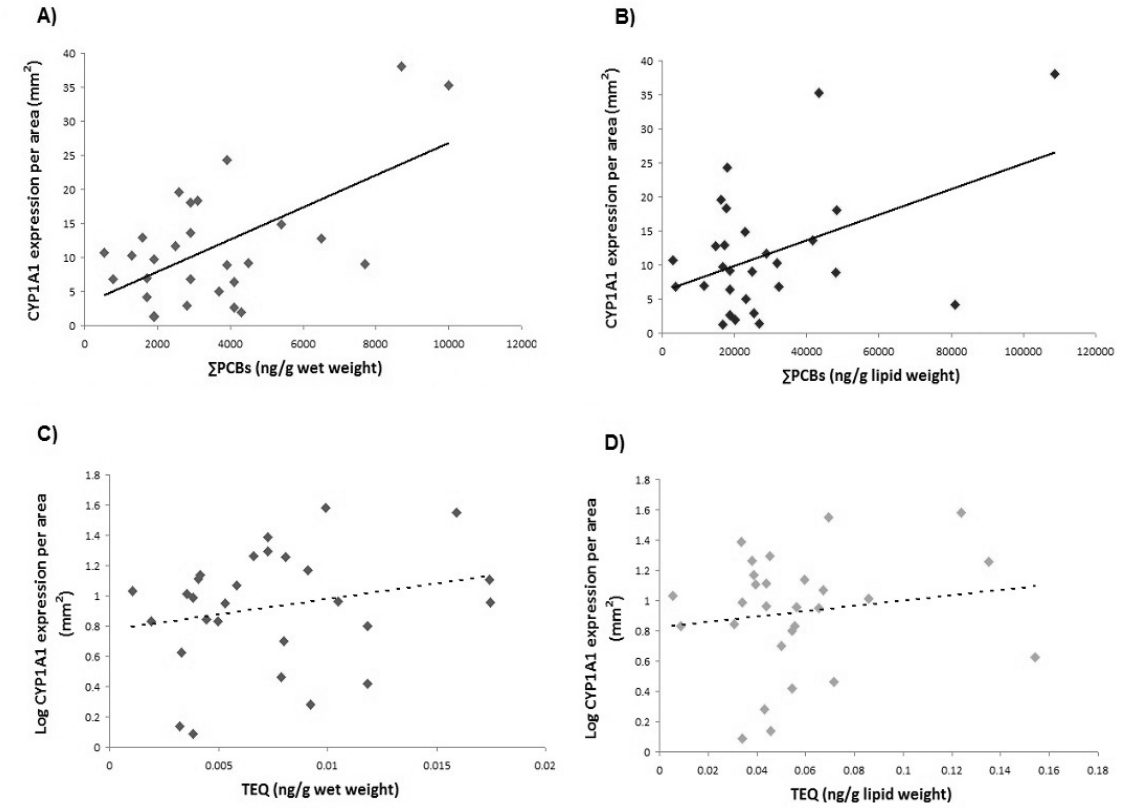

Figure 3.9. Relationship between CYP1A1 expression and ∑PCBs or TEQ (ng/g) in false killer whales. The sample set included $n = 28$ individuals. A) CYP1A1 expression/area correlated significantly with ∑PCBs (ng/g wet weight) ($p < 0.05$) as indicated by the solid line ($R^2 = 0.36$, $p = 0.001$). B) Significance was found between CYP1A1 expression/area and ∑PCBs (ng/g lipid weight) ($R^2 = 0.20$, $p = 0.015$). C) No significance was found between CYP1A1 expression/area and TEQ (ng/g wet weight) as indicated by the dashed line ($R^2 = 0.09$, $p = 0.226$). D) No significance was found between CYP1A1 expression/area and TEQ (ng/g lipid weight) ($R^2 = 0.04$, $p = 0.430$).

3.4. Discussion

Analysis of CYP1A1 expression in blubber biopsies from free-ranging animals provides a glimpse at the molecular effects related to contaminant exposure in presumably healthy, active odontocetes. In order to analyze CYP1A1 expression in these samples, however, tissue area or depth should be accounted for because of highest CYP1A1 expression found in the deep blubber layer (Chapter 2, Montie et al. 2008). This study examined CYP1A1 expression/area (mm^2) in blubber biopsy samples from free-ranging Hawaiian odontocetes.

