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Polycyclic aromatic hydrocarbons in blood related to lower body mass in common loons

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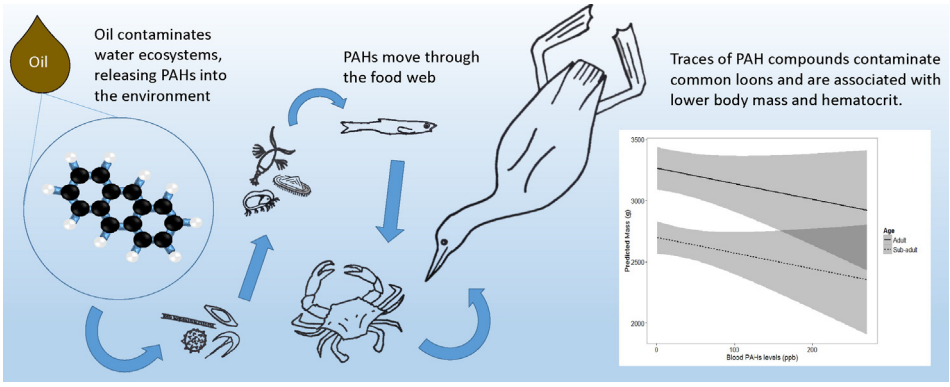
HIGHLIGHTS

- PAHs were found in loon blood in 4/5 years following the Gulf oil spill.
- PAH signatures changed, with light-weights predominating in all years but 2013.
- High PAH levels correlated with low body mass and hematocrit for all birds.
- High PAH levels correlated with high total blood proteins in adult birds.

GRAPHICAL ABSTRACT



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ABSTRACT

We captured 93 wintering adult and subadult Common Loons (*Gavia immer*) in coastal Louisiana from 2011 to 2015 following the Deepwater Horizon oil spill. We tested blood samples for exposure to polycyclic aromatic hydrocarbons (PAHs) and measured physiological variables including hematocrit, hemoglobin and total blood protein. PAH concentrations in loon blood differed from year to year and by age class. High PAH concentrations were significantly related to lower body masses in both adult and subadult birds and higher serum protein levels in adults only. PAH concentrations had marginal relations with both hematocrit and hemoglobin levels. The types of PAHs detected also underwent a major shift over time. The PAHs detected in 2011, 2012, and 2015 were primarily low molecular weight (three carbon rings); however, in 2013, most detected PAHs were high molecular weight (four carbon rings). It is unclear what events led to the increase in PAH concentrations and the shift in type of PAHs over time.

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1. Introduction

1.1. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are global pollutants derived from fossil fuels and produced during pyrogenic and petrogenic processes. Pyrogenic PAHs are derived from forest fires, engine emissions, and coke production, while petrogenic sources include petroleum combustion and discharge (Albers, 2006; Eisler, 1987). PAHs have been detected in air, soils, drinking water, and groundwater, and although they break down when exposed to UV light or oxygen, they can persist in the sediment for decades (Albers, 2006). Lipid solubility of PAHs is high, but animals vary in their ability to assimilate these compounds. While many invertebrates cannot metabolize PAHs and thus accumulate them in the body, most vertebrates can rapidly metabolize PAHs (<2 weeks) with a well-developed mixed-function oxygenase system in their liver (Albers, 2006; Lawrence and Weber, 1984; Varanasi et al., 1989). Thus, although PAHs move through the food chain, potential for biomagnification of PAHs in vertebrates is thought to be low (Boehm et al., 2004; Broman et al., 1990; Kayal and Connell, 1995; Meador et al., 1995). PAH concentrations in marine organisms after a major oil spill have varied from no detectable increase to initially elevated levels that decrease to baseline within a few months to a few years (Al-Yakoob et al., 1993; Boehm et al., 2004; Xia et al., 2012). Several studies suggest that avian predators may be a good indicator of PAH contamination after a major oil spill due to the ingestion of contaminated prey (Alonso-Alvarez et al., 2007a; Paruk et al., 2014a; Seegar et al., 2015; Zuberogoitia et al., 2006).

