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Cytochrome P4501A1 expression in blubber biopsies of endangered false killer whales (*Pseudorca crassidens*) and nine other odontocete species from Hawai'i

Kerry M. Foltz · Robin W. Baird · Gina M. Ylitalo · Brenda A. Jensen

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Abstract Odontocetes (toothed whales) are considered sentinel species in the marine environment because of their high trophic position, long life spans, and blubber that accumulates lipophilic contaminants. Cytochrome P4501A1 (CYP1A1) is a biomarker of exposure and molecular effects of certain persistent organic pollutants. Immunohistochemistry was used to visualize CYP1A1 expression in blubber biopsies collected by non-lethal sampling methods from 10 species of free-ranging Hawaiian odontocetes: short-finned pilot whale, melon-headed whale, pygmy killer whale, common bottlenose dolphin, rough-toothed dolphin, pantropical spotted dolphin, Blainville's beaked whale, Cuvier's beaked whale, sperm whale, and endangered main Hawaiian Islands insular false killer whale. Significantly higher levels of CYP1A1 were observed in false killer whales and rough-toothed dolphins compared to melon-headed whales, and in general, trophic position appears to influence CYP1A1 expression patterns in particular species groups. No significant differences in CYP1A1 were found based on age class or sex across all samples. However, within male false killer whales, juveniles expressed significantly higher levels of CYP1A1 when compared to adults. Total polychlorinated biphenyl ($\sum$PCBs) concentrations in 84 % of false killer whales exceeded proposed threshold levels for health effects, and $\sum$PCBs correlated with CYP1A1 expression. There was no significant relationship between PCB toxic equivalent quotient and CYP1A1 expression, suggesting that this response may be influenced by agonists other than the dioxin-like PCBs measured in this study. No significant differences were found for CYP1A1 expression among social clusters of false killer whales. This work provides a foundation for future health monitoring of the endangered stock of false killer whales and other Hawaiian odontocetes.

Keywords Cytochrome P4501A1 (CYP1A1) · Pseudorca · Hawaiian cetacean · Polychlorinated biphenyls (PCB) · Blubber dart biopsy · Biomarker

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K. M. Foltz · B. A. Jensen (✉)
College of Natural and Computational Sciences, Hawai'i Pacific University, 45-045 Kamehameha Highway, Kaneohe, HI 96744, USA
e-mail: bjensen@hpu.edu

K. M. Foltz
e-mail: kmcfoltz@gmail.com

R. W. Baird
Cascadia Research Collective, 218 1/2 4th Ave W, Olympia, WA 98501, USA
e-mail: rwbaird@cascadiaresearch.org

G. M. Ylitalo
Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Blvd. East, Seattle, WA 98112-2097, USA
e-mail: gina.ylitalo@noaa.gov

Introduction

Persistent organic pollutants (POPs) are ubiquitous throughout the marine environment. POPs are generally lipophilic, which causes them to bioaccumulate throughout an animal's lifetime and biomagnify with increasing trophic level. POPs are known to be transported long

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distances to remote regions via atmospheric deposition and evaporation events (Wania and Mackay 1996).

Particular classes of POPs can exhibit their toxic effects in humans and wildlife 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 certain of 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 dioxin-like polychlorinated biphenyls (PCBs) (Poland and Knutson 1982). In vitro studies on leukocytes have suggested that high levels of PCBs are correlated with decreased immune function in odontocetes (De Guise et al. 1998; Lahvis et al. 1995). Furthermore, other studies have reported high levels of PCBs in animals that succumbed to infectious disease (Jepson et al. 2005) and epizootic outbreaks (Aguilar and Borrell 1994).

PAHs, the other group of established AHR agonists, are also known to cause adverse health effects in wildlife. PAHs have anthropogenic and natural sources that include fossil fuel combustion, refuse burning, forest fires, and volcanic activity (Harvey 1991). Evidence suggests that high levels of PAHs in the St. Lawrence estuary are likely involved in etiology of cancer in beluga whales (*Delphinapterus leucas*) in this heavily impacted region (Martineau et al. 2002).

Cytochrome P4501A1 (CYP1A1) is an enzyme involved in many of these toxic responses, and thus is also a sensitive molecular biomarker of organic contaminant exposure and effect. CYP1A1 is induced by PAH or HAH binding to the AHR. Depending on the chemical nature of the original ligand, CYP1A1 can lead to detoxification or bioactivation (Parkinson 1996). Bioactivation occurs when the detoxification pathways produce reactive metabolites that disrupt or damage cells via several potential pathways (Conney 1982; Miller and Miller 1981; Phillips 1983; reviewed in Whitlock 1999). Examples of 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 interact with the thyroid hormone (TH) system (Letcher et al. 2000), and have been shown to have negative effects on human and marine mammal health (Brouwer et al. 1989, 1998; Zoeller et al. 2002). PAHs can be metabolized to epoxide intermediates by CYP1A1, and further metabolized with the aid of epoxide hydrolase to highly reactive diol-epoxide intermediates capable of binding to DNA and initiating carcinogenesis (Conney 1982; Shimada and Fujii-Kuriyama 2004). Thus, CYP1A1 presence indicates that the individual is responding to contaminant exposure at the molecular level, potentially resulting in negative physiological consequences.