3.4.1. Differences in CYP1A1 expression among species

This study compares CYP1A1 expression across ten different species, and it suggests an influence of trophic ecology on CYP1A1 expression. Significant differences in CYP1A1 expression between false killer whales and melon-headed whales in this study may indicate that these species feed at different trophic levels, and thus have different contaminant burdens. Relatively few stomach contents of melon-headed whales have been analyzed, but they are thought to mainly consume pelagic and mesopelagic fish and squid (Best and Shaughnessy 1981, Clarke and Young 1998). Analysis of stomach contents of stranded melon-headed whales in Hawaiʻi reveal that these animals eat a variety of squid and mesopelagic fish species, such as *Nansenia* sp., *Diaphus* sp., *Lampadena* sp.; stomach contents also revealed bathypelagic species such as Sloane's viperfish *(Chauliodus sloani*), black snaggletooth *(Astronesthes indicus)*, and short fin pearl eye (*Scopelarchus analis*) (K. West, pers. comm.). False killer whales are known to eat large pelagic fish (Baird et al. 2008a, Chivers et al. 2007), many of which are

commercially caught. Documented prey include yellowfin tuna (*Thunnus albacares*), albacore tuna (*Thunnus alalunga*), skipjack tuna (*Katsuwonus pelamis*), broadbill swordfish (*Xiphias gladius*), dolphin fish (or mahimahi, *Coryphaena hippurus*), wahoo (or ono, *Acanthocybium solandri*), and lustrous pomfret (or monchong, *Eumegistus illustrus)* (Baird et al. 2008a). Based on the differences in prey consumption between false killer whales and melon-headed whales, it is likely that false killer whales feed at a higher trophic level than melon-headed whales.

CYP1A1 expression in other species groups may also reveal trophic differences. For example, Blainville's beaked whale with a small sample size (n = 3) displayed the highest average expression, and feed primarily on bathypelagic cephalopods (Baird et al. 2006, McSweeney et al. 2007). Although these prey species are found in the remote deep-sea environment, recent studies indicate that the deep-ocean is a sink for hydrophobic organochlorines such as PCBs (de Brito et al. 2002). Considerable levels of ∑PCBs are found in deep-sea organisms such as cephalopods (de Brito et al. 2002, Ueno et al. 2003). Moreover, preliminary data indicate that Blainville's beaked whale feed at a higher trophic level than short-finned pilot whales (Baird et al. 2009a), and higher average CYP1A1 expression was found in Blainville's beaked whale in this study compared to short-finned pilot whales. Bottlenose dolphins also had relatively high CYP1A1 expression in this study. Although little is known about the diet of bottlenose dolphins in Hawaiʻi, individuals have been observed feeding on large (> 50cm) unidentified fish (Baird et al. 2009b).

Species groups with middle-range CYP1A1 expression included pantropical spotted dolphins, rough-toothed dolphins, and short-finned pilot whales. Pantropical

spotted dolphins are thought to forage in association with the deep-scattering layer (Baird et al. 2001), and they are known to eat mesopelagic fish such as myctophids and squid species (particularly, *Noploteuthis* sp. and *Abraliopsis* sp.) (Clarke and Young 1998). Rough-toothed dolphins are thought to feed primarily on near-surface species. They have been observed chasing needlefish and flying fish species, circling groups of small (5-15 cm in length) fish, and holding squid parts in their mouths (Baird et al. 2008b). Little is known on the diet of short-finned pilot whales, yet they have been observed carrying pieces of squid remains in their mouths (Baird, pers. comm. with Mahaffy 2012).

In this study, pygmy killer whales (n = 5) displayed relatively low CYP1A1 expression, and data is sparse on this species' diet. Because of lack of foraging observations in Hawai‘i, it is hypothesized that pygmy killer whales feed at great depth, or during the night on prey that travel closer to the surface in association with the deep-scattering layer (McSweeney et al. 2009). Their diet may be similar to that of melon-headed whales, since many of the prey of melon-headed whales (mentioned previously) travel closer to the surface at night. Similar diets between these two species would explain their similar levels of CYP1A1 expression. The two Cuvier's beaked whales and the sperm whale in this study (n = 1) also displayed low CYP1A1 expression (the sperm whale having the lowest expression overall). Both of these species feed primarily on squid (Baird et al. 2006, Clarke et al. 1993, Evans and Hindell 2004, McSweeney et al. 2007). Stomach contents of sperm whales (n = 2) that stranded in Hawai‘i contained *Histioteuthis hoylei*, *Ommastrephes bartrami*, and *Architeuthis* sp. in highest percentages (Clarke and Young 1998).