PAHs are toxic at low concentrations (e.g., ng/g), and they can be carcinogenic or mutagenic to wildlife and humans (Albers, 2003; Menzie et al., 1992; Wang et al., 2002). Chronic exposure to sublethal PAH concentrations can cause several physiological impairments, leading to a number of health effects. These effects include liver damage (Leighton, 1993; Miller et al., 1978), hemolytic anemia (Leighton, 1993; Nisbet et al., 2013; Troisi et al., 2007; Yamato et al., 1996), impaired osmoregulation (Meador et al., 1995; Miller et al., 1978), weight loss (Burger and Tsipoura, 1998; Leighton, 1986), gastrointestinal damage (Leighton, 1986; Miller et al., 1978;), endocrine disruption (Franci et al., 2014), and immune suppression (Briggs et al., 1996; Nicolas, 1999; Reynaud and Deschaux, 2006; Rocke et al., 1984). Individuals with physiological impairments and poorer body condition may also experience lower reproductive success (Ainley et al., 1981; Day et al., 1997; Golet et al., 2002).

1.2. The Deepwater horizon oil spill

The Deepwater Horizon oil spill (DHOS) was the largest offshore oil spill in U.S. history. Approximately 780 million liters of crude oil (4.9 million barrels) were released into the northern Gulf of Mexico (nGOM) from early April through mid-July 2010 (McNutt et al., 2012), which is about 20 times the volume released from the 1989 Exxon Valdez into Prince William Sound, Alaska (Atlas and Hazen, 2011). Approximately 1700 km of coastline in the nGOM were impacted by oil (Michel et al., 2013), with the Louisiana coastline receiving the majority: 138 km of marsh shoreline were heavily oiled and an additional 364 km were lightly oiled. The oil released in the DHOS contained approximately 3.9% PAHs by weight. Because of their toxicity, PAHs are one of the principal contaminants of concern during an oil spill. After the DHOS, PAHs moved from the planktonic food web to upper-level trophic consumers, suggesting exposure risk to a wide variety of taxa (Mitra et al., 2005). Due to concern over the effects of exposure on vertebrates, several studies have called for continued monitoring of PAHs in the biota exposed to the DHOS (Carmichael et al., 2012; Gohlke et al., 2011; Sammarco et al., 2013).

1.3. Study species

Common Loons (*Gavia immer*) are long-lived (>25 years), piscivorous waterbirds that breed in freshwater lakes of northern North America and overwinter predominantly along seacoasts (Gray et al., 2014; Paruk et al., 2014a). Although wintering loons are primarily found near shore (Daub, 1989; Haney, 1990; Thompson and Price, 2006), many can be found several miles offshore as well (Jodice, 1993). A substantial portion of the interior North American loon population winters in the nGOM (Gray et al., 2014; Kenow et al., 2002; Paruk et al., 2014b), and adults exhibit a high level of winter site-fidelity (Paruk et al., 2015). Loons typically arrive at their wintering area in November, remain there for 4–5 months, and depart in late March to early April, depending on latitude (Paruk et al., 2015; Spitzer, 1995). Typically, beginning in January, loons undergo a simultaneous wing molt and are flightless for a few weeks (Woolfenden, 1967). Adults generally do not fly during mid-winter, even after wing molt is completed, and first or second-year birds rarely fly during this time (Evers et al., 2010; pers. obs.). As such, any PAHs in wintering loons would likely have been obtained locally. Breeding loons have been used as a bioindicator species for other contaminants (e.g. mercury; Evers, 2006), and they may be useful indicators of marine pollution on their wintering grounds as well (Paruk et al., 2014b).

