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Research Article

Intestinal Polycyclic Aromatic Hydrocarbon-DNA Adducts in a Population of Beluga Whales With High Levels of Gastrointestinal Cancers

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Carcinogenic polycyclic aromatic hydrocarbons (PAHs) were disposed directly into the Saguenay River of the St. Lawrence Estuary (SLE) by local aluminum smelters (Quebec, Canada) for 50 years (1926–1976). PAHs in the river sediments are likely etiologically related to gastrointestinal epithelial cancers observed in 7% of 156 mature (>19-year old) adult beluga found dead along the shorelines. Because DNA adduct formation provides a critical link between exposure and cancer induction, and because PAH–DNA adducts are chemically stable, we hypothesized that SLE beluga intestine would contain PAH–DNA adducts. Using an antiserum specific for DNA modified with several carcinogenic PAHs, we stained sections of paraffin-embedded intestine from 51 SLE beluga (0–63 years), 4 Cook Inlet (CI) Alaska beluga (0–26 years), and 20 beluga (0–46 years) living in Arctic areas (Eastern Beaufort Sea, Eastern Chukchi Sea, Point Lay Alaska) and aquaria, all with low PAH contamination. Stained sections showed nuclear light-to-dark pink color indicating the presence of PAH–DNA adducts concentrated in intestinal crypt epithelial lining cells. Scoring of whole tissue sections revealed higher

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values for the 51 SLE beluga, compared with the 20 Arctic and aquarium beluga ($P = 0.003$). The H-scoring system, applied to coded individual photomicrographs, confirmed that SLE beluga and CI beluga had levels of intestinal PAH–DNA adducts significantly higher than Arctic and aquarium beluga ($P = 0.003$ and 0.02, respectively). Furthermore, high levels of intestinal PAH–DNA adducts in four SLE beluga with gastrointestinal cancers, considered as a group, support a link of causality between PAH exposure and intestinal cancer in SLE beluga. Environ. Mol. Mutagen. 60:29–41, 2019.

Key words: cancer; cetaceans; chemical carcinogens; *Delphinapterus leucas*; PAHs; pollutants

INTRODUCTION

Like humans, whales are long-lived mammals feeding near the top of the food chain, while living in an environment where land and airborne carcinogenic contaminants are often released in large amounts. In this study, we present a unique situation in which a small whale population shares, with people living in the same area, a high rate of cancer epidemiologically associated with extensive exposure to polycyclic aromatic hydrocarbons (PAHs).

Cancer is cause of death for 20% of mature (>19 year old) adult St. Lawrence Estuary (SLE) beluga. This is an order of magnitude higher than in cetacean populations elsewhere in the wild (0.7%–2%). Over half of all cancer cases reported in cetaceans worldwide have been observed in SLE beluga, and no cancer has been reported in other free-ranging beluga populations [Martineau et al., 2002a; Lair et al., 2016; Newman and Smith, 2006]. Adenocarcinoma of the gastrointestinal tract is the most frequent cancer observed in SLE beluga (11 cases or 7% of mature adults examined). In contrast, only three cases of gastrointestinal epithelial cancers have been reported in other populations of cetaceans worldwide, and two of these were gastric adenocarcinomas in harbor porpoises, both from the North Sea [Breuer et al.,1989; Parsons and Jefferson, 2000; Siebert et al., 2010]. Mammary cancer, which has not been reported elsewhere in cetaceans, is second to gastrointestinal tract cancer in SLE beluga, affecting 8 (or 9.9%) of mature adult females examined [Lair et al., 2016]. The types of cancer affecting other free-ranging populations of cetaceans are also different from those seen in SLE beluga. Out of a total of 30 cancers listed, among cancers affecting free-ranging cetaceans worldwide excluding beluga, there were 13 epithelial cancers (or 43%) from various organs, of which only one was found in the gastrointestinal tract, and 12 (or 40%) lymphoid cancers (lymphoma) [Martineau et al., 2002a].

PAHs are a vast family of compounds, some of which have been studied for more than 100 years [Potter, 1963; Hecht, 2003], and a few hundred of these are classified as chemical carcinogens. PAH carcinogenesis is caused by DNA damage resulting from the activity of highly reactive metabolites generated by cytochrome P450 enzymes [Diggs et al., 2011], often in combination with other xenobiotic-metabolizing enzymes. Most environmental PAHs are pyrogenic, derived from incomplete combustion of organic compounds, and produced by industrial processes, which can include metallurgy, motor vehicle use, tobacco smoking, and cooking. In humans, carcinogenic PAHs are implicated in the etiology of lung and colorectal cancers, both of which are major causes of mortality in the Western world [Hecht, 2003; Diggs et al., 2011], as well as skin cancer [Kim et al., 2013]. The major routes of human PAH exposure are diet and inhalation [Dipple, 1985], and dietary intake has been associated with epithelial cancers of the digestive tract in both humans and experimental animals [Culp et al., 1998; Sinha et al., 1999; Poirier, 2004; Diggs et al., 2011]. Furthermore, the presence of PAHs in estuary sediments has been implicated in the etiology of cancer in bottom dwelling fish [Baumann, 1998; Beyer et al., 2010].

During the past century, pyrogenic PAHs were produced persistently by primary aluminum smelters, particularly those using the now antiquated Söderberg process. Smelters located along the Saguenay River in Quebec (Canada) severely contaminated the Saguenay River sediments and ecosystems through smoke fall out, and/or dumping of liquid effluents from chimney scrubbers. From 1926 to 1976 no attempt was made to mitigate the PAH waste [Martel et al., 1986; Smith and Levy, 1990]. Aluminum workers in Saguenay smelters experienced high rates of urinary bladder and lung cancer, which are considered due to occupational PAH exposures. Based on epidemiological data alone, long-term workers affected by urinary bladder or lung cancer were financially compensated by the Quebec worker safety board [Armstrong et al., 1988, 1994; Armstrong and Thériault, 1996; Gibbs et al., 2014]. Because the general population living in the Saguenay area has a rate of digestive system cancers higher than the general population of Quebec province [Lebel, 1998], it has been hypothesized that smelter-generated PAH emissions released into the atmosphere have contributed etiologically to the local cancers of the digestive system by contaminating local surface drinking water [Martineau et al., 2002a,b].

Beluga (*Delphinapterus leucas*) are medium-sized toothed whales which have some life history traits, including lifespan, similar to those of humans. A small isolated population of approximately 900 beluga inhabits a stretch of the SLE roughly centered on the mouth of the Saguenay River. The diet of these whales includes invertebrates (benthic annelids), which live in the river sediment and contain high levels of

PAHs [Pelletier et al., 2009; Martineau, 2012]. Cancers are rare in wildlife, especially in cetaceans (<0.7–2%) [Martineau et al., 2002a; McAloose and Newton, 2009]. However, among the beluga found stranded (Fig. 1) and dead along the shores of the SLE in the 1980s [Martineau et al., 2002a; Martineau, 2012; Lair et al., 2016], there were 11 cases of gastrointestinal adenocarcinoma, which represent 7% of the mature adult beluga systemically examined (n = 156). PAH exposures were therefore suspected to be involved in the etiology of the beluga gastrointestinal cancers, but a direct link between exposure and disease was lacking.