3.4.2. CYP1A1 expression in sex and age groups

No significant differences were found among sex and age class across all species. Within male false killer whales, juveniles had significantly higher CYP1A1 expression compared to adults. Furthermore, within juveniles, males had significantly higher CYP1A1 expression than females. Underrepresentation of females ($n = 3$) is important to note for this analysis. When all age and gender groups were pooled, females had higher average CYP1A1 expression, although this result was not significant.

Although many studies have reported lack of significant CYP1A1 expression trends for sex (Godard-Codding et al. 2011, Jauniaux et al. 2011, Montie et al. 2008, Wilson 2003, Wilson et al. 2007, 2010) and age class (Wilson 2003, Wilson et al. 2007, 2010), a small amount of studies have reported CYP1A1 expression trends in cetaceans with respect to sex and age. Montie et al. (2008) found a significant difference in CYP1A1 expression by age class in the superficial blubber layer of bottlenose dolphins. Subadults (all genders combined) had higher CYP1A1 expression than adults within this blubber layer. This result was explained by the fact that with age, adults experience lipid loss (and subsequent contaminant mobilization) within the deep blubber layer, leading to predominant CYP1A1 expression within this layer. Because the biopsy samples of the present study contain mostly the superficial to middle blubber layers (only 7% contained the deep blubber layer), and the outermost blubber layer becomes less important metabolically with age (Koopman et al. 1996), higher CYP1A1 levels would be expected in juveniles compared to adults. This result may have been confined to male odontocetes because of the variability in metabolic phases of adult females that occurs with cycles of birth and lactation.

Similar to a previous analysis of CYP1A1 in a variety of Hawaiian cetacean species (Fertall 2010), no significant differences in expression for sex and age were found across pooled sex and age classes, across all species. The wide variety of species analyzed in Fertall (2010) and the present study could potentially reduce the ability to distinguish significant differences in CYP1A1 expression among sex and age. For a better assessment of these trends in CYP1A1 expression, future studies should assess expression within particular species groups with large enough sample size and extensive individual data, including reproductive history.

3.4.3. CYP1A1 as a biomarker of contaminant exposure

This study is the first to establish the use of CYP1A1 expression as a biomarker of contaminant exposure in free-ranging Hawaiian odontocetes. Observed trends are consistent with many studies from different regions around the world (Godard et al. 2004, Montie et al. 2008, White et al. 1994, Wilson et al. 2007). In the present study, CYP1A1 expression in false killer whale biopsies significantly correlated with ∑PCBs (ng/g wet weight) and ∑PCBs (ng/g lipid weight) with the exclusion of three individual outliers.

Although ∑PCBs significantly correlated with CYP1A1 expression, TEQ (ng/g wet weight) and TEQ (ng/g lipid weight) did not. Lack of a significant correlation between TEQ and CYP1A1 expression may be because TEQ was determined based only on the three mono-*ortho* congeners PCB 105, 118, 156, all of which have a relatively low TEF of 0.00003 (Safe 1998, Van den Berg et al. 2006). These three mono-*ortho* PCBs were the only known AHR agonists measured in this study. Furthermore, mono-*ortho* PCBs are not the strongest inducers of CYP1A1 compared to the non-*ortho* congeners

(Zeiger et al. 2001) that show more similar patterns of biological responses to TCDD (Safe 1998). Similar findings were reported by Wilson et al. (2007), in which CYP1A1 expression in the lower dermis weakly correlated with total PCBs, yet not with mono-*ortho* PCBs. The significant relationship between ∑PCBs and CYP1A1 expression, yet not TEQ and CYP1A1 expression, may indicate that individuals with higher contaminant burdens overall have a greater likelihood of being exposed to AHR agonists not measured in this study, such as non-*ortho* PCBs and PAHs.