1.4. Objectives and hypotheses

The long-term consequences of oil exposure on migratory birds overwintering in the nGOM have received limited attention despite the enormity of the spill and size of the contaminated area (Franci et al., 2014; Henkel et al., 2012). Acute tests of toxicity in the laboratory are insufficient for ecotoxicological risk assessment, and more field studies are needed in migratory wildlife to fully examine potential sublethal effects of PAHs. Our objective was to evaluate a population of Common Loons for potential exposure to PAHs from the DHOS event. We monitored internal PAH concentrations in loon blood over a period of five years to assess contamination patterns, and we tested for associations between PAH exposure and loon body mass and physiological markers. We hypothesized that birds would show the highest level of contamination in the year following the spill, and that there would be a decreasing trend in blood PAH levels with time. In addition, we hypothesized that PAH exposure may have a deleterious effect on loon physiological markers and body mass.

2. Materials and methods

2.1. Study area

The research area consists of numerous bays and watercourses associated with the much larger Barataria Bay of the Louisiana coast (see Fig. 1). These include: Bay Adams (29.365719°N, −89.620354°W), Bay Batiste (29.458630°N, -89.845540°W), Bastian Bay (29.308186°N, -89.647466°W), Bay Sansbois (29.470335°N, -89.771223°W), Grand Bayou (29.511698°N, −89.765879°W), and Lake Washington (29.385665°N, −89.735918°W). We chose this area because it received moderate to heavy oiling from the DHOS. The watercourses are within 10 km of shore, and are thus considered near-shore environments. The water is typically shallow (1–6 m) and turbid (0.6 m visibility; Paruk et al., 2014a). Other characteristics of the area are described in Paruk et al. (2014a).

2.2. Capture

We captured 93 loons from January–March 2011–2015, using a well-established spotlighting technique in which we boated into bays at night, located rafting or solitary loons, and captured them with a long-handled dip net (Evers, 2001). We did not discriminate between adults and subadults because of the opportunistic nature of this technique. Common Loons were aged by plumage (Evers et al., 2010) as either adult (any loon ≥20 months of age) or subadult (any loon <20 months of age); sexes cannot be determined by plumage. Birds were banded with a U.S. Geological Survey (USGS) aluminum band and a unique combination

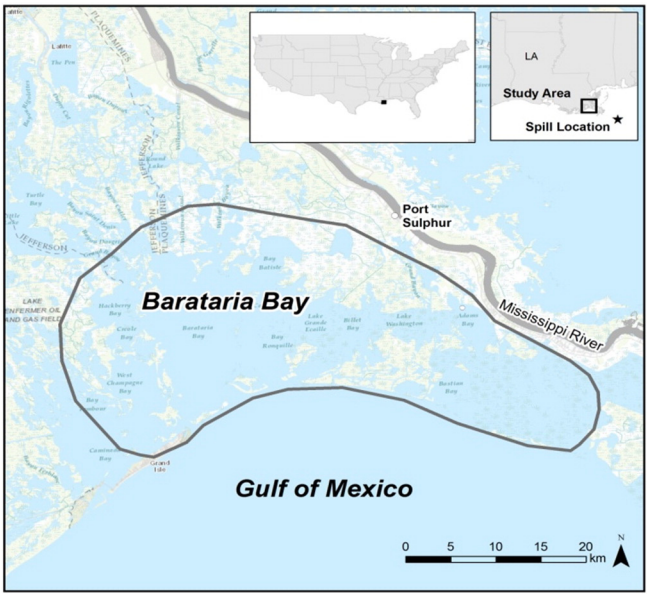

Fig. 1. Study area for wintering Common Loons. Bays and waterways are located near the Mississippi River Delta in southeastern Louisiana.

of plastic colored bands. A suite of morphometric measurements was taken, including bill width, length, and depth, tarsal width and length, and weight. Each bird was inspected for overall health, which consisted of checking the keel condition, alertness, and molt stage. Birds were released within 60 min of capture. Bird capture and handling methods complied with the guidelines of the American Ornithological Council on animal care under permit number #22636 and Louisiana State Wildlife and Fisheries Scientific Collection Permit (LNHP-11-005).