A great deal of published evidence links PAH exposures, PAH–DNA adducts, and cancer risk in experimental animal models and humans [Culp and Beland, 1994; Culp et al., 2000; Poirier, 2004; Gunter et al., 2007; WHO, 2012]. Stranded harbour porpoises in the UK were recently found to have bulky DNA adducts in liver [Acevedo-Whitehouse et al., 2018]. DNA adducts are necessary but not sufficient for a tumor to form, and tumorigenesis is organ-specific, often requiring additional factors such as inflammation and cell proliferation, for mutation fixation in critical genes. Formation of DNA adducts indicates that exposure to a carcinogen has occurred, but in experimental models, and particularly in human cohorts, many individuals form DNA adducts but do not get cancer. There is good evidence in humans that individuals with the highest DNA adduct levels have the highest cancer risk [Poirier, 2012]. However, the relationship between smoking, cigarette-induced DNA damage, and lung cancer, which shows that only about 15% of lifetime smokers develop lung cancer [Koop and Luoto, 1982; Peto et al., 2000], indicates that interindividual variability in metabolic enzymes, DNA repair, and other factors also contribute to cancer risk. Whereas, PAH–DNA adducts generally correlate with the number of cigarettes smoked daily for the members of a whole cohort, the correlation with lung cancer for a particular individual is not absolute [Wiencke, 2002].

In this study, we have evaluated PAH–DNA adduct formation in beluga intestines, comparing whales living in areas with low or no PAH contamination, and those living in the PAH-contaminated SLE. We found PAH–DNA damage in SLE beluga intestines that was significantly higher than the damage found in intestines of beluga inhabiting the low-PAH contamination areas. Moreover, the damage was restricted to epithelial cells located within intestinal crypts that contain stem cells, which are believed to be the origin of epithelial intestinal cancers. The data support the conclusion that environmental PAH contamination leading to PAH–DNA adduct formation plays an important role in the etiology of SLE beluga gastrointestinal cancer.

MATERIALS AND METHODS

Positive Controls and Whale Fibroblast Standard Curve

In order to evaluate the consistency of staining for each new batch of samples, we stained sections from paraffin blocks of small intestine from mice fed benzo[*a*]pyrene (BP) in the diet for 2 years, where the forestomach tumor incidence was 100% [Culp et al., 1998]. Intestinal tissues positive for PAH–DNA from two of the SLE beluga were also very useful as consistent positive controls. In addition, a standard curve was prepared from cultured whale fibroblasts.

Skin fibroblasts were isolated from a tissue biopsy obtained from a free-ranging sperm whale off the coast of North Carolina [Wise et al., 2011; Hernandez-Ramon et al., 2013]. Cells were cultured in a 50:50 mixture of Dulbecco's minimal essential medium and Ham's F-12 medium (DMEM/F-12), supplemented with 15% Cosmic calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM sodium pyruvate. Cells were maintained as adherent subconfluent monolayers, fed at least twice a week, subcultured approximately once a week, and tested routinely for *Mycoplasma* spp. Experiments were conducted on logarithmically growing cells. DMEM/F-12 was purchased from Mediatech Inc. (Herndon, VA). Cosmic calf serum was purchased from Hyclone (Logan, UT). Sodium pyruvate, penicillin/streptomycin, trypsin/EDTA, and Glutamax were purchased from Invitrogen Corporation (Grand Island, NY). Tissue culture dishes, flasks, and plastic-ware were purchased from Corning Inc. (Acton, MA).

For preparation of a standard curve, sperm whale skin fibroblasts were seeded into ten 150-mm tissue culture dishes at 2.2 million cells per dish. Cells were given 48 h to attach and enter a normal growth cycle before exposure to 0, 0.05, 0.15, and 0.30 μM *r*7,*t*8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydro-benzo[*a*]pyrene (BPDE) for 1 h. Dishes were rinsed thoroughly with phosphate-buffered saline (PBS), scraped in 1–2 mL PBS, and collected for analysis. Cells from seven 150-mm dishes were combined, collected by centrifugation, and stored in formalin for 48 h, before being transferred into 70% ethanol. Subsequently, paraffin blocks were made from pelleted cells and these were used for immunohistochemical (IHC) staining as described above. Cells from three 150-mm dishes were combined, collected by centrifugation, and stored frozen at −80°C until DNA was extracted and the BPdG adduct quantified by BPDE–DNA chemiluminescence immunoassay (BPDE–DNA CIA).

DNA was extracted using DNeasy Blood Maxi kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The same BPDE–DNA antiserum was used for *r*7,*t*8,*t*9-trihydroxy-*c*-10-(N^{2-}deoxyguanosyl)-7,8,9,10-tetrahydro-benzo[*a*]pyrene (BPdG) quantification by BPDE–DNA CIA [Divi et al., 2002] as was used for IHC (described above). In brief, the assay was performed in opaque 96-well high-binding plates (Greiner Bio-one, Longwood, FL) coated with 100 pg of sonicated BPDE–DNA (modified to 0.33%), or calf thymus DNA, in 100 μL of Reacti-Bind DNA coating solution (Pierce, Thermo Fisher Scientific, Pittsburgh, PA). For BPDE–DNA CIA analysis, plates were washed with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBST) and 0.02% sodium azide, and blocked with casein (0.25%; Applied Biosystems, Bedford, MA) for 90 min to reduce non-specific binding. Sample DNA (1.0–2.5 μg/well, and standard BPDE–DNA were mixed with an equal volume of PAH–DNA antiserum (rabbit # 31, bleed 08/16/78) diluted 1:3,000,000 in PBST containing casein (0.25%). Standard BPDE–DNA, modified to 1.0 BPdG/10^6 nucleotides, was serially diluted in calf-thymus DNA so that each well contained an equal quantity of DNA but varying amounts of BPdG adduct (0–8.0 fmol/well). After adding samples and standards to wells, plates were incubated for 90 min, washed with PBST, and incubated with biotinylated anti-rabbit antibody (1:2,500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min. After washing, the plates were incubated with streptavidin alkaline phosphatase (15,000; Avidix-AP, Applied Biosystems) for 60 min. Plates were washed sequentially with PBST, distilled water, and Tris buffer (20 mM Tris and 1 mM $MgCl_2$, pH 9.5) before adding CDP Star with Emerald II solution (Applied Biosystems). After an overnight incubation at 37 °C, the plates were brought to room temperature and luminescence was read using a Tropix 717 Microplate Luminometer (Applied Biosystems). For each assay, sample DNA was incubated in three experimental wells and one control well. The Standard Curve 50% Inhibition was at 0.343 ± 0.046 fmol (mean ± SE, n = 18). The lower limit of detection varied based on the amount of DNA and the standard curve but was in the range of 2–5 adducts/10^8 nucleotides.

