Arch Environ Contam Toxicol (2016) 71:35–47
DOI 10.1007/s00244-016-0283-7

Tissue Phthalate Levels Correlate With Changes in Immune Gene Expression in a Population of Juvenile Wild Salmon

Kelly Martins[1] · Birgit Hagedorn[2] · Shareen Ali[3] · John Kennish[3] · Ben Applegate[2] · Matthias Leu[1] · Lidia Epp[1] · Chris Pallister[4] · Patty Zwollo[1]

Received: 18 December 2015 / Accepted: 25 April 2016 / Published online: 13 May 2016

Abstract Phthalates have detrimental effects on health and have been shown to dysregulate the immune system of mammals, birds, and fish. We recently reported that di(2-ethylhexyl) phthalate exposure reduces the abundance and inhibits the proliferation of rainbow trout (*Oncorhynchus mykiss*) IgM^+ B lymphocytes and expression of secreted immunoglobulin heavy-chain mu transcripts in an in vitro culture system. We proposed that phthalates act as immunomodulators by modifying the normal B cell-activation pathways by accelerating B cell differentiation while suppressing plasmablast expansion, thus resulting in fewer IgM-secreting plasma cells. This hypothesis was tested here in an in vivo field study of juvenile Dolly Varden (*Salvelinus malma*) from a plastic-polluted lake in the Gulf of Alaska. Fish tissues were analyzed for both phthalate levels using liquid chromatography-coupled tandem mass spectrometry and for changes in immune gene expression using reverse transcriptase-real time polymerase chain reaction. Results showed that fish with higher tissue levels of di(2-ethylhexyl) phthalate, di(*n*-butyl) phthalate, and/or dimethyl phthalate expressed significantly fewer secreted and membrane-bound immunoglobulin heavy-chain mu and Blimp1 transcripts in their hematopoietic tissue. This suggests that in vivo uptake of phthalates in fish changes the expression of B cell-specific genes. Chronic exposure to phthalates likely dysregulates normal B-lymphoid development and antibody responses in salmonids and may increase susceptibility to infection. Given the conserved nature of B-lineage cells in vertebrate animals, other marine species may be similarly affected by chronic phthalate exposure.

Marine plastic-debris pollutants are a global health threat to living organisms ranging from plankton (Cole 2013), fish, birds (Moore 2008), and humans (Koch et al. 2006). Of these pollutants, phthalates and their metabolites form a particular threat because they are noncovalently bound to polymer products and slowly migrate into the environment. Phthalate exposure occurs through skin as well as food or water intake, whereas absorption takes place in gills and the intestinal system (Mallatt 1985; Schettler 2006). In humans, reported risks from phthalate exposure include androgenic changes, organ and cellular toxicity, endocrine disruption, insulin resistance, and dysregulation of immune function (Schug et al. 2011; Schlezinger et al. 2004).

Phthalates are of particular concern in marine species such as teleosts because their biodegradation in aquatic systems is slow resulting in chronic exposure to these chemicals (Pakalin et al. 2008; Shanker et al. 1985). The presence of di(2-ethylhexyl) phthalate (DEHP), the most studied from the phthalates, has been reported in several wild fish species including yellow croaker (*Larimichthys polyactis*), tilapia (*Oreochromis* spp.), carp (*Cyprinus* sp), and greenback mullet (*Chelon planiceps*) (Huang et al. 2008; Cheng et al. 2013). Chronic exposure to phthalates

Electronic supplementary material The online version of this article (doi:10.1007/s00244-016-0283-7) contains supplementary material, which is available to authorized users.

✉ Patty Zwollo
pxzwol@wm.edu

[1] Department of Biology, The College of William and Mary, Williamsburg, VA 23185, USA

[2] Environment and Natural Resources Institute, The University of Alaska Anchorage, Anchorage, AK 99508, USA

[3] Department of Chemistry, The University of Alaska Anchorage, Anchorage, AK 99508, USA

[4] Gulf of Alaska Keeper, Anchorage, AK 99516, USA

causes long-term damage to multiple biological systems including endocrine and immune systems (Barse et al. 2007). Within the mammalian immune system, phthalates affect inflammation and apoptosis dynamics, dendritic differentiation, and cytokine production as well as the development and proliferation of lymphoid cells (Wu et al. 2012; Hansen et al. 2015; Koike et al. 2009; Schlezinger et al. 2002; Sakazaki et al. 2002.

How do phthalates affect immune cells? One important pathway involves activation of the peroxisome proliferator-activated receptor, PPARγ (Feige et al. 2007; Bility et al. 2004; Hurst and Waxman 2003), a ligand-induced transcription factor in the superfamily of nuclear hormone receptors that is expressed in B lymphocytes and myeloid cells (Garcia-Bates et al. 2009; Wu et al. 2012). Phthalates are strong agonists of PPARγ, and as such, they efficiently compete for binding with PPARγ's natural ligands including conjugated linoleic acids (CLAs) and prostaglandin PGJ2 (reviewed in Houseknecht et al. 2002). PPARγ has a wide variety of functions: It modulates adipogenesis, lipid metabolism, immune development, and inflammation (Glass and Ogawa 2006; Rosen et al. 1999). In mammalian B-lymphocytes, PPARγ has prodifferentiation and antiproliferation regulatory functions (Garcia-Bates et al. 2009; Schlezinger et al. 2007; Setoguchi et al. 2001). The gene encoding PPARγ has been isolated in brown trout (*Salmo trutta*) (Batista-Pinto et al. 2005), thus supporting its presence in salmonids.

The B-lymphoid program is highly conserved among vertebrates including the rainbow trout (*Onchorhynchus mykiss*). Several molecular markers have been used to identify developmental and differentiation stages of teleost B-lineage cells—including two transcription factors, B lymphocyte-induced maturation protein (Blimp1), and paired box protein Pax5—as well as the secreted and membrane-bound forms of the immunoglobulin heavy-chain mu gene (reviewed in Zwollo 2011). Mature teleost B cells express both Pax5 and the membrane form of heavy-chain mu (memHCmu), whereas terminally differentiated plasma cells do not but instead secrete high levels of secreted heavy-chain mu (secHCmu) (Zwollo et al. 2005).

Blimp1 is an essential regulator driving B- and T-lymphocyte differentiation, and it is expressed in vertebrate species including rainbow trout (John and Garrett-Sinha 2009). Within the B lineage, Blimp1 inhibits plasmablast proliferation and promotes terminal differentiation into plasma cells (reviewed in John and Garrett-Sinha 2009 and Zwollo 2011). Furthermore, mammalian Blimp1 has been shown to shift HCmu expression from the membrane-bound form toward the secreted form, thus resulting in the production of antibodies (Lin et al. 2002), and this function is likely conserved in teleosts. Activation of PPARγ has been shown to increase expression of Blimp1 (Garcia-Bates et al. 2009), whereas mice with a B cell-specific targeted deletion of PPARγ had reduced Blimp1 levels (Ramon et al. 2012). Together this suggests that phthalate exposure activates PPARγ causing overexpression of Blimp1, which in turn leads to fewer plasmablasts and consequently fewer plasma cells during a B-cell response.

Evidence that phthalates can functionally affect the teleost immune system was recently reported by our group. Short-term (2- to 7-day) exposure of rainbow trout immune cells to di(2-ethylhexyl)phthalate (DEHP) had significant effects on B-lineage cells using an in vitro culture system: DEHP inhibited plasmablast proliferation, reduced the abundance of IgM^+ immunoglobulin-secreting cells (ISCs), and reduced the expression of *secHCmu* transcripts in anterior kidney cell cultures from rainbow trout (Martins et al. 2015). These effects were particularly significant because the anterior kidney comprises the bone marrow equivalent in teleosts, and as such it is the main site for hematopoiesis (Zapata 1990). In addition, anterior kidney is the site for the storage of long-lived plasma cells (LLPCs), antibody-secreting cells that play important roles in continued protection against previously encountered pathogens (Bromage et al. 2004).

To further test the effects of phthalates on the teleost immune system, we undertook an in vivo study focused on juvenile Dolly Varden, anadromous salmonids in the Char family. These fish are naturally exposed to phthalates in a marine debris-polluted lake on Elizabeth Island, off the remote southwest tip of the Kenai Peninsula, an undeveloped and remote wilderness area typical of the northern Gulf of Alaska coast. Otherwise pristine, the lake, like countless estuaries and near-coastal lakes along the northern Gulf of Alaska, has been inundated by tons of plastic marine debris that has drifted across the Pacific from Western Pacific countries (http://www.goak.org).