Hawai'i is far from continental sources of POPs, yet it is not invulnerable to high levels of contaminant exposure. Reports now confirm a variety of POPs in various Hawaiian habitats and organisms. PAHs in sediments from Hawaiian watersheds of mixed (urban and agricultural) land use were in the highest twenty-fifth percentile of surveyed areas across the United States (Brasher and Wolff 2004). PCB profiles resembling one of the technical mixtures of PCBs, Aroclor 1254, were found in tilapia (*Oreochromis mossambicus*) inhabiting the Manoa stream and the Ala Wai canal located on the island of O'ahu (Yang et al. 2008). Summed PCBs ($\sum$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*) from the insular population around the main Hawaiian Islands (Ylitalo et al. 2009). This level of exposure exceeds 14,800 ng/g lipid weight, the suggested threshold concentration over which marine mammals may experience adverse health effects (Schwacke et al. 2002). PCB concentrations exceeding this threshold concentration have also been quantified within stranded Hawaiian cetaceans (Bachman et al. 2014).

We examined CYP1A1 expression in the blubber biopsies collected by non-lethal sampling methods from 10 species of free-ranging odontocetes from around the main Hawaiian Islands using immunohistochemistry, a method established in several previous studies for measuring CYP1A1 induction in cetacean integument (Angell et al. 2004; Hooker et al. 2008; Montie et al. 2008a; Wilson et al. 2007). The specific aims of this study were to: (1) examine species, age class, and sex related trends in CYP1A1 expression levels; (2) compare CYP1A1 expression levels in false killer whales to summed PCB concentrations measured in blubber; and (3) explore differences in CYP1A1 expression among false killer whale social clusters established by Baird et al. (2012).

Methods

Sample collection

Blubber biopsies were collected from 113 individuals in October and December of 2009, and April through December of 2010, as part of ongoing studies of odontocete cetaceans in the main Hawaiian Islands (Baird et al. 2013).

Table 1 Complete sample list of Hawaiian odontocetes

Species	Sample size[a]
Oceanic dolphins (Delphinidae)	
False killer whale (*Pseudorca crassidens*)[b]	36
Short-finned pilot whale (*Globicephala macrorhynchus*)	10
Melon-headed whale (*Peponocephala electra*)	15
Pygmy killer whale (*Feresa attenuata*)	5
Rough-toothed dolphin (*Steno bredanensis*)	15
Common bottlenose dolphin (*Tursiops truncatus*)	3
Pantropical spotted dolphin (*Stenella attenuata*)	23
Beaked whales (Ziphiidae)	
Blainville's beaked whale (*Mesoplodon densirostris*)	3
Cuvier's beaked whale (*Ziphius cavirostris*)	2
Sperm whales (Physeteridae)	
Sperm whale (*Physeter macrocephalus*)	1
Total	113

[a] Sample size indicates the number of individuals for which CYP1A1 was measured in blubber biopsies from free-ranging Hawaiian odontocetes

[b] All individuals sampled were from the main Hawaiian Islands insular population

Samples were collected off the west sides of the islands of Hawai'i and O'ahu, 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 fitted with a high-density foam collar to prevent dart penetration greater than 18 mm. The amount of tissue retrieved from the darts varied. All biopsies included the epidermis, hypodermis, superficial and middle blubber layers. Seven percent (8 of 113) of the biopsy samples contained the deep blubber layer that overlies the subdermal sheath (for a detailed description of blubber morphology and layering, see Montie et al. 2008b).

Biopsy samples were stored in a cooler with ice packs while in the field, and later 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. 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). Biopsied individuals were photographed and photos were compared to individual photo-identification catalogs of most species (McSweeney et al. 2007, 2009; Baird et al. 2008a, b, 2009; Aschettino et al. 2011; Mahaffy 2012) 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 1.

Sample analysis

Age determination

Age class (juvenile, subadult, adult) of biopsied individuals were noted in the field and based on photos using 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 *Stenella attenuata*), the degree of scarring (which accumulate over time for some species) and erupted teeth (for beaked whales). In some cases, age class could not be determined with certainty; these probable estimates were excluded in ANOVA analyses of CYP1A1 expression by age.

Sex determination

Sex was determined genetically by the Southwest Fisheries Science Center from skin samples from the biopsies for false killer whales, common bottlenose dolphins (*Tursiops truncatus*), pygmy killer whales (*Feresa attenuata*), and melon-headed whales (*Peponocephala electra*), using a Real-Time PCR (Stratagene) assay of the zinc finger genes, as described in Morin et al. (2005). For rough-toothed dolphins (*Steno bredanensis*), sex was identified by Oregon State University for individual dolphins by the amplification of the male-specific SRY gene multiplexed with the ZFX gene as a positive control as described by Gilson et al. (1998).