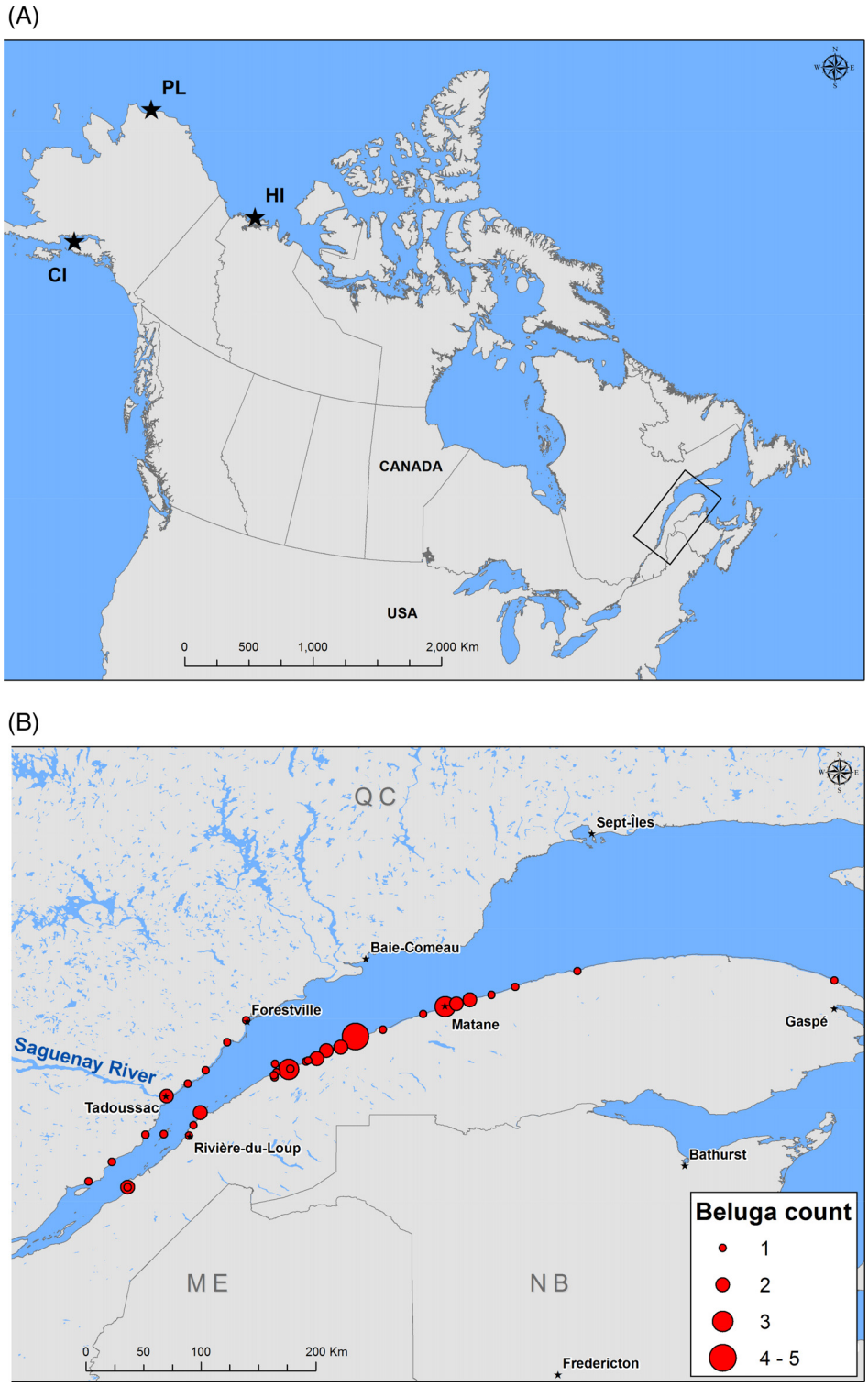

Fig. 1. Maps showing the geographic locations of the beluga sampled in this study. (**A**) Map of North America. Beluga from Eastern Beaufort Sea (n = 5) were collected on Hendrickson Island (HI). Beluga from Alaska were collected in the Eastern Chukchi Sea at Point Lay (PL) (n = 3). CI (n = 4). Boxed area (shown in B) includes the Saint Lawrence Estuary (SLE) where dead SLE beluga (n = 51) were found stranded. (**B**) SLE and Saguenay river with locations of SLE beluga strandings.

Sources of Beluga Populations

Paraffin blocks were obtained from beluga aged 0–63 years inhabiting various areas, with appropriate CITES and National Marine Fisheries Service (NMWF) permissions. Only tissue sections with minimal autolysis were considered for analysis. Histological criteria for minimal autolysis included absence of pyknotic nuclei, and presence of at least one uninterrupted row of ≥4 contiguous epithelial cells present in a single intestinal crypt.

There were 20 beluga from North American aquaria and the Arctic regions (Fig. 1). The aquaria included the Shedd Aquarium (Chicago IL, n = 3), SeaWorld (San Diego, CA, n = 7), the Vancouver Aquarium (Vancouver, BC, Canada, n = 1), and Marineland (Niagara Falls, ON, Canada, n = 1). The Arctic regions were the Eastern Beaufort Sea (Hendrickson Island) in Canada (n = 5) and Point Lay in Alaska (n = 3) (Fig. 1A). From the two Arctic regions, native hunters kindly provided tissues from subsistence harvested whales. The aquarium beluga tissues were from animals dying of natural causes or euthanized due to disease. Because the aquaria and the Eastern Beaufort Sea and Point Lay Arctic regions have low PAH contamination levels [Chen et al., 2018; Stimmelmayr et al., 2018], they have been grouped together as Arctic and aquarium (A&A).

There were 51 beluga from the PAH-contaminated SLE (Fig. 1B), and from Cook Inlet (CI) (Fig. 1A) in Alaska there were 4 beluga. Because there was substantial documentation of PAH contamination in CI [Hite and Stone, 2013], and the CI PAH–DNA staining levels were similar to the SLE beluga, we analyzed the CI whales as a separate group. The SLE beluga and the CI beluga were subjected to complete post mortem examination after being found dead, stranded on various shorelines.

IHC Localization of PAH–DNA Adducts

Sections (5-μm thick) were cut from paraffin blocks of whale tissue, and stained using the Ventana (Benchmark XT, Roche Diagnostics Corp., Indianapolis, IN) with rabbit BPDE–DNA antiserum diluted 1:18,000 [Poirier et al., 1980]. This antiserum was elicited against DNA modified with the major adduct of BP, the BPdG, and was shown to cross-react with at least eight carcinogenic PAHs bound to DNA, with no specificity for DNA alone or individual PAH–DNA adducts hydrolyzed from DNA [Pratt et al., 2011]. Anti-rabbit Fast Red-conjugated secondary antiserum was used to reveal pink-colored nuclei containing DNA damage caused by covalent DNA binding of carcinogenic PAHs [van Gijssel et al., 2002; Pratt et al., 2011]. Paraffin blocks were cut in pairs, so that one section was stained with the specific BPDE–DNA antiserum, and an adjacent section was stained with BPDE–DNA antiserum absorbed with the immunogen BPDE–DNA. The absorbed serum section served as a negative control, demonstrating the specificity of the PAH–DNA signal. Subsequently, the section stained with absorbed serum was stained with hematoxylin to reveal the location and number of nuclei.

Sections from all blocks were stained at least once, but for each whale, sections from at least one block were stained on two or more separate occasions, to verify reproducibility. Two whale blocks that showed very strong positive staining were repeatedly stained to serve as positive internal controls for the consistency of the staining. Additional positive controls included cultured whale fibroblasts exposed to the activated form of BP, the BPDE, and intestines from mice fed BP in the diet for 2 years [Culp et al., 1998]. The generation of these positive controls will be described elsewhere.