Levels of six phthalates were determined in muscle, gastrointestinal, and liver tissues of Dolly Varden using liquid chromatography-coupled tandem mass spectrometry, and immune gene expression levels were determined in three functionally distinct immune tissues from each fish using reverse transcriptase-real time polymerase chain reaction (RT-qPCR). Anterior kidney tissue was used to explore effects of phthalates on hematopoeisis and/or immunological memory in the form of antibody-secreting LLPCs. Effects on the posterior kidney were investigated because it houses high percentages of activated B cells and plasmablasts (Zwollo et al. 2005). Last, the spleen was used to test the effects of phthalates on an immune organ housing mostly resting, mature B cells (Barr et al. 2011). Hence, investigating these three tissues should shed light

on the effects of phthalates in three critical and independent immune sites in fish.

Results showed highly significant negative associations between tissue phthalate levels and expression of secHCmu, memHCmu, and Blimp1 with anterior kidney being the most sensitive to phthalate presence. To our knowledge, this is the first in vivo study showing that the levels of phthalates in tissues of a natural fish population are associated with significant changes in immune gene expression.

Materials and Methods

Animals and Collection

Juvenile Dolly Varden (5–28 g) were collected at Elizabeth Lake (Lake No. 242-10-10,270-0010 [ADF&G 2015]) and at the mouth of Elizabeth Creek (Stream No. 242-10-10270 [ADF&G 2015]) approximately 50 m upstream from Elizabeth Lake (at 59.15345 N, −150.80337 W) (Fig. 1). Fish were captured during the summer 2013 (August 12 [$N = 15$]) and 2014 (July 26 [$N = 21$]). Juvenile Coho salmon (*O. kisutch*) (7–23 g) were also collected from the same locations in 2012 (August 14 [$N = 9$]). Elizabeth Lake is a small (0.3 km in diameter), shallow (1–3 m deep), near-shore, near-costal lake. The water temperature on collection days varied based on time of day and location but was in the range of 11 to 13.5 °C. Alaska Department of Fish and Game (ADF&G) research permits were obtained according to ADF&G regulations (Permit Nos. ADF&G 2012-285, ADF&G 2013-173, and ADF&G 2014-006). Fish were collected using baited Gee's-type minnow traps. Fish were killed using MS-222 in accordance with IACUC protocol 2012-06-14-8016-pxzwol. Whole fish and tissue weights were recorded for each individual. For immune tissue analysis, approximately 0.1 g tissue samples were taken immediately from spleen, anterior kidney, and/or posterior kidney and stored in RNAlater at −20 °C for future processing.

For chemical analysis, muscle (3–6 g), liver (0.3–1.0 g), and/or gastrointestinal (GI) tissues (not flushed, 0.7–1.8 g) were collected in glass vials that were ashed at 440 °C for 2 h or in aluminum foil that was wiped clean with acetone. All samples were stored at −20 °C for future processing. Liver and muscle samples were also obtained from adult Coho salmon and Pacific halibut (*Hippoglossus stenolepis*). Juvenile Coho salmon samples collected included 16 GI and 3 muscle. Juvenile Dolly Varden samples collected in 2013 included 15 GI

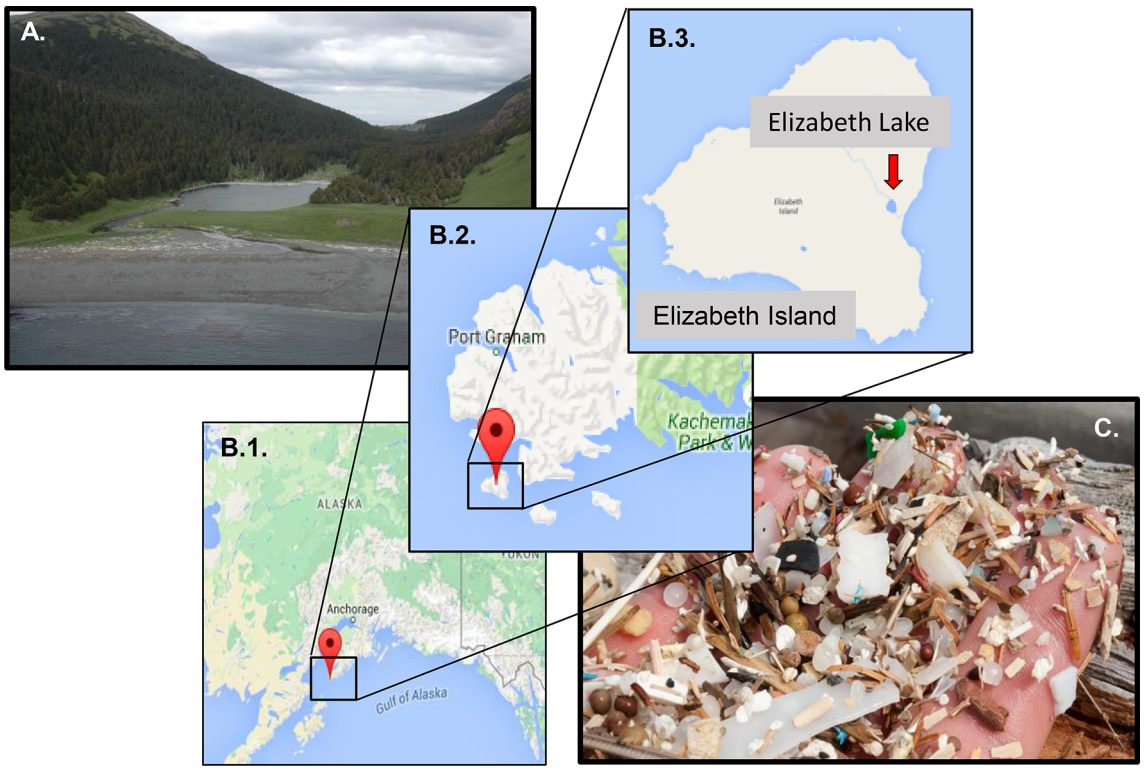

Fig. 1 Location of field study. **a** Photograph of Elizabeth Island Lake. **b** Location of Elizabeth Island Lake relative to (*B.1*) South Central Alaska, (*B.2*) Kenai Peninsula, and (*B.3*) Elizabeth Island Lake (*arrow*). Images adapted from Google Maps. **c** Illustration of small plastic marine debris collected from Elizabeth Island Lake. Photos reprinted with permission from GoAK

and 14 muscle. Dolly Varden samples collected in 2014 included 18 liver and 9 muscle. Adult Coho salmon samples collected included 10 liver and 1 muscle, and for adult halibut included 10 liver and 10 muscle samples. Water and sediment samples were collected in glass containers treated as the vials (see previous text) and stored frozen until processing.

RNA, cDNA, and Real-Time PCR

RNA was isolated using RNAzol-RT (Molecular Research Center Inc.). All cDNA was made using an iScript random labeling cDNA kit (BioRad), and RT-qPCR was performed as previously described (Martins et al. 2015). Salmonid-specific qPCR primers were used to target secreted HCmu (secHCmu), membrane HCmu (memHCmu), Pax5, Blimp1, and α-tubulin sequences (Martins 2015; Diaz-Rosales et al. 2009). C_T values were uploaded into DataAssist Software (ABI). Expression of individual genes from each sample was normalized to expression of the housekeeping gene trout α-tubulin (Martins et al. 2015) within the same experiment. The relative fold change was calculated according to the Fold Change $= 2^{-\Delta CT}$ equation (Livak and Schmittgen 2001).