Sex of Blainville's beaked whale (*Mesoplodon densirostris*), Cuvier's beaked whale (*Ziphius cavirostris*), short-finned pilot whales (*Globicephala macrorhynchus*), and the sperm whale (*Physeter macrocephalus*) were determined by morphology in the field and photo-identification; in some 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).

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.

Analysis of CYP1A1 by immunohistochemistry

Blubber biopsy samples were processed then embedded in paraffin and cut at 5 μm using a Microm 330 rotary

Table 2 PCBs measured in blubber biopsies of 31 free-ranging false killer whales from the endangered main Hawaiian Islands insular population

Congeners	Description	Mean/(SD)[a] ng/g[b]	Range: min value; max value (max–min) ng/g[b]	Percent of ΣPCB ng/g[b] (%)
ΣPCBs 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 99, 101, 110, 128, 138, 149, 151, 153, 158, 170, 171, 177, 180, 183, 187, 191, 194, 195, 199, 205, 206, 208, 209	Mono and di-*ortho* substituted PCBs *not* known to be AHR agonists	28,000 (23,000)	2,200; 100,000 (97,800)	93.3
PCB 153 2,2′,4,4′,5.5′-hexachlorobiphenyl	Di-*ortho*, major contributor to ΣPCB in biota	7,000 (6,200)	450; 29,000 (28,550)	23.3
PCB 138 2,2′,3,4,4′,5′-hexachlorobiphenyl	Di-*ortho*, major contributor to ΣPCB in biota	4,600 (4,000)	320; 17,000 (16,680)	15.3
PCB 105 2,3,3′,4,4′-pentachlorobiphenyl	Mono-*ortho* TEF = 0.00003[c]	410 (270)	36; 1,100 (1,064)	1.4
PCB 118 2,3′,4,4′,5-pentachlorobiphenyl	Mono-*ortho* TEF = 0.00003[c]	1,400 (910)	130; 4,000 (3,870)	4.7
PCB 156 2,3,3′,4,4′,5-hexachlorobiphenyl	Mono-*ortho* TEF = 0.00003[c]	120 (80)	4.0; 320 (316)	0.4
TEQ Toxic equivalency quotient	Total calculated dioxin equivalents	0.06 (0.04)	0.0051; 0.16 (0.155)	6.3
ΣPCB	Total PCBs	30,000 (24,000)	2,200; 110,000 (107,800)	

[a] SD is standard deviation of the mean of the sample set

[b] ng/g lipid weight

[c] From Van den Berg et al. (2006)

microtome (Histology Consultation Services, Inc). Blubber sections were mounted on Leica Biosystems 1 mm X-tra® Slides for both hematoxylin and eosin (H&E) and CYP1A1 staining. After slides were deparaffinized with several changes of xylene, then rehydrated with decreasing concentrations of ethanol, and Milli-Q® purified water incubations, slides underwent the immunohistochemical staining procedure outlined by Vector laboratories (Vectastain® ABC-AP kit, rabbit IgG, Cat. No. AK-5001). Primary antibody slides were treated with polyclonal anti-CYP1A1 antibody (Chemicon International, Cat. No. AB1247; diluted 1:4500 in 0.9 % phosphate buffered saline) for two hour-long incubations, each separated by rinse procedures with 0.9 % phosphate buffered saline. Subsequent steps, including staining with biotinylated secondary antibody and ABC-AP reagent, were performed according to the Vectastain® kit protocol. Slides of cetacean tissues known to express CYP1A1 expression (Fertall 2010) were included as positive controls. Serial (or near-serial) cut slides processed without the use anti-CYP1A1 antibody served as negative controls.

CYP1A1 expression has been described in multiple cell types in cetacean blubber, with most occurring in the vasculature. The highest levels of CYP1A1 typically occur in the endothelial cells of the arterial system (Angell et al. 2004; Montie et al. 2008a; Wilson et al. 2007). In this study, CYP1A1 expression was quantified in all vessels observed from the boundaries of 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 blood vessels expressing CYP1A1). This product was normalized per area of blubber biopsy (mm^2) as described by Montie et al. (2008a). Area of tissue was determined through use of ImageJ 1.45 m software (National Institutes of Health, USA). Background staining of negative controls was subtracted from CYP1A1 expression determined for slides stained with primary antibody.

Contaminant analysis of blubber biopsies

Individual and summed 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). The individual PCB congeners measured are listed in Table 2.