Slides stained with specific and absorbed BPDE–DNA antiserum were first scanned into the Aperio system at ×20 magnification (Aperio ScanScope AT2 digital slide scanner, Leica Biosystems, Inc., Buffalo Grove, IL) to create whole-slide image data files at 0.5 μm/pixel resolution. Image files were stored as ".svs" files in an eSlide Manager Image Management System, and viewed using Aperio ImageScope software (Leica Biosystems, Inc., Buffalo Grove, IL). By Aperio it was possible to identify the same region in each image, allowing simultaneous comparison of staining of a single region with specific antiserum, absorbed antiserum, and hematoxylin.

PAH–DNA IHC: Whole Tissue Evaluation

This approach involved scoring of each complete tissue section visualized by Aperio. Because whole tissue images were examined, it was not possible to perform this scoring in a blinded fashion. Each tissue from a single block was examined thoroughly and scored either 0, 2, or 4, where 0 indicated no nuclear pink color (no PAH–DNA adducts), 2 indicated a sample that had positive pink nuclei, and 4 indicated a sample that had very strongly positive, dark pink nuclei. Additional scoring criteria were as follows: most blocks were sectioned several times but some blocks were cut only once; some sections from a single block were stained on ≥2 occasions to verify reproducibility; scoring values for sections stained on separate occasions were averaged; the final scoring was based on a single block from each whale, the one that had the best combination of good morphology and high score.

PAH–DNA IHC: H-Scoring

H-scoring is a widely accepted semi-quantitative scoring system where staining intensity and percentage of positive cells are combined, and where each staining intensity is weighted: [1 × (% cells with weak staining) + 2 × (% cells with moderate staining) + 3 × (% cells with dense staining)]. Total possible scores range from 0 to 300 [Kinsel et al., 1989].

In this study, analysis by H-score was performed on representative photographs (×20 magnification) of coded samples where only the area visible in the photos was used for the analysis. The coded photos were scored by one of the authors (DM) who did not have access to the whale identity. Each photograph contained images stained with specific antiserum, absorbed antiserum, and hematoxylin, showing the same region in two adjacent sections, one stained with absorbed serum and hematoxylin and one stained with specific antiserum. Nuclei of crypt-lining epithelial cells, which stained positive for PAH–DNA adducts, were counted visually. The regions chosen for analysis were selected from representative areas with the best preservation of tissue architecture and with the highest concentration of positive nuclei. The total number of nuclei counted per photograph ranged between 100 and 400.

For each photograph, positive nuclei were counted visually, and individual nuclei were identified in each photograph representing a different stain. Adjacent sections were stained with the specific PAH–DNA serum and the absorbed serum. Subsequent to the Aperio scanning of the absorbed serum section, it was stained with hematoxylin and scanned again. Therefore, it was often possible to identify the same nucleus in photographs of all three adjacent sections. Individual nuclei in the photo showing specific PAH–DNA staining were considered positive when more than 50% of the nuclear area showed a uniform, pink to red staining, and when the corresponding nuclei stained with absorbed serum showed substantially less or no staining. A punctiform pattern of small pink dots, likely associated with bacterial contamination, was not considered valid PAH–DNA staining because each dot was much smaller than a nucleus, and because it could be seen on tissue stained with absorbed serum.

Nuclei were subdivided into three groups (Groups 1, 2, and 3) based on nuclear staining intensity. Group 1 nuclei were pale pink (weak staining), Group 2 nuclei were darker pink (moderate staining), and Group 3 nuclei had prominent, dense, dark pink to red staining (dense staining).

Statistical Analyses

Comparison of SLE and A&A beluga populations for whole tissue scoring relied on the Mantel–Haenszel test stratified by age (≤30 years, n = 33, and > 30 years, n = 38). Comparison of CI and A&A beluga populations was restricted to individuals younger than 30 years old and relied on the exact chi-square test.

For H-scoring, a linear model with population of origin (SLE vs. A&A) as a fixed factor, and age as a co-factor, was used, taking into

account the unequal variances in the two populations. The model also included the interaction between population of origin and age, but this was dropped in the final model because it was not statistically significant. The same type of model was used to compare the CI and A&A populations for the H-score. To compare levels of PAH–DNA damage determined by H-score in adult SLE beluga (8–63 years) with gastrointestinal cancers (n = 4), and other adult SLE beluga (n = 31) with either no cancer (n = 25) or with cancer other than gastrointestinal tract cancer (n = 6), a linear model was used with group (SLE adult beluga gastrointestinal tract cancer vs. all other SLE adult beluga) as a fixed factor and age as a co-factor. The level of statistical significance was set at $P \leq 0.05$ throughout.

RESULTS

Whale Fibroblast Standard Curve

In order to validate the PAH–DNA IHC staining of whale tissue, for which there was no previous literature, we prepared a standard curve of whale fibroblasts exposed to different concentrations of BPDE, and assayed for BPdG adduct both by analysis of extracted DNA using BPDE–DNA CIA, an ELISA, and by IHC. Whale fibroblasts were exposed to 0, 0.05, 0.15, and 0.30 μM BPDE, with the expectation that the major adduct would be BPdG, and the DNA adduct staining is shown in Figure 2A–D (respectively). Simultaneously-exposed whale cells were used for both extraction of DNA and formalin-fixed paraffin embedding. The paraffin bocks were stained multiple times, simultaneously with the whale sample. Figure 2E shows the DNA adduct values determined by CIA (o) and the IHC values determined by H-scoring (●), with a strong concordance between the two when presented as a function of BPDE dose. This provides proof-of-principle for the validity of the IHC staining in whale intestine.

Beluga Populations and PAH–DNA IHC

Visualization of pink-colored nuclei in the beluga intestinal crypt lining cells stained with antiserum elicited against DNA modified with BPDE was considered as a proof for the presence of carcinogenic PAH–DNA adduct formation when an adjacent section stained with immunogen-absorbed BPDE–DNA antiserum had little or no pink colored nuclei. The BPDE–DNA antiserum is specific for a family of carcinogenic PAHs bound to DNA, with antiserum specificity previously published [Pratt et al., 2011]. Since non-carcinogenic PAHs do not bind to DNA, immunohistochemical staining with this antiserum is considered to identify members of a family of carcinogenic PAHs bound to DNA.

Whole tissue sections were stained with specific or absorbed BPDE–DNA antiserum, and hematoxylin (Fig. 3A–C), to localize PAH–DNA adducts and nuclei, respectively. Not only did we find PAH–DNA damage in nuclei of beluga intestinal epithelium, but there was clear evidence that the majority of the damage was concentrated in the crypt lining epithelial cells.

PAH–DNA IHC: Whole Tissue Evaluation

In order to approximate the intensity of nuclear pink color staining, indicating PAH–DNA adduct level, we first evaluated each whole tissue section visually in the Aperio software. Scoring of the nuclear pink color intensity was based on areas having moderate or good morphology, and reflected an impression of such areas throughout the tissue section. The color was graded as either non-existent (0), moderate (2), or intense (4) (Fig. 4A–C, respectively, and Table I). This approach allowed us to evaluate the overall condition of the tissue in that section and to choose a representative area for preparation of the coded photographs used for H-scoring (see below).