Chemical Analysis

Concentrations of six phthalates were determined: BBP [butyl benzyl phthalate], DBP [di-*n*-butyl phthalate], DEHP [di(2-ethylhexyl)phthalate], DEP [diethyl phthalate], DMP [dimethyl phthalate], and DnOP [di-n-octyl phthalate]. All equipment was of glass and either washed with hexane and acetone or ashed at 440 °C for 2 h before use. A Quentcher method with primary secondary amine (PSA), Supelclean (Sigma-Aldrich 52738-U), magnesium sulfate, and sodium chloride was used for extracting phthalates from animal tissue. Samples were weighed into 10-mL Kimex glass centrifuge tubes together with a mixture of $MgSO_4$ and NaCl. One hundred nanograms of the surrogates DMP-d_4 and DEHP-d_4 and 2 mL of acetonitrile were added to the samples. Samples were then vortexed for 1 min and placed in an ultrasonic bath for 20 min and kept at 4 °C for 24 h to complete the extraction. Next, samples were vortexed for 5 min and centrifuged. The liquid was syphoned off and placed in a second glass culture tube that had been previously filled with $MgSO_4$ and PSA sorbent resin to remove other organic compounds. The mixture was vortexed for 3 min and centrifuged. Next, 0.5 mL of the liquid was transferred to a 2-mL autosampler vial with Teflon septa, and internal standards DBP-d_4 and DnOP-d_4 were added at a concentration of 25 ng/mL.

Instrumental Analysis

Samples were analyzed by Agilent high-pressure (600-bar maximum) 1200 series HPLC connected to an Agilent 6410B tandem mass spectrometer with ESI source under the following conditions: HPLC settings: *guard column* = XDB-C18 4.6 × 12.5, 5 µm; rapid-resolution column = Zorbax SB-C18 2.1 to 30 mm, 3.5 µm; *solvent A* = water with 10 mM formic acid; *solvent B* = methanol with 10 mM formic acid; ramp: B = 25–85 % in 5 min, 85–90 % in 9 min; *B* = 90 % for 9 min; mass spectrometer settings: *gas temperature* = 300 °C; *gas flow* = 6 L/min; *nebulizer* = 15 psi; *capillary voltage* = 4000 V. The precursor and fragmentation molecules that were tracked for quantification and as qualifying mass are listed in Supplemental Table 1.

For quality control, laboratory method blanks and continuing calibration verification standards were analyzed every 10th sample, and sample duplicates were analyzed with every extraction batch (approximately 20 samples). In addition, spiked samples and blanks were carried through the entire extraction procedure to determine method recovery. Limit of detection (LOD) was calculated from seven-level calibration (Harris 2010). Method limit of detection (MLOD) was determined from SD of analysis of seven replicates of homogenized salmon tissue. If high blanks were encountered in a batch, counts were blank-corrected before calculating concentrations. Chemical structures of these phthalates are shown in Fig. 2.

Statistical Analysis

Student *t*-tests were used to compare phthalate levels between years and between fish caught in lakes *versus* the upstream creek. Dolly Varden caught in the lake did not have significantly different levels of phthalates in their tissues than those caught in the creek ($t = 1.05$, $df_{1,22}$, $p = 0.31$) suggesting that fish move back and forth between the sites (a summary of all Dolly Varden tissue samples used, including their location in lake or creek, is listed in Supplementary Table 2). As such, lake and creek fish samples were pooled for statistical analyses. Pearson product-moment correlation coefficients were used to test for linear correlations between phthalate levels in a given tissue. Associations between tissue phthalate concentrations and each of the four immune genes (secHCmu, memHCmu, Blimp1, and Pax5) were investigated using linear regression analyses (LM package) in R (R Foundation for Statistical Computing, version 3.2.0). Because gene expression levels and phthalate concentrations related exponentially, the relative fold-change values were log-transformed (natural log) for each genetic marker (indicated in Figs. 3, 4 as relative [fold change of] "target

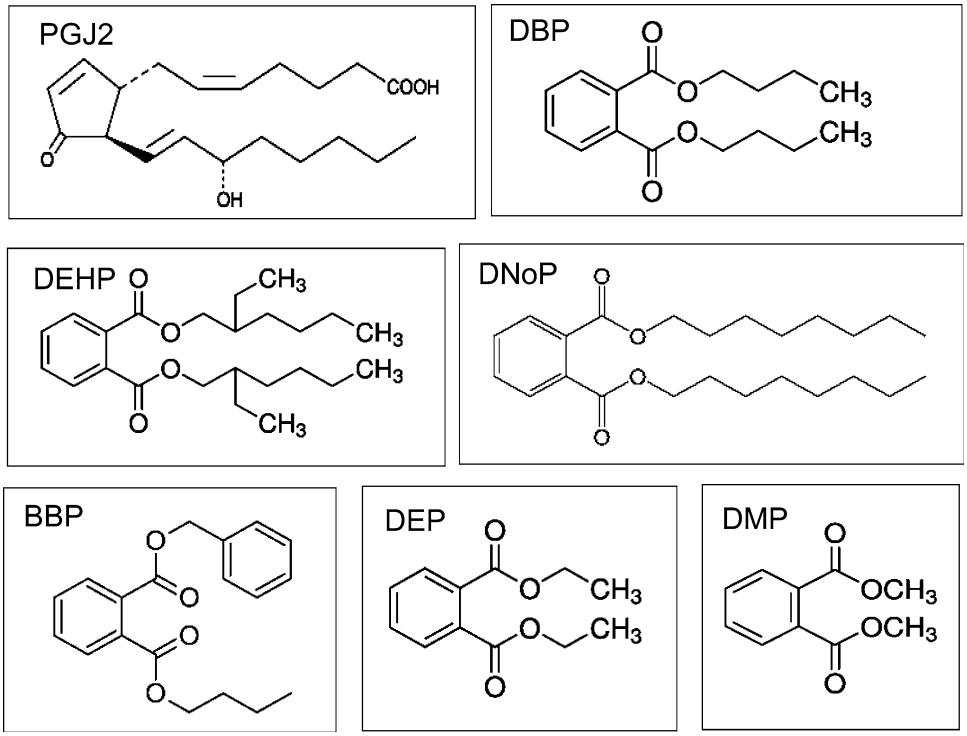

Fig. 2 Chemical structures of natural PPARγ ligand PGJ2 and the six phthalate esters investigated in the study. (Adapted from http://www.sigmaaldrich.com/catalog)

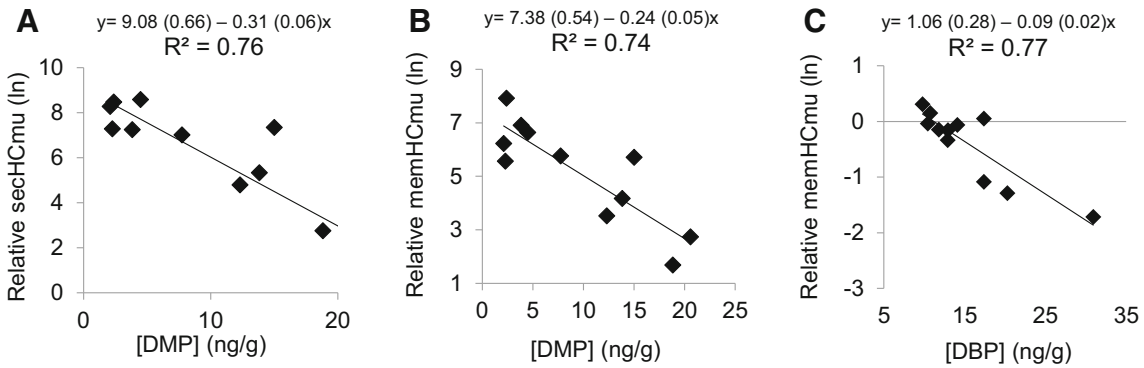

Fig. 3 Scatterplots illustrate associations between phthalate tissue levels and transcript levels of secreted (sec) and membrane (mem) *HCmu* from 2013 samples using RT-qPCR. Values on the *Y* axes indicate the log-transformed (ln) relative fold-change values for *secHCmu* and *memHCmu*. Phthalate concentrations are in ng/g. R^2 values for each scatterplot, as well as the regression equation (SD), are indicated. **a** GI DMP levels associated with *secHCmu* in anterior kidney. **b** GI DMP levels associated with *memHCmu* in anterior kidney. **c** Muscle DBP levels associated with *memHCmu* in posterior kidney

gene" [ln]). Associations were considered significant if $p < 0.05$; however, all p values that were close to being significant ($p < 0.1$) were also listed because of the small sample size used. When applicable, mean values ±SD were reported.

Results

The main goal of the study was to determine whether chronic phthalate exposure affects B-lineage cells in vivo using populations of juvenile Dolly Varden residing in a plastic marine-debris polluted lake in Alaska. The study focused on juvenile fish because developing organisms are typically more sensitive to environmental toxins compared with adults.