Percent lipid was determined in biopsies by thin-layer chromatography with flame ionization detection (Ylitalo et al. 2005). Lipid weight ∑PCB concentrations (ng/g lipid) were determined for each individual, along with Toxic Equivalency Quotients (TEQ) based on the 2005 international mammalian toxic equivalency factors (TEFs) (Van den Berg et al. 2006). TEQ took into account the only AHR agonists measured in this study: the mono-*ortho* PCBs 105, 118, and 156. For PCB 156, $n = 4$ were below the lower limit of quantitation (LOQ). Therefore, we divided the LOQ by two for an estimate of the contaminant concentration for these individuals.

Statistical procedures

Expression of CYP1A1 (score/mm^2) was transformed by the fourth root to meet ANOVA assumptions of data normality and homogeneity of variances. One-way ANOVA was performed for analysis of expression by species, and a Tukey–Kramer post hoc test was used to determine pairwise differences among the groups. Factorial ANOVA was carried out for age and sex, excluding individuals with undetermined sex or probable age estimates. Because there was no interaction between age and sex for the analysis of all samples pooled, one-way ANOVAs were conducted for age and sex individually.

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 meet ANOVA assumptions of 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 weight), TEQ (ng/g wet weight), and TEQ (ng/g lipid weight) (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 aforementioned 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 and the highest CYP1A1 expression score. The other two excluded individuals were HIPc184, an adult male, and HIPc266, a probable subadult female. Both individuals had the highest ∑PCBs, and very low CYP1A1 expression scores. Further explanation for the two analyses with and without individual outliers is given below.

For all ANOVA and linear regression analyses in this study, $\alpha = 0.05$. 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 and transformed similarly to data for analysis (fourth root). Data were analyzed using JMP 9.0.2 Statistical software (©2010 SAS Institute Inc).

Results and discussion

In order to examine the molecular effects related to contaminant exposure in free-ranging odontocete cetaceans, we measured CYP1A1 expression in blubber biopsies from apparently healthy, active individuals. Because integument is an extremely heterogeneous tissue, we analyzed CYP1A1 expression in these samples using an immunohistochemical method that has been used successfully to examine CYP1A1 induction in cetacean integument in several studies (Angell et al. 2004; Hooker et al. 2008; Montie et al. 2008a; Wilson et al. 2007). Since biopsy sizes tend to vary, and because the highest levels of CYP1A1 expression have been found in the deep blubber layer (Foltz 2012; Montie et al. 2008a), CYP1A1 expression was normalized per unit area (mm^2) in order to account for variable depth of biopsy dart penetration (Foltz 2012).

Species-specific differences in CYP1A1 expression

CYP1A1 expression was examined in 10 different species. CYP1A1 was expressed specifically in vessels within the blubber (Fig. 1), and exhibited variability among individuals. Six out of the 10 species had large enough sample sizes ($n > 4$) for statistical analysis of CYP1A1 expression by species. For these species, mean CYP1A1 expression was highest in false killer whales, followed by rough-toothed dolphins, pantropical spotted dolphins, short-finned pilot whales, pygmy killer whales, and melon-headed whales. CYP1A1 expression in false killer whales and rough-toothed dolphins differed significantly from levels of expression in melon-headed whales ($p = 0.009$) (Fig. 2).

Differences in trophic position influence contaminant burdens, and consequently, CYP1A1 expression levels. It is generally established that lipophilic contaminants biomagnify with increasing trophic level (Jarman et al. 1996; Ross et al. 2000). However, feeding habits and trophic levels are less known in Hawaiian odontocetes. The significant differences in CYP1A1 expression between melon-headed whales and false killer whales, and melon-headed

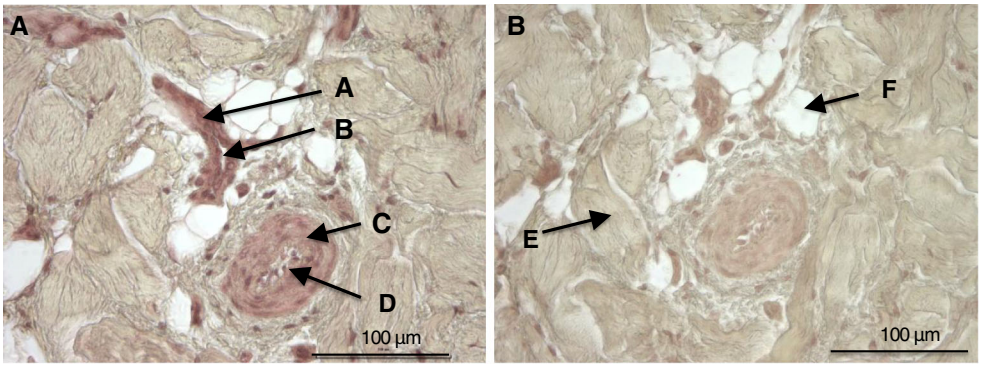

Fig. 1 CYP1A1 expression in blubber biopsies. Representative CYP1A1 expression is demonstrated in serial sections of blubber from an adult false killer whale. **a** Blubber section immunohistochemically stained with anti-CYP1A1 antibody. *Red stain* indicates CYP1A1 expression. *Arrows* indicate specific staining in *A* smooth muscle cells and elastic fibers of the tunica media and *B* endothelium in venule, *C* tunica media and *D* endothelium in arteriole. **b** Negative control staining in serial section with *arrows* indicating *E* connective tissue and *F* adipocyte. Photos were taken at ×400 magnification with a 100 µm *bar* to indicate scale (Color figure online)