A comparison of populations of beluga stratified by age was performed (Table I). Overall, beluga in the SLE population (n = 51) had significantly higher scores (P = 0.003) than those in the A&A population (n = 20) (Table I). Similar to the SLE population, the CI beluga (n = 4 whales all <30 years old) had high PAH–DNA adduct staining and were compared separately to the <30 years old A&A beluga (n = 15). While all four (100%) of the CI beluga showed a score of 2, only 13% of the A&A beluga had a similar score, a significant difference (P = 0.004, Table I).

PAH–DNA IHC: H-Score

Unlike the analysis of whole tissue sections, the blind analysis by H-score was performed on photographs where only the area visible in the pictures was analyzed, and coded photos were scored by one of the authors (DM). The H-score analysis of whales of all ages, which compared the SLE (n = 51, 0–63 years) and A&A populations (n = 20, 0–46 years), showed that there was significantly more PAH–DNA damage in the SLE population than in the A&A population, controlling for age (P = 0.03) (Fig. 5A). Furthermore, in both the SLE and the A&A groups there was a significant increase in PAH–DNA damage with age (P = 0.003). Even though the SLE PAH–DNA values were higher at all ages, the rate of increase was not different in the two populations as indicated by the non-significant interaction between population of origin and age (P = 0.36) (Fig. 5A). When the PAH–DNA damage levels in the CI beluga and the A&A beluga were compared, the mean H-score was significantly higher in the CI beluga (P = 0.02), controlling for the non-significant effect of age (P = 0.19) (Fig. 5A). A single captive 36-year-old aquarium beluga showed high levels of PAH–DNA damage, the possible causes of which are discussed below.

A further analysis by H-score evaluated PAH–DNA adducts in adult (8–63 years) SLE beluga (n = 35), and compared SLE beluga with gastrointestinal cancer (n = 4, ●), to SLE beluga without gastrointestinal cancer (n = 31, o), six of which had other types of cancer (Fig. 5B). There

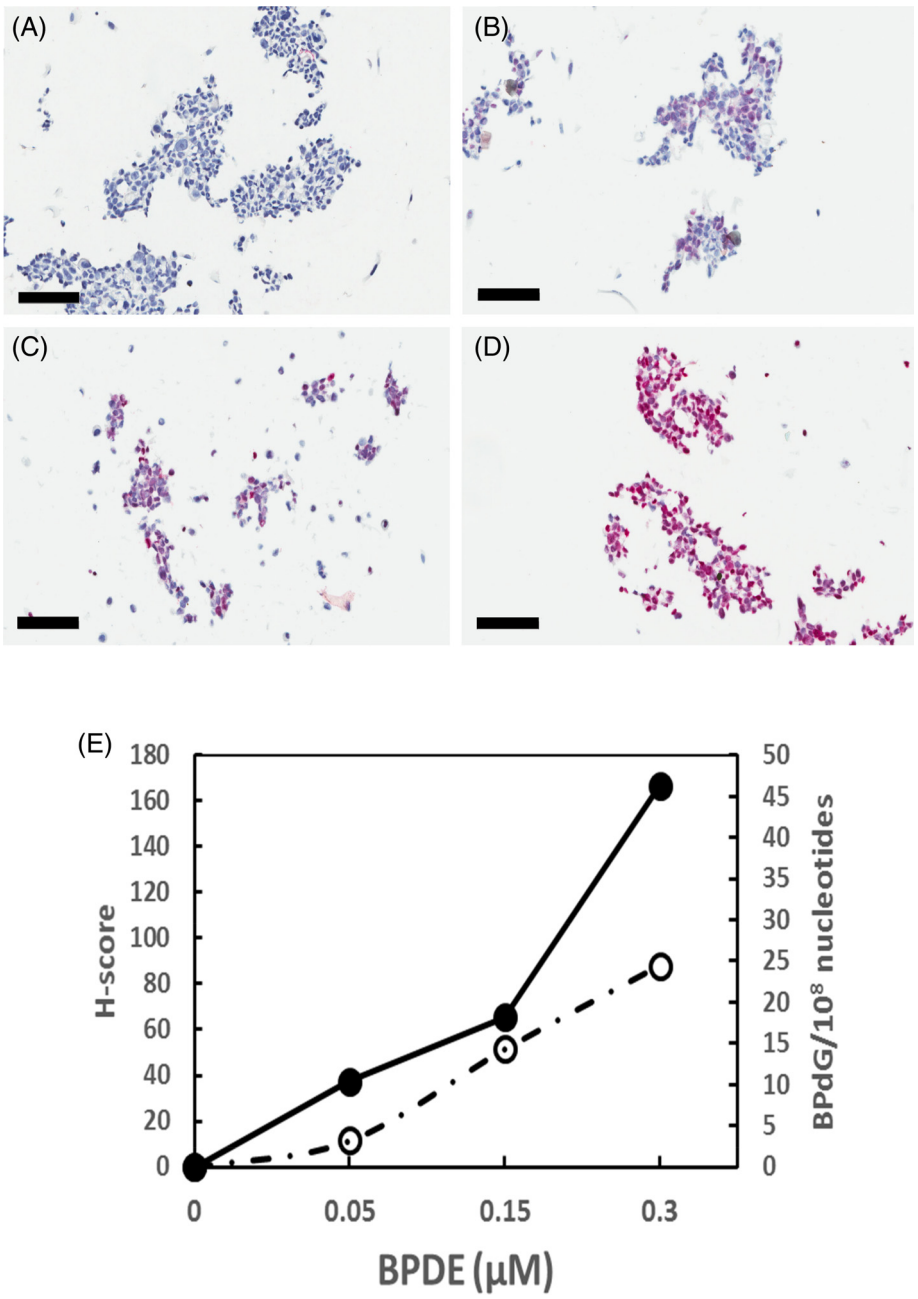

Fig. 2. Whale fibroblast standard curve photomicrographs (Bar = 100 μM) showing IHC for cells exposed to 0, 0.05, 0.15, and 0.30 μM BPDE and stained with anti-BPDE–DNA antiserum (pink nuclei) and hematoxylin (**A–D**, respectively). (**E**) Number of BPdG adducts/10^8 nucleotides determined by BPDE-DNA CIA in extracted DNA (o), and H-scoring for the cells shown in A–D (●), presented as a function of BPDE dose.

was a significantly higher (P = 0.006) level of PAH–DNA in the four adult SLE whales with gastrointestinal cancer, compared to the 31 adult SLE beluga without gastrointestinal cancer, controlling for age. In addition, in the gastrointestinal cancer group (n = 4), H-scores increased significantly with age (P = 0.0002), but in the other group (n = 31) there was no significant increase with age (P = 0.70).