2.3. Blood collection and processing

We drew blood from either the metatarsal or jugular vein and stored it in 5–10 cm^3 vaccutainers with sodium heparin, hematocrit tubes, and EDTA tubes. Samples were processed in the laboratory within 3–6 h of collection. Blood stored in vacutainers was centrifuged at 3000 rpm for 10 min, and the plasma was separated from the cell elements and placed into multiple containers for shipping to laboratories. We extracted a small amount of plasma (0.1–0.2 mL) and placed it onto a refractometer to determine the total protein concentration (g/dL). Plasma was frozen for subsequent chemical analyses (Section 2.4). Plasma samples were shipped to the Avian and Wildlife Laboratory at the University of Miami to analyze cell blood chemistry and to the University of Connecticut for PAH analyses.

We measured both hematocrit (packed cell volume) and hemoglobin to assess oxidative blood cell damage from contaminant exposure. Hematocrit tubes were spun for 5 min at 12,000 rpm using a hematocrit rotor (Haefele et al., 2005). Hematocrit was determined using a hematocrit reader. To determine hemoglobin levels, blood stored in the EDTA tube was gently inverted 6–8 times before 25ul was transferred via pipette to a microcuvette. The microcuvette was inserted into a HemoCue analyzer (Fisher Scientific, 22-601-003) and held for 30 s before reading the display in g/dL. Baseline hematocrit measurements for breeding Common Loons averaged 47% (n = 117, range 27–62) for adults and 40% for sub-adults (n = 13, range 32–50; Haefele et al., 2005). In our study, adult loons with hematocrits <40%, and sub-adult loons with hematocrits <35%, were considered anemic.

2.4. PAH analysis

The plasma samples were shipped on dry ice to the University of Connecticut for PAH analysis at the end of the field season. In accordance with the U.S. Environmental Protection Agency (EPA) recommendations, the 16 most toxic parent PAHs were measured (Keith and Telliard, 1979) for all samples. The concentrations of naphthalene, acenaphthylene, acenaphthene, flourene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo(*a*)anthracene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*) pyrene, dibenzo(*a*,*h*)anthracene, benzo(*g*, *h*, *i*)perylene, and indenol(1, 2, 3-*c*-*d*)pyrene were determined. In addition, alkylated PAH concentrations were examined in 2012 and 2013, with a target compound list of 2-methylnaphthalene, 2.6-dimethylnaphthalene, 3-methylphenanthrene, 9-methylphenanthrene, and 1.7-dimethylphenanthrene. The detection limits were 1.0 ng/g in 2011 and 2012, and 5.0 ng/g in 2013, 2014 and 2015. Because of this change, we have removed individuals with <5.0 ng/g to standardize comparisons of between year frequencies. However, the lower concentrations from birds during 2011 and 2012 are still used in the models of relations between PAH levels and physiological measures and other numerical comparisons. The sum of concentrations from all individual PAHs was used to estimate the degree of oil contamination for individuals.

We extracted 0.25 g samples of plasma for parent and alkylated PAH compound analysis using liquid-liquid extraction in 2.5 ml hexane, vortex-mixed it for 5 min at 2500 RPM, and centrifuged it for 3 min at 4000 RPM (Pleil et al., 2010; Yeudakimaua et al., 2013). The hexane extraction was repeated three times, with the top organic layer transferred to a clean vial after each step. Once completed, the combined extract was concentrated to 2.0 ml under a nitrogen stream, followed by sample clean-up using SEP Pak Alumina-N, 6 cm^3, solid-phase extraction columns (Waters, Inc., Milford, MA) with methylene chloride as the elution solvent and reduced to a volume of 0.5 ml. Acetonitrile (4.0 ml) was added to each sample, concentrated to a final volume of 0.5 ml, and the internal standard of chrysene-d12 (Sigma Aldrich, Inc., St. Louis, MO) was added. Following extraction, the samples were analyzed for alkylated PAHs using an Agilent (Norwalk, CT) 6890 gas chromatograph equipped with a Restek (Bellefonte, PA) Rxi-5Sil MS column (30 m) using splitless injection coupled to a Waters (Milford, MA) QuattroMicro triple quadrupole mass spectrometer (GC/MS/MS). Parent PAHs were quantified suing a Waters (Milford, MA) Acquity ultra performance liquid chromatograph (UPLC) with fluorescence and photo diode array, which was equipped with an Acquity UPLC BEH Shield RP18 column (1.7 μm, 2.1 × 50 mm). All peaks were quantified against the internal standard, and the extraction efficiency was evaluated using a surrogate standard of naphthalene-d8 (Sigma Aldrich, Inc., St. Louis, MO). Standard quality assurance procedures were employed, including analysis of duplicate samples, method blanks, post-digestion spiked samples, laboratory control samples (LCS), and standard reference materials where available (SRM-2974a and SRM-1947; NIST Canada).