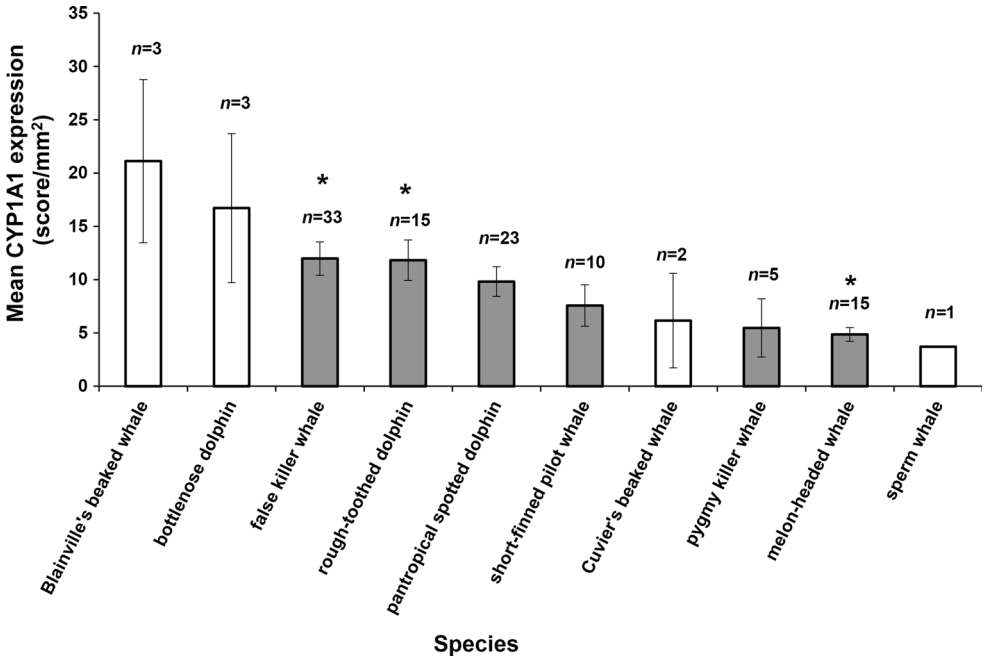

Fig. 2 Average CYP1A1 expression in Hawaiian odontocetes. Quantitative CYP1A1 expression scores were normalized per unit area (mm^2). The sample set analyzed included 110 individuals. The *asterisks* show that rough-toothed dolphins and false killer whales differed significantly from melon-headed whales in the Tukey–Kramer test ($p = 0.009$). Species represented by *white bars* were excluded from the ANOVA and Tukey–Kramer test because of small sample size ($n < 5$). *Error bars* represent standard error

whales and rough-toothed dolphins, may indicate that melon-headed whales feed at a lower trophic level than the other two species. Relatively few stomach contents of melon-headed whales have been analyzed, but these whales 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 Hawai'i 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 fish species such as Sloane's viperfish (*Chauliodus sloani*), black snaggletooth (*Astronesthes indicus*), and short fin pearleye (*Scopelarchus analis*) (K. West, pers. comm.). False killer whales, on the other hand, are known to consume large pelagic fish (Baird et al. 2008a), many of which are also commercially caught. Documented prey species include yellowfin tuna (*Thunnus albacares*), albacore tuna (*Thunnus alalunga*), skipjack tuna (*Katsuwonus pelamis*), broadbill swordfish (*Xiphias gladius*), mahi–mahi (*Coryphaena hippurus*), wahoo (or ono, *Acanthocybium solandri*), and lustrous pomfret (or monchong, *Eumegistus illustrus*) (Baird et al. 2008a). 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), as well as feeding on mahi–mahi (R. W. Baird unpublished). Previous studies on

eastern North Pacific killer whales have examined carbon and nitrogen stable isotope measurements together with contaminant data to help describe the feeding ecology and trophic position of these whales (Herman et al. 2005; Krahn et al. 2007, 2009). Although stable isotope measurements were not conducted in the current study, these measurements could provide information on trophic positions of the different Hawaiian odontocetes.

Species with middle-range CYP1A1 expression included pantropical spotted 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 consume mesopelagic fish such as myctophids and squid species (particularly, *Enoploteuthis* sp. and *Abraliopsis* sp.) (Clarke and Young 1998). Little is known about the diet of short-finned pilot whales in Hawai'i, although several species of squid (*Alloposus mollis* and *Stigmatoteuthis hoylei*) have been recovered near pilot whale groups (R.W. Baird and W.A. Walker, unpublished).