DISCUSSION

Considered together, the following observations further support an etiological role for exposure to PAHs in the high rate of gastrointestinal cancers found in the SLE beluga [Martineau et al., 2002a]. First, this study comprised 51 SLE beluga, four of which had gastrointestinal cancers. As a group, these four whales had significantly higher

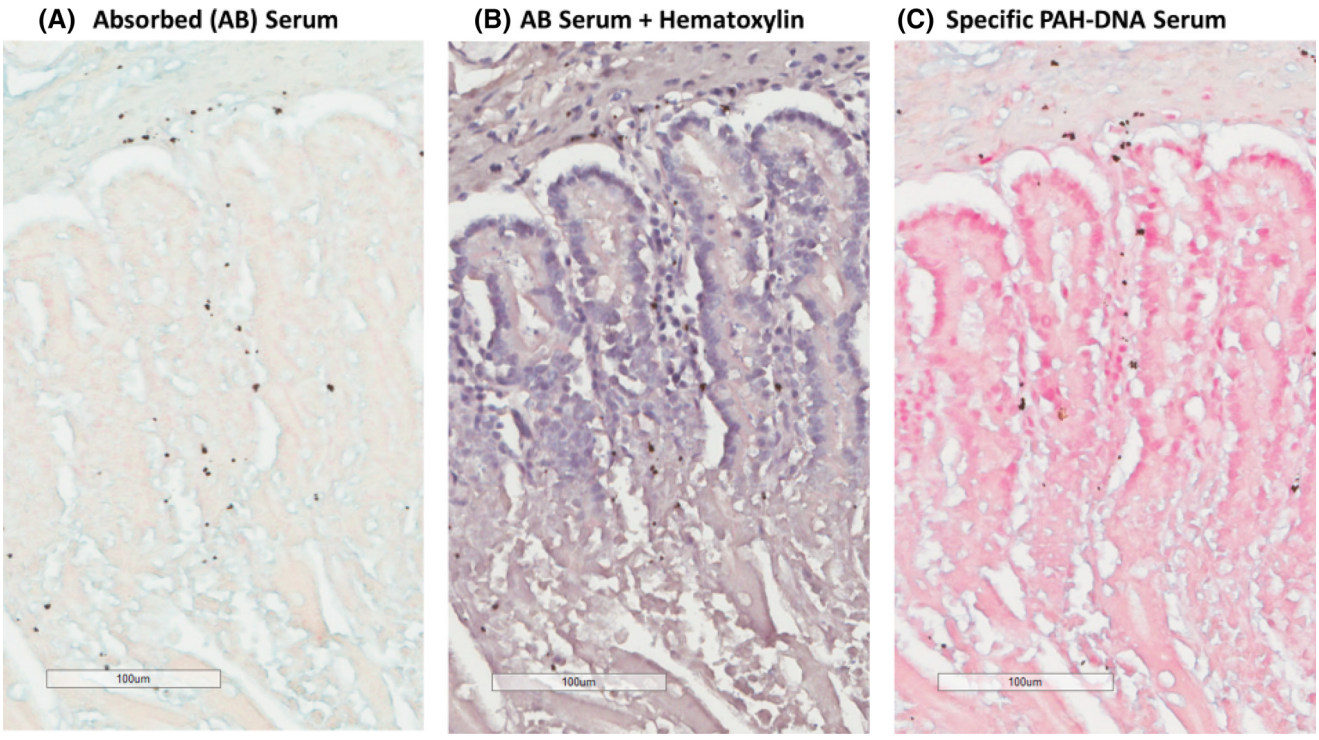

Fig. 3. Staining of SLE beluga small intestinal crypts for PAH–DNA adducts shows: (**A**) no nuclear staining with absorbed antiserum. (**B**) Crypt lining nuclei stained blue with hematoxylin in same section as (A); (**C**) nuclei of crypt lining cells stained pink with primary specific anti-BPDE–DNA serum, recognizing DNA adducts of carcinogenic PAHs, in a section adjacent to (A). Hematoxylin staining reveals that the PAH–DNA staining is nuclear.

levels of intestinal PAH–DNA adducts (P = 0.006) compared to SLE beluga with no cancer or with other types of cancers. One of the four beluga had a lower level of PAH–DNA damage than the others. This was expected since interindividual variability in PAH–DNA damage is common, the damage level at death may not reflect the maximum damage level during years of exposure, and PAH–DNA damage in an individual is necessary but not sufficient for a cancer to occur [Wiencke, 2002]. Second, the A&A beluga, coming from areas with low contamination, showed significantly lower levels of intestinal PAH–DNA, and no evidence of gastrointestinal cancer, compared to the SLE beluga. Third, in the whale intestine, PAH–DNA adduct localization concentrated in epithelial cells lining the intestinal crypts, cells which, in the mouse, are known to metabolize xenobiotics [Brooks et al., 1999]. Further, stem cells, which are part of the normal intestinal crypt cell population, are thought to be the cells from which intestinal carcinomas arise [Barker et al., 2009]. Finally, in mice, a single oral administration of BP has been shown to induce mutations in the small intestinal crypt stem cells [Brooks et al., 1999]. Thus, the presence of PAH–DNA adducts in SLE beluga small intestinal crypt epithelial cells provides an essential link between documented PAH exposures and gastrointestinal cancers in SLE beluga. To our knowledge this is the first report of a validated biomarker, localized in the target tissue for tumor induction, supporting an etiological relationship between chemical carcinogen exposure in free ranging marine mammals and elevated cancer risk.

Overwhelming evidence supports the carcinogenicity of various PAHs in humans and experimental animal models [Hecht, 2003; WHO, 2012]. For instance, long-term ingestion of PAH-rich coal tar causes high rates of carcinomas in small intestine and forestomach in mice [Goldstein, 2001; Poirier, 2004]. DNA adduct levels found in forestomach of mice fed BP for 28 days correlated well with the high incidence of forestomach tumors (100%) in mice fed BP for 2 years [Culp and Beland, 1994]. Extrapolation to humans is reasonable because the metabolic capacity of most mammals for metabolism of carcinogenic PAHs is similar to that of humans [Fitzgerald et al., 2004; WHO, 2012]. Furthermore, mutational patterns and mutation sites caused by PAH exposure in in vitro models are similar to those observed in human lung cancers associated with smoking [Pfeifer et al., 2002]. Indeed, an epidemiologic evaluation of levels of PAH–DNA adducts in white blood cell DNA of US Army Officers showed that individuals with the highest PAH–DNA adduct levels had the highest habitual consumption of PAHs in heavily-cooked meats, and a threefold higher risk of colon adenomas compared to Officers with the lowest PAH–DNA adducts [Gunter et al., 2007]. The study reported here employs PAH–DNA adduct