2.5. Statistical analyses

2.5.1. Temporal trends in PAH concentrations

We hypothesized that loons might show the highest concentration of PAHs in the first year following the DHOS, and also that PAH contamination would vary by age class. To test this, we used a generalized linear model to describe the temporal changes in PAH concentrations for each age class. For the response variable, we rounded the blood PAH concentrations to the nearest whole number and modeled these data using a negative binomial distribution. The large number of zeroes caused these data to be highly non-normally distributed and overdispersed, making traditional methods of modeling (e.g. log-transforming the data, linear models, Poisson regression) poor fits for our data. While rounding to the nearest whole number lost a bit of information, using negative binomial regression allowed us to correct for overdispersion in the data and best matched the distribution of our response variable. We tested whether age (subadult or adult), year (as a categorical variable) and the interaction between the two were associated with PAH

levels by testing two possible models. The first included age and year and the second included those terms and the interaction between the two. If the interaction term was statistically significant (using an F-test with $p < 0.05$), then we used that model to describe the relationship, otherwise we used the model with age and year to describe the pattern. For five individuals that were recaptured during the study, the data from the second capture were removed to avoid repeated sampling of individuals.

2.5.2. PAH signatures

We also tested if type of PAH (alkyl vs. parent) changed between the two years that we sampled (2012 and 2013) among the birds that were exposed to PAHs. To do this, we created a linear model comparing the log-transformed PAH concentration and year (as a categorical variable) for each type of PAH. Significance was assessed using an F-test.

2.5.3. Physiological variables for sublethal effects

To test the hypothesis that PAH exposure influenced loon physiology, we quantified the relationships between four different physiological endpoints as dependent variables — body mass, hematocrit, hemoglobin and total blood proteins — against blood PAH concentrations as the independent variable. We also included age class as a second independent variable to test whether the relationship between physiological endpoint and PAH concentration was age-specific. In body mass, we included tarsus width as an additional variable in all models to control for body size related increases in body mass.

Due to a large number of individuals below the detection threshold of 5 ng/g (n = 65), blood PAH concentration was a difficult term to use as an explanatory variable. While explanatory variables are not required to follow any distribution, the large amount of data clumped around zero led to data that was not ideal for linear regression, even if log-transformed. So we tested three ways of analyzing the PAH data: (1) PAH concentrations as a continuous variable, (2) PAH concentrations as presence/absence data where a value less than the lower detection limit (5 ng/g) was considered zero and any value above was considered 1, and (3) a combination of the previous two methods when both variables were added to the model. This third option forces the linear effects of continuous PAHs to be conditional upon exposure, allowing us to document if there are effects from being exposed or not, then, if exposed, describing the effect of increased exposure. We tested models that included all three types of PAH data and their interactions with age class. To select the best model, we first picked the combination of PAH variables that explained the most variance (via an F-test), then tested the best PAH variable combination with age class as an additive effect and interaction. If the interaction was significant, then the model with concentration, age class, and the interaction term was selected; if not, the model with age class and concentration was selected. All analyses were run using functions 'lm' and 'glm.nb' in the R Statistical Computing Environment (R Core Team, 2015).