Pygmy killer whales ($n = 5$) expressed low levels of CYP1A1 (Fig. 2), similar to melon-headed whales. Information on the diet of pygmy killer whales is sparse. Because of lack of foraging observations in Hawai'i, it is hypothesized that pygmy killer whales feed at depth or during the night on prey that travel closer to the surface in association with the deep-scattering layer (McSweeney et al. 2009). Similar diets between these two species would explain their similar levels of CYP1A1 expression, however differences in relative tooth size between the two species suggest they may feed on different sized prey. The two Cuvier's beaked whales also showed low CYP1A1 expression and the sperm whale ($n = 1$) in this study had the lowest expression overall. Both of these species feed primarily on squid (Clarke et al. 1993; Evans and Hindell 2004; MacLeod et al. 2003). 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).

CYP1A1 expression in other species groups may also reflect specialized diets. The representation of Blainville's beaked whales in this study is small ($n = 3$), and they were thus left out of statistical comparisons. However, it is worth noting that two of these individuals represented some of the highest CYP1A1 observations in this study. Blainville's beaked whales in Hawai'i are thought to feed primarily on fish and squid species found between 500 and 1,500 meters (Baird et al. 2006). The deep-water species that compose the diet of Blainville's beaked whale may have substantial contaminant levels. Considerable levels of $\sum$PCBs have been found in deep-sea organisms such as cephalopods (de Brito et al. 2002; Ueno et al. 2003). Although these prey species are found in the remote deep-sea environment, studies indicate that the deep-ocean is a sink for hydrophobic organochlorines, such as PCBs (de Brito et al. 2002; Hargrave et al. 1988). Other species with high CYP1A1 expression in this study included common bottlenose dolphins. Although little is known about the diet of common bottlenose dolphins in Hawai'i, individuals have been observed feeding on large (>50 cm) jacks including almaco jack (*Seriola rivoliana*) (R.W. Baird unpublished).

Age class and sex differences in CYP1A1 expression

No interaction was found between age and sex in this study across all samples ($p = 0.765$), so differences in CYP1A1 expression for age class and sex were examined over the entire data set, including false killer whales. No significant differences were found among age class ($p = 0.321$) or sex ($p = 0.787$) across all species. The power to detect significant differences at $\delta = 0.10$ and $\delta = 0.50$ in the sample set of 99 individuals (age class) or 80 individuals (sex) was 1.00 and 1.00 respectively for both analyses, suggesting that these conditions would be adequate to see differences in these groups if they were present.

An age and sex interaction ($p = 0.015$) for CYP1A1 expression occurred within the false killer whale samples, so comparisons were made within these groups. Among males, juveniles had higher average CYP1A1 expression compared to adults ($p = 0.016$) (Fig. 3). 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, $n = 3$). Among adults, no significant differences occurred for sex ($p = 0.381$). When analyzing contaminant levels for false killer whales, no significant interaction was found between age and sex, and no significant differences were found for age and sex across all samples pooled (for detailed information on all false killer whale analyses, including power to detect significant differences, see Supplemental Tables 1 and 2).

Although several studies have reported a lack of CYP1A1 expression trends based on sex (Godard-Codding et al. 2011; Jauniaux et al. 2011; Montie et al. 2008a; Wilson 2003; Wilson et al. 2007, 2010) and age class (Wilson 2003; Wilson et al. 2007, 2010), there are a small number of reports that have observed sex and age-related CYP1A1 expression trends in cetaceans. In particular, Montie et al. (2008a) found that subadult bottlenose dolphins (both sexes combined) had higher CYP1A1 expression in the superficial layer compared to adults, suggesting that adults experience lipid loss (and subsequent contaminant mobilization) within the deep blubber layer as the animal ages, which renders the outermost blubber layer less important metabolically (Koopman et al. 1996). Because

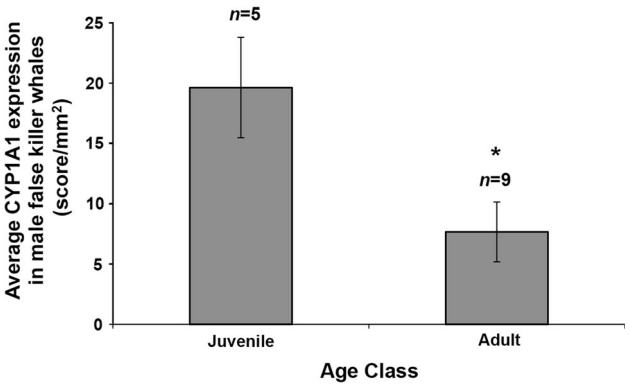

Fig. 3 Average CYP1A1 expression by age class within male false killer whales. The *asterisk* represents significant differences found between the two age classes, $p = 0.016$. The sample set included 14 individuals. *Error bars* represent standard error

the biopsy samples in this study contain mostly the superficial to middle blubber layers, the higher CYP1A1 expression levels that we observed in juveniles compared to adults would be expected. This result may have been more prominent in male odontocetes because of the variability in metabolic phases of adult females that occurs with cycles of birth and lactation.