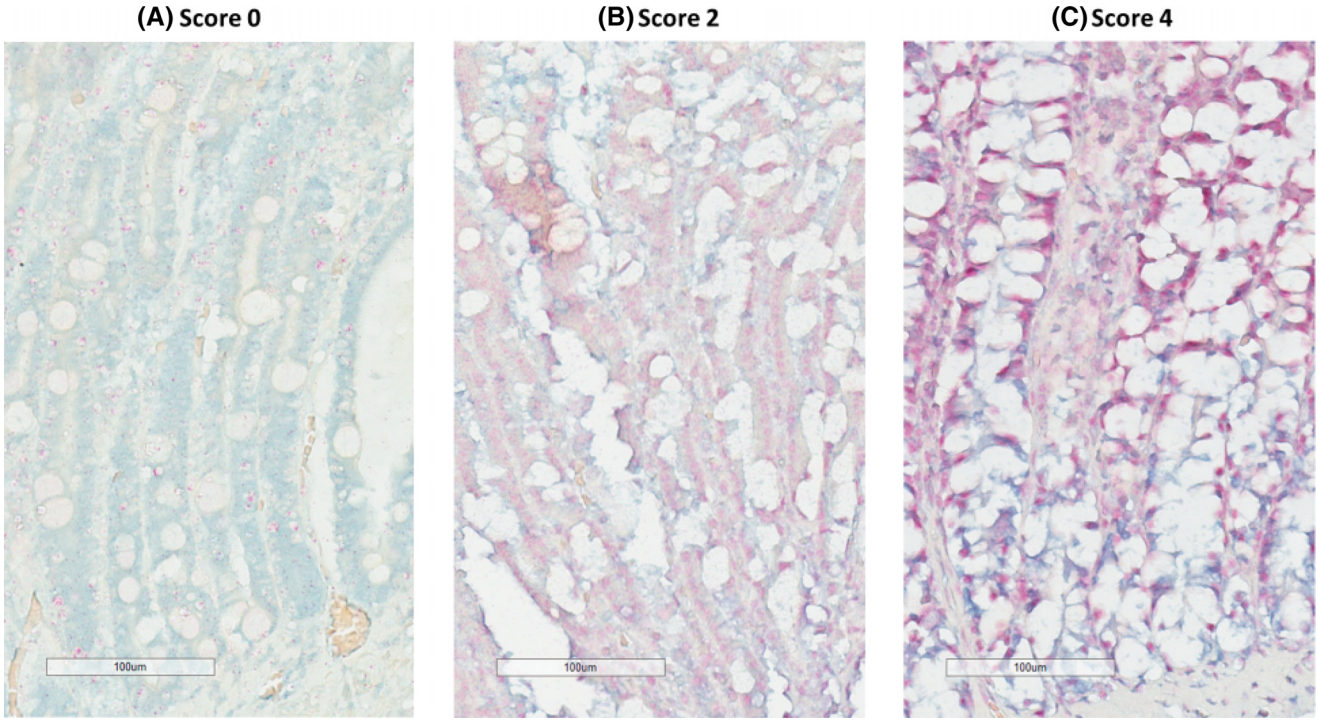

Fig. 4. PAH–DNA staining of beluga small intestine with specific antiserum shows: (**A**) a beluga with no staining, or a score of 0. (**B**) A beluga with moderate positive staining scored 2; and (**C**) a beluga with very strong nuclear staining scored 4.

analysis to extend the correlation to yet another species, the beluga whales, suggesting that many free-ranging marine mammals may also be susceptible to cancer induction by environmental PAH exposure.

Extensive exposure to PAHs of industrial origin has been documented in SLE beluga [Smith and Levy, 1990; Martineau et al., 2002a,b]. The aluminum industry released massive amounts of PAHs into the water and atmosphere of the Saguenay River region for approximately 5 decades (1926–1976), and these compounds heavily contaminated the water, river sediments, and river drainage basin [Martel et al., 1986; Smith and Levy, 1990; Martineau et al., 2002a,b; Pelletier et al., 2009; Martineau, 2012]. Maximum contamination of sediments occurred between 1956 and 1976, when PAH-rich liquid effluents were directly released, without treatment from chimney scrubbers, into the Saguenay River [reviewed in Martineau, 2012]. As a result, tissues of terrestrial mammals (woodchucks) and fish (halibut) revealed high levels of PAH contamination [Blondin and Viau, 1992; Hellou et al., 1994]. BP levels increased 200 times in the tissues of blue mussels within a month after they were transplanted from an unpolluted area to a polluted area of the Saguenay River [Cossa et al., 1983]. PAH contaminated fish and benthic annelids of the Saguenay River are both beluga prey [Seaman, 1983; Hellou et al., 1994; Béland, 1995; Martineau et al., 2002a,b; Pelletier et al., 2009; Martineau, 2012; Lesage, 2014; Quakenbush et al., 2015]. Beluga also ingest sediments, directly during diving and suction feeding of benthic annelids, as variable amounts of sediment are often present in the beluga stomach [Dalcourt et al., 1992; Quakenbush et al., 2015].

All 32 beluga found dead (Fig. 1B) with cancer in the SLE, from 1983 to 2012 [Martineau et al., 2002a: Martineau, 2012; Lair et al., 2016], lived during the period of maximum PAH contamination of the Saguenay River sediments, and the cancer rate was highest in adult beluga born in the 1940s and 1950s. Of the 11 gastrointestinal cancers found in SLE beluga from 1983 to 2004, eight were epithelial cancers of the proximal intestine and three were epithelial cancers of the gastric mucosa [Lair et al., 2016]. No gastrointestinal cancer has been observed in beluga with estimated years of birth higher than 1958 and/or in beluga found stranded dead after 2004 [Lair et al., 2016; Lair, unpublished]. This temporal pattern of cancer incidence presumes initiation during the years of maximal contamination, with cancers found during the subsequent 50 years. Furthermore, 28 years after the company stopped releasing PAH from the chimney scrubbers directly into the Saguenay River (in 1976), no additional gastrointestinal cancers were found in the beluga. This chronological sequence of events further supports the etiological involvement of PAHs in the formation of gastrointestinal cancers affecting the SLE whales.

TABLE I. PAH–DNA Scoring of Whole Tissue Sections Comparing SLE and CI Beluga With A&A Beluga.

Beluga population	Number of beluga	% of Beluga with:		
		Score 0	Score 2	Score 4
SLE (0– < 30 years)[1]	23	56.5	[2]*39.1	*4.3
A&A (0– < 30 years)[1]	15	86.7	13.3	0
SLE (30–63 years)[1]	28	25.0	*42.9	*32.1
A&A (30–46 years)[1]	5	80.0	20.0	0
CI (0– < 30 years)[3]	4	0	*100	0
A&A (0– < 30 years)[3]	15	86.7	13.3	0

[1]The scores, by Cochran–Mantel–Haenszel test, are significantly shifted to higher values in the SLE populations than in the A&A populations ($P = 0.003$).

[2]Percentages marked with * are significantly ($P \leq 0.05$) higher than the paired A&A group at the same scoring level.

[3]All Cook Inlet beluga were 0– < 30 years. By exact chi-square test proportionally more CI beluga scored 2 (100%), compared to the A&A beluga belonging to the same age class (13.3%, $P = 0.004$).

This study involved beluga that were either hunted in the Arctic wilds, euthanized after unsuccessful treatment or dead of disease in aquaria, or found stranded dead along the shores of the SLE or CI. All were examined and cancer was observed only in SLE beluga. The biomarker of interest in this study is the formation of DNA adducts of carcinogenic PAHs, which are minor metabolites formed by the activity of multiple Cytochrome P450 mixed function oxidases and other metabolic enzymes. This metabolism requires that the tissue be alive, and no adducts are expected to form after death. A major cause of death among beluga in the wild is bacterial or parasitic infection, which leads to inflammation. There are no studies on the potential direct effects of disease and/or inflammation on the formation of PAH DNA adducts. However, inflammation is known to decrease cytochrome P450 activity, which is central to PAH metabolism [Harvey and Morgan 2014; Morgan, 2009; Zanger and Schwab, 2013]. Consequently, a potential bias caused by inflammation would result in: (1) decreased formation of PAH DNA adducts in SLE and CI beluga, both of which are or have been exposed to PAH; and (2) an increased formation of these adducts in presumably healthier hunted beluga. Furthermore, PAH–DNA adducts present at any given time reflect the steady state conditions of adduct formation and removal that have been ongoing for months. Whereas it would be ideal, it is not possible to have all of the beluga in the study living under the same conditions. However, because the PAH exposure level is the primary determinant of our end point we believe that this comparison is valid.