3. Results

3.1. Frequencies and concentrations of PAHs

3.1.1. Parent PAHs

The percentage of loons with elevated PAH levels varied each year. In 2011 and 2014 (years one and four post-spill), no individuals had above the minimum detectable concentration; in 2012, about one-fourth of the birds had detectable PAHs; and in years 2013 and 2015, more than half of individuals had detectable PAH concentrations. The percentages of loons with detectable PAH concentrations are listed in Table 1.

The PAH concentrations in loon blood differed from year to year and by age class, but the pattern of differences between the age classes was similar across years (overall model pseudo-$r^2 = 0.15$). The dispersion parameter theta in the negative binomial model was found to be a significant improvement over the Poisson model ($\chi^2 = 4119$, df = 6, $p < 0.001$) and there were no high-leverage outliers. Overall, year was important for explaining variance in PAH concentration ($F_{2,64} = 8.4$, $p = 0.02$; Fig. 2). A pairwise comparison of years using the Tukey HSD test found that PAH concentrations in 2013 and 2015 were higher than 2012. Concentrations in all of these years were higher than in 2011 and 2014, which had no concentrations higher than 5 ng/g. Adults had slightly higher exposure to PAHs than subadult loons ($F_{1,79} = 3.1$, $p = 0.08$), and this relationship did not change among years.

Five loons were recaptured one to two years after originally being caught (4 adults, 1 subadult). All of the recaptured loons had low (<10 ng/g) initial parent PAH concentrations and low subsequent PAH concentrations in the recapture year.

3.1.2. Alkyl and parent PAHs, 2012–2013 subset

Loons were exposed to both parent and alkyl PAHs in 2012 and 2013. Slightly more than a third of the loons sampled for alkyl PAHs had above the minimal detection limit in both 2012 and 2013 (Table 2). Of the 50 loons captured during those years, 40 (80.0%) had detectable levels of PAHs. The great majority of the birds (92.5%, 37/40) had only one type of PAH (either parent or alkyl), and it was uncommon (7.5%, 3/40) for a loon to be exposed to both types. Of the loons exposed to only one PAH type, slightly more than half (56.8%, 21/37) had only parent PAHs and just under half (43.2%, 16/37) had alkyl PAHs only. There was a borderline significant difference in alkyl PAH concentrations between years ($F_{1,17} = 4.1$, $p = 0.06$; overall $r^2 = 0.19$) in exposed birds. The alkyl PAH concentrations were 0.7 ng/g [−0.03, 1.5] higher in 2013 compared to in 2012. There was no overall difference in the parent PAH concentrations in loons between 2012 and 2013, ($F_{1,17} = 0.8$, $p = 0.38$; overall $r^2 = 0.04$).

3.2. Signature of PAHs

3.2.1. Parent PAHs

The type of parent PAHs detected in loon blood varied over time (Table 3). The only detected parent PAH in 2012 was anthracene, which is a 3-carbon ring with a low molecular weight (LMW ≤ 178). In contrast, medium to high molecular weight (MMW = 202, HMW ≥ 228) were detected in 2013, and anthracene was entirely absent (Table 3). The most common parent PAH detected was fluoranthene (MMW, 100%, 15/15), followed by the HMW compounds benzo(*b*)fluoranthene (80%, 12/15), benzo(*a*)pyrene (13%, 2/15), and chrysene (13%, 2/15). No PAHs were detected in loons in 2014 (0%, 0/13). All parent PAHs detected in 2015 were lightweight aromatics (100%, 7/7). Anthracene was, again, the most common LMW parent PAH detected (100%, 7/7), followed by phenanthrene (44.4%, 3/7) and acenaphthene (15.4%, 1/7).

3.2.2. Alkyl PAHs

Only LMW alkyl PAHs (2-methylnaphthalene, 142.20 g/mol) were detected in 2012 and 2013; however, the PAHs that predominated in 2013 were higher molecular weight (methylphenanthrenes, 192.26 g/mol). The 2012 alkyl PAH profile consisted of 2-methylnaphthalene (89%; 8/9 samples), 3-methylphenanthrene (22%; 2/9) and 9-methylphenanthrene (11%; 1/9); but in 2013, 2-methylnaphthalene was not detected (0%, 0/10), and 3-methylphenanthrene (60%, 6/10) and 9-methylphenanthrene (100%, 10/10) were much more prevalent.