3.4.4. Analysis of CYP1A1 expression among false killer whale social cluster

False killer whale social clusters reveal differential habitat use (Baird et al. 2012), so different levels of contaminant responses might be expected. No significant differences in CYP1A1 expression among social cluster were observed in this study. However, on average, cluster 3 animals had higher CYP1A1 expression and contaminant levels, followed by cluster 1 and cluster 2 animals respectively (refer back to Figure 3.7). Although Cluster 3 animals share a similar overall range with Cluster 1 animals, they have different patterns of habitat use (Baird et al. 2012). Cluster 3 animals primarily use the area north of Maui to northwest of Molokaʻi, while Cluster 1 animals have high density in the north end of the island of Hawaiʻi (but appear to commonly use the north and northwest side of Molokaʻi as well, and southwest side of Lanaʻi). Based on photo-identification data, Cluster 2 animals are sighted most often off the island of Hawaiʻi (Baird et al. 2012), but since they are seen there only rarely, it is not known where they spend most of their time (R.W. Baird, pers. comm.). Therefore, the CYP1A1 expression trends may be a result of different high density areas of habitat utilization and variable

diet among clusters. Prey organisms on the north and northwest side of Molokaʻi (primarily used by Cluster 3 animals, and sometimes Cluster 1 animals) may represent higher average trophic level, since this region has been observed to contain the highest density of apex predators in comparison to a variety of locations in the main Hawaiian Islands (south shore of Lanaʻi, north shore of Maui and Molokini Crater, and the east and northeast shores of Oʻahu) (Coles et al. 2008). The remote nature of the north and northwest side of Molokaʻi may result in low fishing pressure that allows for greater numbers of species at higher trophic levels, although some fishing occurs in this region during calm periods (E. Brown, pers. comm. with Coles et al. 2008).

3.4.5. Conclusions

CYP1A1 was detected in all free-ranging odontocetes examined in this study. This confirms a biological response to contaminant exposure in all species of Hawaiian cetaceans examined. The results of this study indicate that trophic position influences CYP1A1 expression patterns, yet more information is needed in regard to habitat use and diet preferences for Hawaiian odontocete species. For false killer whales, a larger sample size and more information on habitat use may reveal patterns of CYP1A1 expression among social clusters. In this study, average levels of expression differed among social cluster, and the trends of expression and contaminant levels were consistent with each other.

The significant correlation between ∑PCBs and CYP1A1 expression, yet not with TEQ and CYP1A1 expression, suggests that CYP1A1 expression is caused by a broader suite of AHR agonists than was measured for this study. Future contaminant analysis,

including a larger suite of dioxin like HAH and PAHs, will be useful for determining whether or not CYP1A1 expression in blubber biopsies is an effective biomarker of AHR agonist exposure for Hawaiian odontocetes. Data on reproductive history in females will be critical for future analysis, because post-partum females should be excluded from contaminant and CYP1A1 linear regression analysis due to the influence of contaminant offloading during lactation.

Importantly, the insular false killer whale stock in Hawaiʻi is now classified as endangered under the Endangered Species Act (Department of Commerce and NOAA 2012). Fisheries interactions and high levels of POPs are critical anthropogenic stressors to this population (Baird 2009, Ylitalo et al. 2009). In the current study, false killer whales showed evidence of contaminant impacts with higher CYP1A1 expression levels compared to melon-headed whales, and higher average CYP1A1 expression relative to other species overall. Future analysis of CYP1A1 in blubber biopsies is encouraged to continue monitoring the health of Hawaiian odontocetes, particulary for the endangered insular false killer whale stock.

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Chapter 4: Scientific Contribution of this Study and Future Research Suggestions

4.1. Contribution of this study

Prior to this work, there was no information on CYP1A1 expression in free-ranging Hawaiian odontocetes. For stranded and free-ranging animals, full depth blubber and dart biopsy samples alike, CYP1A1 was present and significant differences existed among species. Furthermore, there were physiological trends in cellular distribution throughout the blubber of stranded animals, and it was further established that the blubber is multifunctional with distinct layering of adipocytes, endothelial cells, and CYP1A1.

This study primarily focused on two different analyses: cellular and CYP1A1 stratification in the blubber of stranded Hawaiian odontocetes (Chapter 2), and CYP1A1 presence in free-ranging Hawaiian odontocetes (Chapter 3). Assessment of cellular stratification and CYP1A1 stratification contributed to an understanding of how these processes are interrelated, with significant variability of adipocyte area in the deep blubber layer among condition groups indicating lipid mobilization, leading to stratification of CYP1A1 observed in the blubber. Patterns of cellular and CYP1A1 stratification in stranded Hawaiian odontocetes are most likely due to the fact that these animals experienced stress and lipid mobilization prior to stranding; however, a variety of other factors may explain stratification patterns such as reproduction/lactation in females, food availability, and potentially small-scale seasonal changes in water temperature observed in Hawai‘i (Montie et al. 2008a, NOAA 2007).