We examined intestine from four whales found stranded in CI, Alaska (Fig. 5A). CI is adjacent to Prince William Sound where the Exxon Valdez oil spill occurred in 1989, and where oil reached into the lower regions of the Inlet. CI is also the site of several oil platforms and a commercial shipping terminal, and it is adjacent to the largest population center in Alaska, though there is comparatively less human activity compared to the SLE. Despite this, there are very few data on PAH levels in CI beluga [Burek-Huntington et al., 2015]. In our study, the CI beluga were analyzed as a separate group, and their number was too small to provide robust evaluation of, for example, increase in PAH–DNA adducts with age. The fact that cancer was not observed in these whales may be related to the very small number of animals examined, the age of the population, the length of time that the environment has been contaminated, and/or other factors [Burek-Huntington et al., 2015].

The cause of the PAH–DNA positivity seen in a single 36-year-old captive beluga (Fig. 5 A) remains elusive. Possible routes of exposure include environmental exposure prior to collection or exposure during sea pen housing while in managed care. This animal was housed in a pool environment with no likely PAH exposure for 7 years prior to death.

In 1988 using a BPdG-specific method, PAH–DNA damage was detected in the range of 7.3–21.5 BPdG adducts/10^8 nucleotides in brains of three SLE beluga aged 14.0–22.5 years, while no detectable BPdG adducts in liver and brain DNA from four beluga were found in the Mackenzie Delta in the Canadian Arctic [Martineau et al., 1988]. Subsequently, other researchers [Ray et al., 1991] used ^{32}P-postlabelling, a method detecting bulky (or aromatic) DNA adducts without chemical characterization, and found similar levels of bulky-DNA adducts in livers of beluga from the East Hudson Bay, the Mackenzie Delta, and the SLE. That study involved 4–8 whales per group, and because the ^{32}P-postlabelling method does not identify specific DNA adducts, there is no certainty that only PAH–DNA adducts were being measured in each group. Therefore, direct comparability is in doubt. The current study is a much more robust demonstration of the connection between PAH–DNA adduct formation and cancer in beluga whales.

The observations of this study support a link of causality between documented PAH exposures and epithelial cancers found in the small intestine and stomach of SLE beluga. First, PAH–DNA adducts were significantly more abundant in the crypt cells of SLE beluga small intestine than in intestines of beluga from aquaria and the Arctic, areas with low PAH contamination and no cancers. Second, the immunohistochemical localization of these adducts was consistent with

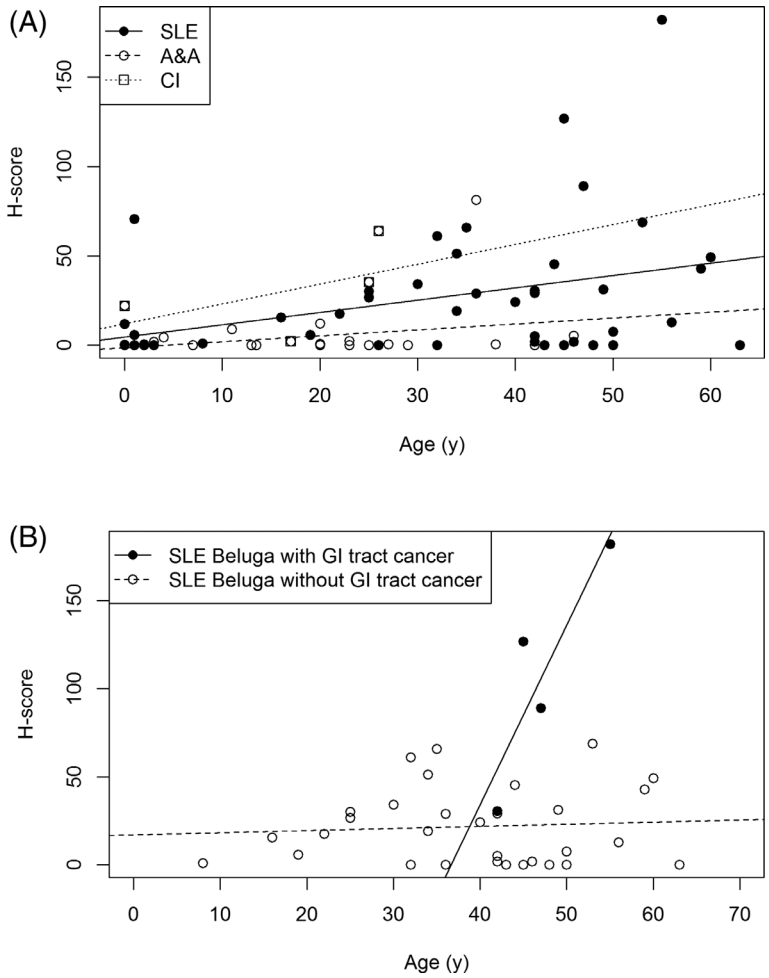

Fig. 5. H-score values for PAH–DNA staining in beluga small intestine as a function of age. (**A**) Controlling for age, the H-score for the SLE beluga (n = 51) was significantly higher than for the A&A beluga (n = 20) residing in less polluted areas (P = 0.03). In both groups there was a significant increase in PAH–DNA adduct level with age (P = 0.003), and the rate of increase with age was similar in both groups (P = 0.35) despite the higher level of PAH–DNA damage in the SLE group. The H-score for the CI beluga (n = 4) was significantly higher than for the A&A beluga (P = 0.02), controlling for the non-significant effect of age (P = 0.19). (**B**) H-score values for PAH–DNA staining in adult SLE beluga (8–63 years) small intestine as a function of gastrointestinal (GI) cancer status. Controlling for age, the H-score in the four SLE beluga with gastrointestinal cancer was significantly larger than in the group without gastrointestinal cancer (P = 0.006). There was a significant increase in the H-score with age in the gastrointestinal cancer group (P = 0.0002), but not in the group without gastrointestinal cancer (P = 0.70). Adult SLE beluga (n = 31), cancer-free adult SLE beluga (n = 25); SLE adult beluga with other types of cancer (n = 6).

the current understanding of the mechanisms leading to small intestinal epithelial cancer [Barker et al., 2009]. Further study will be necessary to assess the identity of affected crypt cells in SLE beluga, using stem cell markers such as Lgr5 [Barker et al., 2009]. As mentioned above, there is evidence that various PAHs are carcinogenic in humans, and this study extends that association to marine mammals.

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AUTHORS CONTRIBUTIONS

Conception and design: Poirier, Lair, Michaud, Hernandez-Ramon and Martineau. Development of

methodology: Poirier, Hernandez-Ramon, Divi, Dwyer, Ester, Si, and Ali. Acquisition of data (provided whale samples and standard curve materials): Lair, Loseto, Martineau, Raverty, St. Leger, Van Bonn, Colegrove, Burek-Huntington, Stimmelmayr, Suydam, J. Wise and S. Wise. Analysis and interpretation of data, including statistical analysis: Beauchamp, Lair, Michaud, Poirier and Martineau. Evaluation of the manuscript: All authors.

CONFLICT OF INTEREST

All the authors have declared no conflict of interest.

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