Phthalate Levels in Fish Tissues

During the 3-year study period, three different tissues were analyzed: 1) GI tissue to assess phthalate exposure due to ingestion, (2) liver tissue to assess phthalate presence after ingestion, and (3) muscle tissue to assess levels of accumulated phthalates. The majority of tissue samples

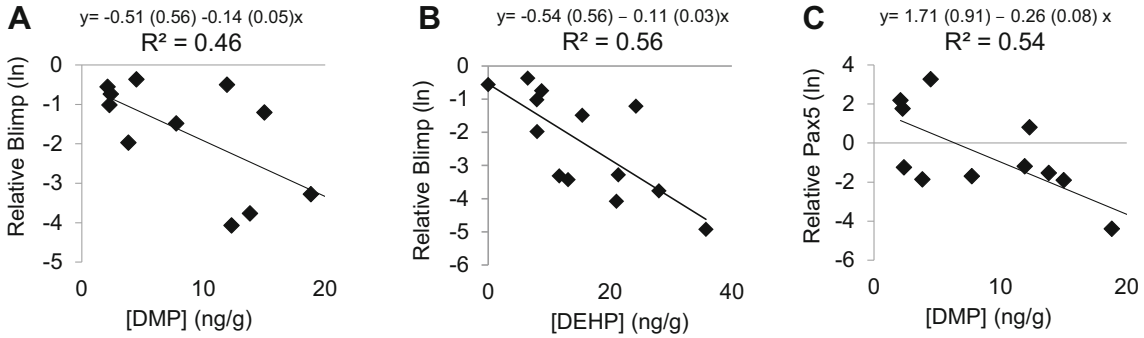

Fig. 4 Scatterplots illustrate associations between phthalate levels and transcript levels of B lymphoid-specific transcription factors in anterior kidney in 2013 samples using RT-qPCR. Values on the *Y* axes indicate the log-transformed (ln) relative fold-change values for *Blimp1* and *Pax5*. Phthalate concentrations are given in ng/g. R^2 values for each scatterplot, as well as the regression equation (SD), are indicated. **a** Gastrointestinal DMP levels associated with *Blimp1*. **b** Muscle DEHP levels associated with *Blimp1*. **c** GI DMP levels associated with *Pax5*

contained significant levels of phthalates. Average levels are summarized in Table 1.

The first year of collections was performed for juvenile Coho salmon only, which were the most abundant species in our traps in 2012. In 2013 and 2014, residents comprised mostly Dolly Varden, and thus this species was chosen for analysis. In 2012, only DEHP was measured, whereas a total of six phthalates were measured in 2013 and 2014: DEHP, DMP, DBP, BBP, DEP, and DNoP.

Average tissue levels of DEHP (Table 1) were similar in liver and GI of juvenile Dolly Varden (97.0 ± 32.0 and 91.5 ± 37.0 ng/g, respectively). In comparison, juvenile Coho salmon had lower (39.8 ± 3.9 ng/g) levels of DEHP in GI tissues. The average DEHP levels in Dolly Varden muscle were significantly different between 2013 and 2014 (15.6 ± 2.4 and 122.3 ± 24.2 ng/g, respectively ($t = -5.8322$, $df_{1,20}$, $p < 0.001$). Overall, DEHP was the most abundant phthalate closely followed by DBP. DMP levels in GI tissue were approximately 10-fold lower than levels of DEHP (Table 1). In 2013, average DEP levels were very low in GI (7.8 ± 4.0 ng/g) lower then the LOD in muscle, but they were high in liver (50.4 ± 16.7 ng/g). Muscle levels of DBP and BBP in 2014 were similar to those in 2013 samples. Overall, this suggests that exposure levels of phthalates can vary widely from year to year as well as between fish. It should be noted that during summer 2013, after winter storms lowered the lake's outlet to the ocean, the lake level dropped approximately 60 to 70 cm. This may have affected phthalate levels in those 2014 juvenile fish that hatched in 2013.

We also determined whether there were any correlations *between* phthalate levels in each tissue. For many of the samples, this was not the case, but several correlations were found. One strong correlation was observed between DBP and DMP levels in GI samples collected in 2013. In addition in GI samples, BBP levels correlated with levels of all other phthalates. For 2014 muscle samples, BBP levels correlated significantly with DBP levels. A strong correlation was also seen between DNoP and DEHP levels in GI tissues. These data are summarized in Supplemental Table III.

Next, the "total" phthalate tissue levels (total levels for the six phthalates) were calculated for each fish for both years (Table 1). In 2013, total phthalate levels in Dolly Varden muscle varied from 18.2 to 80.3 ng/g (average 42.1) and in GI from 35.3 to 940 ng/g (average 238.9). In 2014, Dolly Varden muscle total phthalate levels varied from 32.9 to 277.3 ng/g (average 140.2) and in liver from 30.4 to 320.2 ng/g (average 162.5). Muscle total phthalate levels were significantly higher in 2014 compared with 2013 ($t = -4.409$, $df_{1,22}$, $p = 0.0002$).

To compare the phthalate levels in juvenile salmon with those in adult ocean-residing fish species, two different adult fish species were used: Coho salmon and halibut (Table 1). No tissue samples could be obtained for adult Dolly Varden during our field trips (Table 1). In liver, levels of DEHP, BBP, and DEP were lower than LOD in both adult species. Similarly, muscle levels of DMP, BBP, DEP, and DNoP were lower then LOD in adults. However, DEHP and DBP levels in muscle were essentially the same in juveniles and adults (Table 1).

Phthalate Levels in Water and Sediment Samples

Water concentrations of the six phthalates (DEHP, DMP, DBP, BBP, DEP, and DNoP) were determined in Elizabeth Island Lake in summer 2010 and 2012. Five independent samples were taken each year. Of the six phthalates tested, only DEHP and DBP were detected in all water samples. Total average phthalate levels were 8.5 ng/mL in 2010 and

Table 1 Phthalate levels in fish tissues

Juveniles												
	GI Coho EI 2012	LOD	GI Dolly V EI 2013	LOD	Liver Dolly V EI 2014	LOD	Liver Coho EI 2012	LOD	Muscle Dolly V EI, 2013	LOD	Muscle Dolly V EI, 2014	LOD
DEHP (ng/g tissue)	39.8 ± 3.9	6.2	91.5 ± 37.0	6.8	97.0 ± 32.0	1.36	10.6 ± 8.5	6.2	15.6 ± 2.4	6.8	122.3 ± 24.2	9.8
DMP (ng/g tissue)	ND	ND	9.0 ± 1.6	6.3	ND	ND	ND	ND	<LOD	6.3	11.1 ± 0.58	4.1
DBP (ng/g tissue)	ND	ND	87.7 ± 21.6	2.1	ND	ND	ND	ND	15.9 ± 1.5	2.1	5.6 ± 1.3	3.9
BBP (ng/g tissue)	ND	ND	18.7 ± 8.2	4.3	15.1 ± 5.0	0.96	ND	ND	5.2 ± 0.9	4.3	1.2 ± 0.28	0.84
DEP (ng/g tissue)	ND	ND	7.8 ± 4.0	5.6	50.4 ± 16.7	1.39	ND	ND	<LOD	5.6	<LOD	1.3
DNoP (ng/g tissue)	ND	ND	24.2 ± 7.5	3.6	<LOD	1.8	ND	ND	5.4 ± 1.0	3.6	ND	
Total (ng/g tissue)	ND		238.9		162.5		ND		42.1		140.2	

Adults								
	Liver Coho PWS 2010	LOD	Liver Halibut CI 2010	LOD	Muscle Coho PWS 2010	LOD	Muscle Halibut CI 2010	LOD
DEHP (ng/g tissue)	<LOD	2.4	<LOD	2.4	3.0 ± 0.92	2.4	4.9 ± 1.4	2.4
DMP (ng/g tissue)	2.5 ± 0.8	1.5	<LOD	1.5	<LOD	1.5	<LOD	1.5
DBP (ng/g tissue)	32.6 ± 29.1	0.13	51.8 ± 25.2	0.13	8.7 ± 5.1	0.13	35.8 ± 25.8	0.13
BBP (ng/g tissue)	<LOD	2	<LOD	2	<LOD	2	<LOD	2
DEP (ng/g tissue)	<LOD	0.67	<LOD	0.67	<LOD	0.67	<LOD	0.67
DNoP (ng/g tissue)	<LOD	1.6	<LOD	1.6	<LOD	1.6	<LOD	1.6
Total (ng/g tissue)	35.1		51.8		12.0		41.2	

Values are in ng/g tissue and are averages ± SE. *GI* gastrointestinal, *DV* Dolly Varden, *EI* Elizabeth Island, *CI* Cook Inlet, *PWS* Prince William Sound, *ND* not done, *LOD* limit of detection

32.8 ng/mL in 2012 (Table 2), and they were not significantly different between years ($t = -1.65$, $df_{1,10}$, $p = 0.13$). Sediment samples were taken in 2012 only: Only DEHP, DBP, and BBP were detected with an average total phthalate level of 13.5 ng/g (Table 2).