It is unknown whether foraging at depth contributes to stratification of lipids and CYP1A1 expression in the blubber. However, this is an important consideration for future research because bottlenose dolphins from Charleston Harbor, SC, and Hawaiian odontocetes (that both display stratification of CYP1A1) potentially feed at greater depth

relative to dolphins from the shallow Indian River Lagoon (that did not show significant CYP1A1 stratification) (Indian River Lagoon National Estuary Program, Montie et al. 2008a, South Carolina Ports). In particular, many Hawaiian odontocetes are known to feed at mesopelagic and bathypelagic depths (Baird et al. 2006, Benoit-Bird and Au 2003, Clarke and Young 1998, McSweeney et al. 2007, Norris et al. 1994, Shomura and Hida 1965). Future stratification analyses should take into account diving behavior to see if time spent at depth affects cellular and CYP1A1 stratification.

Correlations of CYP1A1 expression among the blubber layers of stranded animals in Chapter 2 suggests that dart biopsy samples from free-ranging odontocetes are useful for analysis of CYP1A1. This is an important contribution toward the ability to monitor the health of free-ranging Hawaiian odontocetes, with special consideration for threatened populations such as the insular false killer whale (Baird 2009). Moreover, this study suggests procedures and considerations for future quantification of CYP1A1 expression in dart-biopsies. It is very important to take into account area of tissue when assessing CYP1A1 expression in dart biopsies, because depth of tissue retrieved varies depending on angle of biopsy entry (Todd et al. 1997), and CYP1A1 is found to be highest in the deep blubber layer (Chapter 2, Montie et al. 2008a). Only 7% of the dart biopsy samples in this study captured the deep blubber layer, yet, this percentage is high enough to influence CYP1A1 patterns observed among different species, gender, or age groups. Therefore, dividing CYP1A1 expression by area (mm^2) reduces the influence of higher expression found in greater depth biopsy samples.

For the stranded animal analysis in Chapter 2, we assessed CYP1A1 expression per area, but also examined CYP1A1 expression per total number of endothelial cells.

Both scoring methods correlated significantly with one another. Blubber condition was quite variable among stranded animals, and smaller adipocytes were found in the deep blubber layer of thin animals relative to robust animals. Because more endothelial cells were also quantified in thin animals, we realized the importance of dividing CYP1A1 expression by total number of endothelial cells quantified to account for the potential greater ability to see CYP1A1 expression in thin animals. Less variability in blubber condition was visually distinguished within the free-ranging dart biopsy samples, yet the majority of these samples did not obtain the deep blubber layer in which adipocyte cell area fluctuates significantly based on animal condition. Therefore, we conclude that CYP1A1 expression/total number of endothelial cells is the most biologically sound method for quantifying CYP1A1 expression in stranded animal samples, and CYP1A1 expression/area is the most reasonable method for quantifying expression in free-ranging dart biopsy samples of variable depth.

This study also investigated some previously established trends in blubber dynamics seen within individual blubber layers. Montie et al. (2008b) predicted that the middle blubber layer shows ontogenetic changes with growth (decrease in adipocyte quantities) and changes with seasonal water temperature (adipocyte shrinkage), while the deep blubber layer varies primarily based on nutritive condition. In Chapter 2, we found significant differences in adipocyte area and quantity for age class only within the middle blubber layer, and significant differences in adipocyte area for animal condition solely within the deep blubber layer. Therefore, not only does the blubber show variability in cellular characteristics and CYP1A1 presence throughout depth, but there appear to be specific functions for each blubber layer. Many other studies have indicated that the deep

blubber layer is an active source of energy storage and supply (Koopman et al. 1996), yet few studies have described the changes that occur in the middle blubber layer with age or seasonal changes in water temperature. Decreases in surface-area-to-volume and increased demand for energy with growth (Dunkin et al. 2005, McLellan et al. 2002, Struntz et al. 2004) may explain changes in blubber composition with age in this study (larger adipocytes with increasing age).

4.2. Future assessments

The results of Chapter 2 of this thesis were beneficial for gaining a better understanding of blubber physiology: lipid and contaminant mobilization leading to CYP1A1 induction. Future research on cellular stratification should examine enzymes and receptors important for lipid storage and retrieval (e.g. hormone-sensitive lipase, adipose triglyceride lipase, and Type 2 deiodinase) (Watanabe et al. 2006, Zimmerman et al. 2004, reviewed in Montie et al. 2008b) to better establish physiological processes within the blubber of odontocetes and the different functions of the blubber layers.