PCB concentrations and CYP1A1 expression in false killer whales

The false killer whale data set is unique because of the opportunity to integrate CYP1A1 expression with concentrations of PCBs in blubber, as well as genetic and social cluster information from extensive field observations over several years. In addition, the sample size of animals in which CYP1A1 expression ($n = 36$) and PCB levels ($n = 31$) were measured was large enough to examine trends within this species group. A summary of the sample sets, significance values, and power analyses is provided in Supplemental Tables 1 and 2.

Measured $\sum$PCB confirmed and extended previous observations that PCB burdens are high in this population of Hawaiian cetaceans. $\sum$PCBs in 26 of 31 (84 %) of false killer whale biopsies surpassed the 14.7 μg/g lipid health effect threshold proposed by Schwacke et al. (2002) to assess risk of maternal failure in bottlenose dolphins, and 22 of 31 (71 %) surpassed the threshold proposed by Kannan et al. (2000) for thyroid and immune system dysfunction in aquatic mammals. The upper range of 110,000 ng/g lipid compares to levels measured in blubber of a stranded killer whale in Hawai'i (Bachman et al. 2014) and in blubber biopsies of the fish-eating resident killer whale populations in the Pacific Northwest (Krahn et al. 2009; Ross et al. 2000).

Analyses of the relationship between PCB concentrations and CYP1A1 expression 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 sum PCB levels: 310 ng/g wet weight, 2,200 ng/g lipid weight; concentrations 9 and 10 times less than the lower 95 % mean of 2,700 ng/g wet weight and 21,000 ng/g lipid weight respectively. HIPc220 had the highest CYP1A1 expression score, 3 times greater than the upper 95 % mean of 16.70/mm^2. The low contaminant levels and high CYP1A1 expression seen in HIPc220 could be a result of this individual recently giving birth and lactating, resulting in lipid mobilization and offloading of lipophilic contaminants to offspring as seen in other studies of marine mammals (Addison and Brodie 1987, Borrell et al. 1995), leading to CYP1A1 induction as a result of the movement of AHR agonists from deep blubber storage (Montie et al. 2008a). This particular individual was only sighted twice throughout field studies (once in 2005 and once in 2010), without indication of a calf in close proximity (R.W. Baird, unpublished). However, with such a sparse sighting history, it is possible that HIPc220 could have given birth in her recent past. The other two excluded individuals were HIPc184, an adult male, and HIPc266, a female (based on genetics) considered a probable subadult. Both individuals had the highest $\sum$PCBs (respectively, 4 and 2.7 times greater $\sum$PCBs (ng/g wet weight) than the upper 95 % mean of 6,400 ng/g, 1.5 and 2 times greater $\sum$PCBs (ng/g lipid) as the upper 95 % mean of 39,000 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 on liver tissues (Schlezinger and Stegeman 2001; Wilson et al. 2005).

With the exclusion of the three individual outliers, CYP1A1 expression in the blood vessels correlated positively with $\sum$PCBs (ng/g wet weight) measured in the blubber ($R^2 = 0.36$, $p = 0.001$) (Fig. 4a), along with lipid-normalized $\sum$PCBs (ng/g lipid weight) ($R^2 = 0.20$, $p = 0.015$). No significant relationships were found between CYP1A1 expression and TEQ wet weight ($R^2 = 0.09$, $p = 0.226$) (Fig. 4b), and CYP1A1 expression and TEQ lipid weight ($R^2 = 0.04$, $p = 0.430$).

The significant correlation between $\sum$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 in this study. 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 (Van den Berg et al. 2006).