Immune Gene Expression in Anterior Kidney

As predicted, based on our previous in vitro study, immune cells from anterior kidney were highly sensitive to tissue levels of phthalates. This is perhaps not surprising because this hematopoietic tissue contains multiple lineages of immune cells including developing B-cell, myeloid, and erythroid lineages (Zapata 1990) as well as LLPCs (Bromage et al. 2004). Multiple significant negative associations were observed between tissue phthalate levels and the expression of specific immune genes in anterior kidney (Tables 3, 4). The strongest associations with immune gene expression were found for DEHP, which is in agreement with detectable levels of DEHP in both tissues and water samples at Elizabeth Lake. DMP and DBP also had significant associations with selected immune gene markers.

Levels of these phthalates in GI and, to a lesser extent, muscle and liver were associated with a reduced expression of immunoglobulin genes secHCmu and memHCmu in anterior kidney (Tables 3, 4). Lower secHCmu transcripts correlated with higher DEHP concentrations in GI and liver and were close to being significant for muscle in both years (Tables 3, 4). Similarly, higher DMP concentrations in GI (2013) and muscle (2014) were associated with reduced secHCmu levels (Tables 3, 4; Fig. 3a). The same phthalates (DEHP and DMP) also showed strong inverse associations with memHCmu expression (Tables 3, 4; Fig. 3b). Both GI and muscle samples (2013 only) showed strong associations between DBP and a reduction in memHCmu expression (Tables 3, 4; Fig. 3c).

Effects of tissue phthalate levels on the expression of transcription factor Blimp1 showed that increased levels of

Table 2 Levels of six phthalates in water and soil samples collected from Elizabeth Island Lake in 2010 and 2012

	Water			Water			Sediment		
	2010			2012			2012		
	Average	LOD	Range	Average	LOD	Range	Average	LOD	Range
DEHP (ng/mL)	7.02	2.40	1.9–14.1	10.92	3.20	0.6–35.3	7.42	3.20	4.4–17.8
DMP (ng/mL)	<LOD	1.50	NA	<LOD	1.40	NA	<LOD	1.40	NA
DBP (ng/mL)	0.64	0.13	0.3–0.7	2.70	0.78	0.1–6.3	0.83	0.78	0.0–3.3
BBP (ng/mL)	<LOD	2.00	NA	13.21	3.20	0.0–44.3	5.27	3.20	0.0–25.7
DEP (ng/mL)	0.76	0.67	0.6–0.76	<LOD	0.90	0.0–1.3	<LOD	0.90	NA
DnoP (ng/mL)	<LOD	1.60	0.0–0.2	6.69	2.10	0.0–46.2	<LOD	2.10	NA
Total (ng/mL)	8.5 ± 2.5		2.8–16.1	32.8 ± 17.0		0.7–133.4	13.5 ± 5.7		4.4–46.8

LOD limit of detection, *na* not applicable. Total, combined level of the 6 phthalates

most of the phthalates tested in GI (DEHP, DMP, DBP and BBP) were associated with reduced overall levels of Blimp1 in anterior kidney (Table 3; Fig. 4a). Similarly, DEHP and, to a lesser extent, DBP levels in muscle were associated with reduced expression of Blimp1 (Tables 3, 4; Fig. 4b). Associations with Pax5 expression were not significant with one exception, DMP levels in GI (Table 2; Fig. 4c). Therefore, this marker was not used for the 2014 samples.

Because DEHP levels were significantly higher in the muscle of Dolly Varden collected in 2014 compared with 2013 (Table 1), we wished to determine whether expression levels of the immune genes also significantly differed by collection year. We did not find a significant correlation between gene expression and sampling year for secHCmu ($p = 0.23$) or memHCmu ($p = 0.43$); however, Blimp1 expression levels were significantly different between years ($p = 0.014$).

In summary, increased levels of phthalates DEHP, DMP, and DBP in juvenile Dolly Varden were strongly associated with lower expression of membrane and secreted forms of immunoglobulin heavy-chain mu as well as Blimp1 transcripts. These data suggest that the higher the concentration of phthalates in a fish tissue, the lower the abundance of IgM^+ B cells (memHCMu $^{\pm}$ $secHCmu^-$), plasmablasts (memHCMu $^{\pm}$ $secHCmu^+$ $^{\pm}$ $Blimp1^+$), and/or plasma cells ($memHCMu^-/secHCmu^{++}$ $^{\pm}$ $Blimp1^{++}$) in the anterior kidney of that fish.

Immune Gene Expression in Posterior Kidney and Spleen

Posterior kidney and spleen both lack developing B cells. Although posterior kidney contains mostly activated and/or proliferating B cells (coexpressing memHCmu and secHCmu), spleen contains mostly resting mature B cells (expressing only memHCmu) (Zapata 1990; Zwollo 2011). Spleen did not show any significant effects of any of the six phthalates on immune gene expression for the four genes tested (Table 3). This suggests that resting mature B cells are relatively insensitive to phthalate exposure compared with developing B cells and immunoglobulin-secreting cells.

Immune gene expression in posterior kidney showed associations with phthalate tissue levels albeit weaker than those in anterior kidney. For example, DEHP levels did not predict any significant immune gene changes in posterior kidney, although in muscle tissues associations came close to being significant for memHCmu (Table 3; $p = 0.054$). However, DBP levels in muscle were strong indicators for inhibitory effects on membrane IgM expression: The higher the DBP tissue level, the lower the memHCmu expression ($p = 0.0002$ and 0.0004, respectively [Table 3]). Furthermore, DBP levels in GI tissue associated inversely with the expression of secreted IgM: The higher the DBP levels, the lower the secHCmu expression (Table 3). Last, levels of the phthalate DNoP in muscle tissue were associated inversely with Blimp1 expression in posterior kidney (Table 3). Together, this suggests that activated B cells are likely sensitive to phthalate presence.

Phthalates and Body Weight

Regression tests were also performed comparing phthalate levels with total body weight. Significant negative correlations were found between fish weight and DMP in GI (2013 $R^2 = 0.80$), DBP in GI (2013 $R^2 = 0.81$), DEHP in muscle (2013 $R^2 = 0.65$), and DEP in liver (2014 $R^2 = 0.66$). This suggests that smaller fish had higher levels of phthalates relative to their total body weight compared with larger fish. This pattern will be addressed in the discussion. No significant associations between body weight and expression of *secHCmu, memHCmu,* or *Blimp1* were detected (Table 3).