Regarding the findings of Chapter 3 of this study, more information is needed on AHR agonists within the blubber of Hawaiian odontocetes. The significant correlations between ∑PCBs and CYP1A1 expression found in false killer whales, yet not TEQ and CYP1A1 expression, was most likely a result of only a few measured AHR agonists in the study (PCB 105, 118, and 156) with generally low TEFs. Non-*ortho* PCBs and PAHs, both AHR agonists, were not measured in this study. Therefore, a significant correlation between ∑PCBs and CYP1A1 expression may indicate that individuals (with higher contaminant burdens overall) are potentially exposed to greater concentrations of AHR

agonists that were not measured in this study. Although it appears CYP1A1 is a biomarker of contaminant exposure for Hawaiian odontocetes based on the significant relationship between ∑PCBs and CYP1A1 expression, a significant relationship with TEQ (including more AHR agonists measured) would provide further evidence supporting use of this biomarker. Future studies should assess a variety of non-*ortho* and mono-*ortho* PCBs, while examining differences in the strength of relationships between these contaminant classes and expression. It is important to keep in mind that CYP1A1 induction may also be a result of PAH exposure, which is most likely increasing on the Hawaiian islands because of increased anthropogenic activity (combustion of fossil fuels) and volcanic emissions into the atmosphere (Harvey 1991, Grimmer 1983, U.S. Census Bureau).

Although the analysis of CYP1A1 expression by false killer whale social cluster did not provide significant results in this study, future research should continue to assess differences. Cluster 3, on average, displayed higher CYP1A1 expression and contaminant levels (Chapter 3). Cluster 3 false killer whales also are found at high density in north and northwest Molokaʻi (Baird et al. 2012), a region known to have a high percentage of apex predators (Coles et al. 2008) that would contribute to higher contaminant exposure because of biomagnification. We feel that analysis of contaminant levels and CYP1A1 expression by social cluster with greater sample size remains a vital priority for future research.

Also in Chapter 3 of this thesis, false killer whales had significantly higher CYP1A1 expression compared to melon-headed whales. False killer whales, known to eat large pelagic fish (Baird et al. 2008, Chivers et al. 2007), would be expected to have

high levels of expression relative to species such as melon-headed whales that are known to eat smaller deep-water fish and squid (K. West, pers. comm). Future studies on trophic ecology are vital for research on Hawaiian odontocetes, as relatively little is known except for observations of foraging in the field and stranded animal stomach contents.

Lastly, there appeared to be geographic and trophic patterns of CYP1A1 expression in this study. In Chapter 2, striped dolphins had significantly lower CYP1A1 expression compared to other species analyzed with $n > 5$. Striped dolphins are found primarily in deep, offshore waters of Hawaiʻi (R.W. Baird, pers. comm.) Therefore, striped dolphins may not be exposed to the same particular contaminants as species that are known to live in shallower, near-shore environments (e.g. bottlenose dolphins) (Martien et al. 2011). Godard-Codding et al. (2011) found that sperm whales from regions farthest from continents had lower CYP1A1 expression levels. Future studies should continue to monitor this trend to see if geographic distribution to continents or islands influences CYP1A1 expression levels. Studies on different populations of an individual species (for example, melon-headed whales that reside mostly northwest of Hawaiʻi vs. those that travel and reside throughout the main Hawaiian Islands, Aschettino et al. 2012) would be useful to examine this hypothesis.

The present study recognizes the challenges that exist with unequal group sizes and making inferences across a wide variety of different species. However, the individual data provided for this diverse assemblage of species in Hawaiʻi is very remarkable in respect to the fact that the animals included in this study have very large ranges across the main Hawaiian islands (and for some species, the central tropical Pacific), in which resightings is often a difficult task for free-ranging animals and opportunity to necropsy

can be variable based on access ability, decompositon, etc. The ongoing nature of these studies is truly like no other in the world, and future analysis of CYP1A1 will be valuable to monitor the long-term effects of contaminants to Hawaiian odontocetes. CYP1A1 will be important to continue to assess in the future, as contaminants are known to travel to distant regions of the world and bioaccumulate in tissues throughout time.

Although the current study brought forth many new questions for future research, the results are a great stepping stone for future analysis of contaminant threats to Hawaiian odontocetes. With the ongoing nature of research conducted by Cascadia Research Collective and the Hawai‘i Pacific University Stranding Response Team, much more can be learned regarding CYP1A1 expression and its relation to health and disease in free-ranging and stranded animals.

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