Table 1
Total parent PAH concentrations in Common Loons by year from the Louisiana coast.

Year	# loons caught	# loons w/PAHs	Frequency (%)	$\bar{x} \pm$ SE PAH concentration (ng/g)	Upper PAH value
2011	17	0	0	<5.0	<5.0
2012	21	5	23.8	6.0 ± 0.4	8.0
2013	29	19	61.9	103.4 ± 16.3	270.2
2014	13	0	0	<5.0	<5.0
2015	13	7	53.8	89.9 ± 17.4	163.5
Total	93	31	33	83.1 ± 15.2	270.2

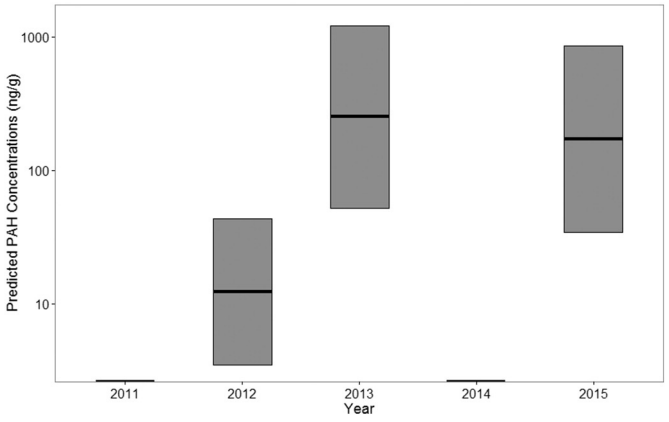

Fig. 2. Predicted PAH levels in adult Common Loons from the Louisiana coast. Black lines represent the predicted means for each year and the gray bars are the 95% confidence interval. PAHs were not detected at levels >5 ng/g in 2011 and 2014, so we were not able to predict PAH concentrations in these years. Overall pseudo-r^2 for this model was 0.15.

3.3. Sublethal effects

3.3.1. Body mass

PAH concentrations were best explained by age class, tarsus width, and categorical PAH (less than the detection limit vs. greater than the detection limit; overall $r^2 = 0.41$). Adults were heavier than subadults by 413 g [216, 609] (the parameter estimate mean [95% confidence interval]) on average ($F_{1,92} = 18.3$, $p < 0.001$), an increase of 1 mm of tarsus width equated to a 190 g [110, 269] increase in body mass ($F_{1,92} = 23.0$, $p < 0.001$), and individuals with PAH exposure were 274 g [69, 480] lighter than those with undetectable amounts ($F_{1,92} = 23.0$, $p < 0.001$; Table 4). This analysis (Fig. 3) did not include recaptures, but as a note, we did have 5 over the course of the study. In this small subset, four of the five recaptured loons (80.0%) had gained body mass (500 g ± 34) upon recapture. One adult, however, lost 50 g (approximately 3.33% of its original weight).

3.3.2. Hematocrit, hemoglobin and protein levels

The best model for explaining variation in hematocrit levels included age and categorical exposure to PAH (overall $r^2 = 0.21$). Both age class ($F_{1,59} = 9.9$, $p < 0.01$) and PAH exposure ($F_{1,59} = 5.7$, $p = 0.02$) were important in the final model (Fig. 4). Subadults had 6% [2.3, 10.1] less hematocrit than adults, and loons with detectable amounts of PAHs had 3% [−1.0, 6.9] less hematocrit than loons without measurable PAHs (Table 4). While both age class and PAH exposure were important to the model, the effect of PAH exposure on hematocrit was individually much weaker, with confidence intervals overlapping zero, lessening the significance of this result. Three subadult loons were considered anemic, with hematocrit (and corresponding PAH concentrations) values of 25 (10 ng/g), 26 (201 ng/g) and 35 (43 ng/g). Two of these individuals were the lightest (<2000 g) subadults captured (n = 60).