Table 3 2013 data

GI	Anterior kidney								Posterior kidney						Spleen					
	SEC		MEM		Blimp		PAX5		SEC		MEM		Blimp		SEC		MEM		Blimp	
	p	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2
DEHP	**0.02**	**0.45**	*0.06*	*0.32*	**0.02**	**0.40**	>0.1	0.04	>0.1	0.01	>0.1	0.09	>0.1	0.02	>0.1	0.22	>0.1	0.02	>0.1	0.07
DMP	**0.0005**	**0.76**	**0.0007**	**0.74**	**0.02**	**0.46**	**0.01**	**0.54**	*0.08*	*0.30*	>0.1	0.11	>0.1	0.01	>0.1	0.19	>0.1	0.00	>0.1	0.01
DBP	*0.06*	*0.28*	**0.01**	**0.46**	**0.01**	**0.42**	>0.1	0.10	**0.03**	**0.40**	>0.1	0.06	>0.1	0.00	>0.1	0.15	>0.1	0.01	>0.1	0.12
BBP	>0.1	0.11	*0.06*	*0.29*	**0.03**	**0.35**	>0.1	0.00	>0.1	0.07	>0.1	0.01	>0.1	0.00	*0.06*	*0.38*	>0.1	0.02	>0.1	0.10
DEP	>0.1	0.02	>0.1	0.17	*0.09*	*0.22*	>0.1	0.01	>0.1	0.13	>0.1	0.12	>0.1	0.14	>0.1	0.09	>0.1	0.11	>0.1	0.13
DnOP	>0.1	0.21	>0.1	*0.25*	*0.06*	*0.34*	>0.1	0.02	>0.1	0.01	>0.1	0.15	>0.1	0.08	*>0.1*	0.22	>0.1	0.02	>0.1	0.03
Muscle	Anterior kidney								Posterior kidney						Spleen					
	SEC		MEM		BLIMP		PAX5		SEC		MEM		BLIMP		SEC		MEM		*BLIMP*	
	p	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2
DEHP	*0.10*	*0.23*	**0.02**	**0.42**	**0.003**	**0.56**	>0.1	0.11	>0.1	0.19	*0.054*	*0.35*	>0.1	0.07	>0.1	0.21	>0.1	0.14	>0.1	0.18
DMP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DBP	>0.1	0.20	**0.02**	**0.39**	**0.04**	**0.33**	>0.1	0.11	>0.1	0.13	**0.0004**	**0.77**	>0.1	0.22	>0.1	0.09	>0.1	0.07	>0.1	0.02
BBP	>0.1	0.04	>0.1	0.11	>0.1	0.00	>0.1	0.06	>0.1	0.05	>0.1	0.10	>0.1	0.03	>0.1	0.00	>0.1	0.08	>0.1	0.00
DEP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DnoP	>0.1	0.00	>0.1	0.11	>0.1	0.07	*>0.1*	*0.19*	>0.1	0.23	>0.1	0.11	**0.002**	**0.70**	>0.1	0.07	>0.1	0.02	>0.1	0.04
Fishweight	*>0.1*	0.21	*0.09*	*0.24*	>0.1	0.14	**0.05**	**0.29**	>0.1	0.114	>0.1	0.17	>0.1	0.04	>0.1	0.07	>0.1	0.01	>0.1	0.00

Immune gene expression in the anterior kidney, posterior kidney, and spleen, against 6 phthalate levels in gastrointestinal (GI) tissue (top three panels) and muscle tissue (bottom three panels). *In bold*: significant *R*-squared values. *In italics: R*-squared values that were close to being significant. All *p* values higher than 0.1 are indicated as $p > 0.1$

SEC SecHCmu, *MEM* MemHCmu, *ND* not performed

Table 4 2014 data

Phthalate	SEC		MEM		BLIMP	
	p	R^2	*p*	R^2	*p*	R^2
Liver						
DEHP	**0.001**	**0.59**	**0.01**	**0.38**	>0.1	0.01
DMP	ND	ND	ND	ND	ND	ND
DBP	ND	ND	ND	ND	ND	ND
BBP	>0.1	0.14	>0.1	0.07	**0.03**	**0.29**
DEP	>0.1	0.05	>0.1	0.01	>0.1	0.0047
DnoP	ND	ND	ND	ND	ND	ND
Muscle						
DEHP	*0.09*	*0.46*	*0.09*	*0.49*	*0.07*	*0.50*
DMP	**0.01**	**0.66**	*0.07*	*0.43*	**0.02**	**0.62**
DBP	>0.1	0.08	>0.1	0.06	>0.1	0.16
BBP	>0.1	0.05	>0.1	0.03	>0.1	0.09
DEP	ND	ND	ND	ND	ND	ND
DnoP	ND	ND	ND	ND	ND	ND

Immune gene expression in the anterior kidney against 6 phthalate levels in liver muscle tissue. *In bold*: significant *R* squared values ($p < 0.05$). *In italics*: *R*-squared values close to being significant. All *p* values higher than 0.1 are indicated as $p > 0.1$. *SEC* SecHCmu, *MEM* MemHCmu, *ND* not done

Discussion

This field study investigated potential immunomodulatory effects of chronic environmental phthalate exposure on the expression of B-lymphoid-specific genes in two wild populations of Dolly Varden. Expression patterns of these immune genes were determined in anterior kidney, posterior kidney and spleen using RT-qPCR, whereas, in parallel, levels of the six phthalates were determined in GI, muscle, and liver tissues in each fish. All fish in the study had detectable levels of one or more phthalate compounds, and numerous significant associations were found between immune gene expression and levels of phthalates detected in the three tissues as discussed below.

The sample size of the study was modest but sufficiently large to draw several important conclusions. It should be kept in mind that the collection of biological samples from Elizabeth Island Lake was a difficult, and often dangerous, undertaking because the rugged coastal shoreline is prone to unpredictable weather and extreme surf conditions, thereby limiting access by boat and aircraft.

Associations Between Phthalate Levels and Expression of Immune Genes

Phthalate-dependent changes in immune gene expression were most significant in anterior kidney (based on their *p* values using R). This is perhaps not coincidental because this is the site where new immune cells are generated, where developing immune cells mature, and where LLPCs are maintained; the anterior kidney clearly is an organ housing immune cells that are especially vulnerable to toxins. The presence of phthalates in three different tissue types, including muscle, suggests that when juvenile fish are chronically exposed to phthalates in vivo, they accumulate in such fish. Therefore, levels of phthalates found in fish tissues were inversely associated with the expression of immune genes that play essential roles during B-cell maturation, activation, and antibody production.

Most strikingly, higher DEHP/DMP tissue levels were associated with reduced expression of the secHCmu transcript, which encodes the immunoglobulin heavy chain for the secreted form of the antibody IgM. Antibodies bind to pathogens with high specificity and are essential for pathogen neutralization. Therefore, our data suggest that the presence of phthalates in tissues may *inhibit* antibody production in juvenile fish. This conclusion is supported by our previous in vitro study in rainbow trout (Martins et al. 2015), which showed a significant dose-dependent reduction of secHCmu transcripts in anterior kidney cell cultures after 7 days of DEHP exposure. In the same study, we also showed that DEHP inhibited the proliferation of IgM^+ plasmablasts. We proposed that DEHP inappropriately induces Blimp1 expression, which accelerates differentiation toward plasma cells and therefore is detrimental to effective antibody responses. This also explains why, in the current study, Blimp1 levels were reduced in fish with detectable phthalate levels: Blimp1 is most highly expressed in plasma cells (Lin et al. 2002); if fewer plasma cells were present, overall levels of Blimp1 would be reduced.

Significant associations between phthalates and the membrane-bound form of HCmu were also detected. Again, anterior kidney appeared to be most affected by phthalates with higher phthalate tissue levels being associated with lower memHCmu expression. What are the consequences of this inhibitory effect? MemHCmu is expressed in late-developing B cells including small pre-B and immature B cells. We speculate that DEHP causes (abnormal) expression of the Blimp1 gene in developing B cells, thus accelerating their differentiation at the cost of proliferative expansion. This is supported by our previous study using pre-B cell line PD31, which showed increased Blimp1 expression after culturing it in the presence of DEHP and furthermore a significant reduction in cell proliferation (Martins et al. 2015). Another group (Schlezinger et al. 2004) reported similar antiproliferative effects of (<25 μM) DEHP in pro-B and pre-B cells from mouse bone marrow. However, the effects of phthalates on Blimp1 expression in developing B cells have not been reported in mammals.

Dividing plasmablasts, which express both membrane and secreted forms of IgM, are likely targets for phthalates with detrimental consequences. As shown in our in vitro study (Martins 2015), DEHP inhibited the expansion of plasmablast populations causing a reduction in both memHCmu and secHCmu transcripts, which identical to what was observed in the current in vivo study. However, one limitation of our current approach is that it determined *overall* levels of such transcripts without identifying individual cells. The latter is necessary to verify that the abundance of IgM + plasmablasts was in fact reduced. Future studies should include flow cytometric analysis to positively identify plasmablasts and other B-lineage cells in phthalate-exposed fish populations.