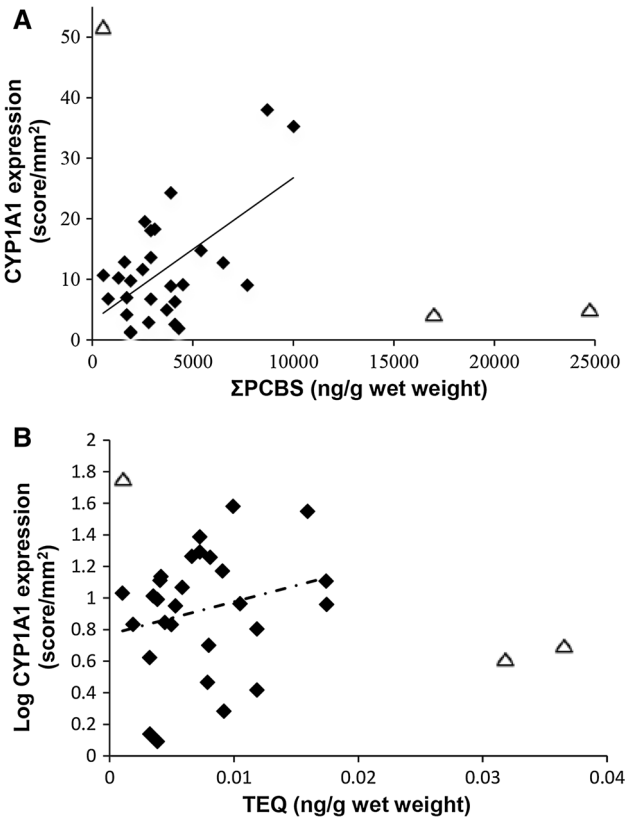

Fig. 4 Relationship between CYP1A1 expression and ∑PCBs or TEQ in false killer whales. The sample set included 28 individuals. Three outliers (*white triangles*) represent individuals excluded from analyses based on unusual patterns of CYP1A1 expression with contaminant burdens. **a** CYP1A1 expression correlated significantly with ∑PCBs (ng/g wet weight) as indicated by the *solid line* ($R^2 = 0.36$, $p = 0.001$). **b** No significance was found between CYP1A1 expression and TEQ (ng/g wet weight) as indicated by the *dashed line* ($R^2 = 0.09$, $p = 0.226$)

Other dioxin-like PCBs (77, 81, 126, 169, 114, 157, 167, 189) usually occur at very low concentrations and are not reliably quantified with this analytical method. Furthermore, mono-*ortho* PCBs are not as strong inducers of CYP1A1 compared to non-*ortho*-substituted PCB congeners, such as PCBs 77, 126 and 169 (Zeiger et al. 2001) that cause biological responses similar to TCDD (Safe 1998). Similar findings were reported by Wilson et al. (2007), in which CYP1A1 expression in the lower dermis correlated weakly with summed 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 overall contaminant burdens have a greater likelihood of being exposed to AHR agonists not measured in this study, such as dioxins, furans, non-*ortho* PCBs, and PAHs.

There is legitimate focus on AHR-dependent mechanisms of toxicity because AHR ligands have hundreds, if not thousands-fold more toxic potency than other non-AHR binding HAHs. Yet less toxic congeners continue to pose a great threat with their persistence and high prevalence. For example, PCB 153 constitutes the highest percentage (22.5 %) of the overall PCB burden in this study, followed by PCB 138 (14.7 %), a very typical observation in marine mammal tissues (Table 2). Mechanistic evidence is becoming available that supports the epidemiological suspicion that the prevalent PCBs are not inert- a recent proteomic experiment reveals that PCB 153 alone can induce endocrine disruption in a cancer cell line as a result of influencing several stress-related signal transduction pathways (Tremoen et al. 2014). Furthermore, a recent review discusses that despite their discontinued industrial use, there is little if any downward trend in overall PCB levels measured in cetaceans since 2008 (Law 2014), confirming that the threat of toxicity from persistent organics is not abating with time.

CYP1A1 and contaminant levels among false killer whale social clusters

Because false killer whale social clusters have different habitat utilization patterns (Baird et al. 2012), we examined whether social clusters influenced CYP1A1 expression. No significant differences in CYP1A1 expression were observed among social clusters 1, 2, and 3 ($p = 0.212$). However, false killer whales from cluster 3 had higher average CYP1A1 expression overall. Contaminant levels did not differ significantly among social clusters: $p = 0.961$ (∑PCBs wet weight), $p = 0.741$ (∑PCBs lipid weight), $p = 0.974$ (TEQ wet weight), and $p = 0.289$ (TEQ lipid weight). Although Cluster 3 had a small sample size ($n = 4$), this social group had higher average ∑PCBs (ng/g lipid weight) and TEQ (ng/g lipid weight) in comparison to Cluster 1 and Cluster 2 animals. As our understanding and available information about these social clusters expands, CYP1A1 expression levels may yet be a tool to reveal diet-driven differences among these groups.

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 suggest that trophic position influences CYP1A1 expression patterns, yet more information is needed in regard to specific habitat use and diet preferences if we are to discern species-specific trends in these high trophic level predators. For false killer whales, a larger sample size and more information on habitat use may reveal distinct patterns of CYP1A1 expression among social clusters. The use of CYP1A1 expression in blubber

biopsies as a biomarker of ∑PCBs burdens is generally effective, but must proceed with caution because of the indication that CYP1A1 expression can be suppressed in the individuals with the highest chemical burdens.

The main Hawaiian Islands insular false killer whale stock is classified as Endangered under the US 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). This study presented further evidence for contaminant-related health impacts on this population by confirming and extending observations that PCB burdens exceed proposed threshold levels for health effects, and by showing that CYP1A1 expression levels correlated with ∑PCBs, and were higher relative to other cetacean species. Future analysis of CYP1A1 in blubber biopsies is encouraged in order to continue monitoring the health of Hawaiian odontocetes, particularly for the endangered insular false killer whale stock.

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Conflict of interest The authors declare that they have no conflict of interest.

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