Interaction Strength of Phthalates With the PPARγ Receptor

Phthalates are known agonists of nuclear hormone receptor, PPARγ, and mimic natural ligands such as PGJ2 and CLA (Hurst and Waxman 2003; Houseknecht et al. 2002). Structural analysis of phthalates (including the six used in this study) showed a striking similarity with natural ligands, particularly DEHP, DBP, and DNoP. Sarath Josh et al. (2014) analyzed functional groups essential for the active site of PPARγ in a recent molecular docking study. Their results showed several conserved amino-acids on the active site that conferred tight hydrogen bonding and pi–pi interactions necessary for firm holding of the ligand and, more importantly, provided direct evidence that the great majority of phthalates have strong binding affinities for PPARγ. In fact, the binding affinity for many phthalates, especially the di-phthalates, is higher than for natural PPARγ ligands such as CLA. This is important for our study because it suggests that when phthalates are present (in immune cells), they will likely outcompete natural ligands, thus resulting in preferential binding of phthalates and therefore chronic dysregulation of immune function.

Phthalate-PPARγ complexes, especially long-branched lipophilic phthalates such as DEHP and DBP, can act as transcription factors with a high transactivation potential (Bility et al. 2004). Hence, DEHP and DBP would be expected to be strong inducers of target genes like *Blimp1*. In agreement with that assumption, DEHP and DBP conferred the stronger associations with immune markers in our study.

Comparative Phthalate Tissue Levels

A limited number of studies have measured phthalate levels in fish. One study measured phthalate muscle levels in multiple fish species from highly phthalate-polluted coastal areas around Hong Kong. Muscle DEHP levels for these fish were in the range of 200–4260 ng/g (average 629 [Cheng et al. 2013]), >10 times higher than DEHP levels found in Elizabeth Lake fish muscle (10.6–122.3 ng/g [average 49.5]). However, levels of DMP were higher in Elizabeth Lake samples compared with the highly polluted Hong Kong samples (1.5–11.1 ng/g at Elizabeth Lake versus undetectable at −3 ng/g in Hong Kong samples), whereas muscle DBP concentrations were at least 100 times higher in the highly contaminated Hong Kong samples (430–2080 ng/g [average 950]) compared with Elizabeth Lake (5.6–15.9 ng/g [average 3.6]), thus illustrating the dramatic variation in relative levels of phthalates between sites. The dose-dependent effects of phthalates on B-cell development and activation pathways, as reported here, are very likely to have exponentially detrimental effects on the antibody response in fish residing in more highly contaminated environments.

Our juvenile fish typically had higher levels of phthalates, particularly for DEHP, in their tissues compared with the two adult species we studied. These data are in agreement with the negative association observed between phthalate levels and body weight within Dolly Varden populations from Elizabeth Lake. This suggests that smaller fish absorb and/or incorporate higher levels of phthalates compared with larger fish. Juvenile Dolly Varden primarily feed on sediment-dwelling insect larvae, invertebrates, and other bottom-dwelling taxa (Steward 2009). Phthalates, especially those with long lipophilic chains, such as DEHP, biodegrade slowly in the anaerobic conditions of sediment (Lertsirisopon et al. 2006; Pakalin et al. 2008; World Health Organization 2003). Hence, juveniles are likely accumulate such phthalates through feeding in the sediments of plastic-polluted waters.

So why are phthalate levels lower in larger fish? Possible explanations include diet, metabolism, and habitat location. As fish mature, they change diet toward food sources that perhaps have lower phthalate levels, e.g., flying insects instead of bottom-dwelling invertebrates. In addition, juvenile fish may simply incorporate more phthalates because they consume more food relative to their body weight compared with adults (Wargo-Rub et al. 2011). Furthermore, mature fish likely metabolize phthalates more efficiently than juveniles.

Exposure to moderate levels of phthalates during the earliest stages of life, when the immune system is still being established, may cause permanent, life-long damage. Virtually nothing is known about the early detrimental effects of phthalates on the developing immune system. However, at least one study has suggested this to be the case: Phthalate exposure during development increased risks of respiratory dysfunction and allergic disease in humans (Kimber and Dearman 2010). Clearly further investigations on phthalate effects during immune

development are required to fully understand the long-term implications of early exposure.

Relevant Levels of Phthalate Contamination in Water

As the most abundantly produced phthalate, there has been extensive research involving the levels of DEHP in biological aquatic systems. The World Health Organization Risk Assessment of DEHP (WHO 2003) has stated that the water guideline value of DEHP is 8 ng/mL. The Unites States Environmental Protection Agency (USEPA) maximum contaminant level (MCL) of DEHP in drinking water has been set to 6 ng/mL (United States Environmental Protection Agency 1991). Because the measured DEHP in collected water samples from Elizabeth Lake varied from 0.6 to 35.3 ng/mL, this puts Elizabeth Lake significantly higher than EPA's maximum contaminant level. However, Elizabeth Lake is not an anomaly of high phthalate levels. A composite study by Zeng et al. (2009) measuring DEHP in water found ranges from 3.1 to 64.3 ng/mL (Klang River Basin, Malaysia), 0.33–97.8 ng/mL (Brandenburg and Berlin, Germany), 0.301–15.1 ng/mL (Talihu Lake, China), and 0.06–23.6 ng/mL (Eastern Cape, South Africa), whereas another composite study (Selvaraj et al. 2014) reported DEHP water levels ranging from 0.9 to 5 ng/mL (Dommel, Netherlands), 0.01–0.04 ng/mL (Hoje, Sweden), 0.12–5.0 ng/mL (Spain), and 0.16–0.39 ng/mL (Seine, France).

Taking all this information into account, it is important to recognize that even moderate levels of phthalates, as found in Elizabeth Island Lake, can lead to long-term effects on the immune system if there is chronic exposure, especially in juvenile organisms, as we show here. Biodegradation of DEHP and other phthalates in sediment and water is slow and temperature-dependent, thus leading to accumulation in polluted waterways. This puts exposed fish in colder climates, such as Alaska, at higher risks.

In summary, this study shows for the first time that chronic natural exposure to phthalates in a wild population of juvenile fish leads to its accumulation in GI, liver, and muscle tissues, and is associated with reduced expression of the genes necessary for antibody response. Together with our previous in vitro data, the results support a model in which chronic exposure to phthalates dysregulates the normal B-cell developmental and activation pathways and subsequently the antibody response in salmonids.

Acknowledgments The authors thank Alissa Moore and Erik Pallister for help with sample collection, Erik Pallister for first observations on marine-debris pollution in Elizabeth Lake, Gulf of Alaska Keeper for donation of ship-boarding time, and Will Frost for helpful comments. This study was funded by a grant from the Exxon Valdez Oil Spill Trustee Council Project 14120116 (C. P.). The chemical analytical determinations were funded in part by a Grant from the National Fish and Wildlife Foundation Marine Plastic Debris Section (K. M. and J. K.) and performed on instrumentation provided through a Major Research Instrumentation Grant of the National Science Foundation (Grant No. MRI 0813850 [K. M. and B. H.]) to the Applied Science Engineering and Technology Laboratory at the University of Alaska Anchorage.

References

ADF&G (2015) Anadromous catalog: nominations. Interactive mapping

Barr M, Mott K, Zwollo P (2011) Defining terminally differentiating B cell populations in rainbow trout immune tissues using the transcription factor XbpI. Fish Shellfish Immunol 31:727–735

Barse AV, Chakrabarti T, Ghosh TK, Pal AK, Jadhao SB (2007) Endocrine disruption and metabolic changes following exposure of Cyprinus carpio to diethyl phthalate. Pestic Biochem Physiol 88:36–42

Batista-Pinto C, Rodrigues P, Rocha E, Lobo-da-Cunha A (2005) Identification and organ expression of peroxisome proliferator activated receptors in brown trout (Salmo trutta f. fario). Biochim Biophys Acta 1731:88–94

Bility MT, Thompson JT, McKee RH, David RM, Butala JH, Vanden Heuvel JP et al (2004) Activation of mouse and human peroxisome proliferator-activated receptors (PPARs) by phthalate monoesters. Toxicol Sci 82:170–182

Bromage ES, Kaattari IM, Zwollo P, Kaattari SL (2004) Plasmablast and plasma cell production and distribution in trout immune tissues. J Immunol 173:7317–7323

Cheng Z, Nie X-P, Wang H-S, Wong M-H (2013) Risk assessments of human exposure to bioaccessible phthalate esters through market fish consumption. Environ Int 57–58:75–80

Cole (2013) Microplastic ingestion by zooplankton. Environ Sci Technol 47:6646–6655

Diaz-Rosales P, Bird S, Wang TH, Fujiki K, Davidson WS, Zou J et al (2009) Rainbow trout interleukin-2: cloning, expression and bioactivity analysis. Fish Shellfish Immunol 27:414–422

Feige JN, Gelman L, Rossi D, Zoete V, Métivier R, Tudor C et al (2007) The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor gamma modulator that promotes adipogenesis. J Biol Chem 282:19152–19166

Garcia-Bates TM, Peslak SA, Baglole CJ, Maggirwar SB, Bernstein SH, Phipps RP (2009) Peroxisome proliferator-activated receptor gamma overexpression and knockdown: impact on human B cell lymphoma proliferation and survival. Cancer Immunol Immunother 58:1071–1083

Glass C, Ogawa S (2006) Combinatorial roles of nuclear receptors in inflammation and immunity. Nat Rev Immunol 6:44–55

Hansen JF, Bendtzen K, Boas M, Frederiksen H, Nielsen CH, Rasmussen ÅK et al (2015) Influence of phthalates on cytokine production in monocytes and macrophages: a systematic review of experimental trials. PLoS ONE 10:0120083

Harris DC (2010) Quantitative chemical analysis. Freeman and Company, New York

Houseknecht KL, Cole BM, Steele PJ (2002) Peroxisome proliferator-activated receptor gamma (PPARgamma) and its ligands: a review. Domestic Anim Endocrinol 22:1–23

Huang P-C, Tien C-J, Sun Y-M, Hsieh C-Y, Lee C-C (2008) Occurrence of phthalates in sediment and biota: relationship to aquatic factors and the biota-sediment accumulation factor. Chemosphere 73:539–544

Hurst CH, Waxman DJ (2003) Activation of PPAR and PPAR by environmental phthalate monoesters. Toxicol Sci 74:297–308

John SA, Garrett-Sinha LA (2009) Blimp1: a conserved transcriptional repressor critical for differentiation of many tissues. Exp Cell Res 31:1077–1084

Kimber I, Dearman RJ (2010) An assessment of the ability of phthalates to influence immune and allergic responses. Toxicology 271:73–82

Koch HM, Preuss R, Angerer J (2006) Di (2-ethylhexyl) phthalate (DEHP): Human Metabolism and internal exposure—an update and latest results. Int J Androl 29(1):155–165

Koike E, Inoue K, Yanagisawa R, Takano H (2009) Di-(2-ethylhexyl) phthalate affects immune cells from atopic prone mice in vitro. Toxicology 259:54–60

Lertsirisopon R, Soda S, Sei K, Ike M, Fujita M (2006) Biodegradability of four phthalic acid esters under anaerobic condition assessed using natural sediment. J Environ Sci China 18:793–796

Lin K-I, Angelin-Duclos C, Kuo TC, Calame K (2002) Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. Mol Cell Biol 22:4771–4780

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–408

Mallatt (1985) Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can J Fish Aquat Sci 42:630–648

Martins (2015) Di(2-Ethylhexyl) phthalate inhibits B cell proliferation and reduces the abundance of IgM-secreting cells in cultured immune tissues of the rainbow trout. Fish Shellfish Immunol 44:332–341

Martins K, Applegate B, Hagedorn B, Kennish J, Zwollo P (2015) Di(2-ethylhexyl) phthalate inhibits B cell proliferation and reduces the abundance of IgM-secreting cells in cultured immune tissues of the rainbow trout. Fish Shellfish Immunol 44:332–341

Moore CJ (2008) Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. Environ Res 108:131–139

Pakalin S, Aschberger K, Cosgrove O, Lund B-O, Paya-Perez A, Vegro S (2008) Bis(2-ethylhexyl)phthalate: summary risk assesment report. Council Regulation EEC No. 793/93

Ramon S, Bancos S, Thatcher TH, Murant TI, Moshkani S, Sahler JM et al (2012) Peroxisome proliferator-activated receptor B cell-specific-deficient mice have an impaired antibody response. J Immunol 189:4740–4747

Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS et al (1999) PPARγ is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell 4:611–617

Sakazaki H, Ueno H, Nakamuro K (2002) Estrogen receptor α in mouse splenic lymphocytes: possible involvement in immunity. Toxicol Lett 133:221–229

Sarath Josh MK, Pradeep S, Vijayalekshmi Amma KS, Balachandran S, Abdul Jaleel UC, Doble M et al (2014) Phthalates efficiently bind to human peroxisome proliferator activated receptor and retinoid X receptor α, β, γ subtypes: an in silico approach: Molecular interactions of phthalates with hPPARs and hRXRs. J Appl Toxicol 34:754–765

Schettler T (2006) Human exposure to phthalates via consumer products. Int J Androl 29:134–139

Schlezinger JJ, Jensen BA, Mann KK, Ryu H-Y, Sherr DH (2002) Peroxisome proliferator-activated receptor-mediated NF-B activation and apoptosis in pre-B Cells. J Immunol 169:6831–6841

Schlezinger JJ, Howard GJ, Hurst CH, Emberley JK, Waxman DJ, Webster T et al (2004) Environmental and endogenous peroxisome proliferator-activated receptor γ agonists induce bone marrow B cell growth arrest and apoptosis: interactions between mono (2-ethylhexyl) phthalate, 9-cis-retinoic acid, and 15-deoxy-Δ12 14-prostaglandin J2. J Immunol 173:3165–3177

Schlezinger JJ, Emberley JK, Bissonnette SL, Sherr DH (2007) An L-tyrosine derivative and PPAR agonist, GW7845, activates a multifaceted caspase cascade in bone marrow B cells. Toxicol Sci 98:125–136

Schug TT, Janesick A, Blumberg B, Heindel JJ (2011) Endocrine disrupting chemicals and disease susceptibility. J Steroid Biochem Mol Biol 127:204–215

Selvaraj KK, Sundaramoorthy G, Ravichandran PK, Girijan GK, Sampath S, Ramaswamy BR (2014) Phthalate esters in water and sediments of the Kaveri River, India: environmental levels and ecotoxicological evaluations. Environ Geochem Health

Setoguchi K, Misaki Y, Terauchi Y, Yamauchi T, Kawahata K et al (2001) Peroxisome proliferator-activated receptor-γ haploinsufficiency enhances B cell proliferative responses and exacerbates experimentally induced arthritis. J Clin Invest 108:1667–1675

Shanker R, Ramakrishma C, Seth PK (1985) Degradation of some phthalic acid esters in soil. Environ Pollut Ser Ecol Biol 39:1–7

Steward D (2009) Fish diets and food webs in the Northwest territories: Dolly Varden. Canadian Manuscript Report of Fisheries and Aquatic Sciences 2912. Central and Arctic Region, Fisheries and Oceans Canada, Winnipeg, MB, Canada

United States Environmental Protection Agency (1991) National primary drinking water regulations. Federal Register; Part 12, 40 CFR Part 141, p 395

Wargo-Rub AM, Sandford BP, Gilbreath LG, Myers MS, Peterson ME, Charlton LL, Smith SG, Matthews GM, Plaza RD (2011) Comparative performance of acoustic tagged and passive integrated transponder tagged juvenile Chinook salmon in the Columbia and Snake rivers, 2008. In: Report of the National Marine Fisheries Service to the US Army Corps of Engineers, Portland District, Portland, Oregon

World Health Organization (2003) Di(2-ethylhexyl)phthalate in drinking water. WHO, Geneva

Wu L, Yan C, Czader M, Foreman O, Blum JS, Kapur R et al (2012) Inhibition of PPAR in myeloid-lineage cells induces systemic inflammation, immunosuppression, and tumorigenesis. Blood 119:115–126

Zapata Cooper (1990) The immune system. Comparative histopathology. Wily, Chichester

Zeng F, Wen J, Cui K, Wu L, Liu M, Li Y et al (2009) Seasonal distribution of phthalate esters in surface water of the urban lakes in the subtropical city, Guangzhou, China. J Hazard Mater 169:719–725

Zwollo P (2011) Dissecting teleost B cell differentiation using transcription factors. Dev Comp Immunol 35:898–905

Zwollo P, Cole S, Bromage E, Kaattari S (2005) B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. J Immunol 174:6608–6616