The best model for explaining hemoglobin included age class and categorical PAH exposure, but model fit was mediocre (overall $r^2 = 0.11$, Fig. 5). Neither age class ($F_{1,60} = 3.7$, $p = 0.06$) nor PAH exposure ($F_{1,59} = 3.6$, $p = 0.06$) was a significant predictor of hemoglobin levels, though both produced borderline effects. Adults had 1.5 ppb [−0.1, 3.0] higher hemoglobin levels than subadults, and birds with PAH exposure had 0.9 ppb [−0.5, 2.3] lower hemoglobin levels than birds with undetectable levels (Table 4).

The total proteins in the blood had a more complex relationship with PAH levels. The interaction between age class and categorical PAH exposure was significant and included in the top model (overall $r^2 = 0.29$). The base terms of the model, while statistically less important due to the included interaction term, suggest that birds exposed to PAHs had about 8 g/dL [2.3, 13.9] more total proteins than those that were not ($F_{1,63} = 0.9$, $p = 0.34$), and subadults had 4.6 g/dL [1.4, 7.9] more than adults ($F_{1,63} = 2.9$, $p = 0.11$). Once we include the interaction, adult birds exposed to PAHs have even more total proteins than exposed subadults by 9 g/dL [3.16], more than expected when using the base model ($F_{1,63} = 8.4$, $p < 0.01$; Fig. 6). The sum of these terms shows that adult birds had increased total protein concentrations when exposed to PAHs while subadults did not (Table 4).

Table 2
Alkyl PAH concentrations in Common Loons by year from the Louisiana coast.

Year	# loons caught	# loons w/PAHs*	Frequency (%)	$\bar{x}$ + SE PAH concentration (ng/g)	Upper PAH value
2012	21	9	42.9	9.3 ± 0.4	91.8
2013	29	10	34.5	20.3 ± 7.2	158.0
Total	50	19	38.0	15.2 ± 4.3	158.0

4. Discussion

4.1. Temporal trends, patterns and sources of contamination

4.1.1. Temporal trends and patterns of parent PAHs

Blood PAH concentrations in Common Loons were highest the third year after the DHOS, contrary to our hypothesis that they would spike immediately after the spill. Also, there was no consistent pattern in blood PAH concentrations in loons from year-to-year. The fact that we did not detect an increase in PAHs during the first two years is consistent with results of other studies in the nGOM. For example, in the first year after the DHOS, only low concentrations of PAHs were found in the water column, oysters, crustaceans, and finfish (Allan et al., 2012; Carmichael et al., 2012; Fry and Anderson, 2014; Gohlke et al., 2011; Xia et al., 2012), as well as in Northern Gannets (*Morus bassanus*; Franci et al., 2014). Common Loons feed on both invertebrates and fish during the winter months (Daub, 1989; McIntyre, 1978), and because they likely obtain PAHs through ingesting exposed prey rather than from the water column, oil may have to settle before effects are seen in loons. Loons in our study area consume a high proportion of detritus-feeding invertebrates (BRI, unpublished data); detritus feeders accumulate sedimented compounds rather than suspended ones. Additionally, a variety of factors such as feeding mode and rate, absorption via ventilation over the gills, and chemical hydrophobicity affect the uptake and accumulation of PAHs in these lower trophic level organisms (Meador et al., 1995), which will change uptake and PAH levels in loons. Poorly understood uptake patterns by invertebrates could cause the unexpected patterns we see in loons.

There was no consistent pattern of blood PAH concentrations in loons from year-to-year. For example, loon PAH concentrations were low in the winter of 2011, 6–8 months after the DHOS, remained low in 2012, spiked in 2013, were low again in 2014, and spiked again in 2015 (Table 2). Without pre-spill data, it is impossible to know the previous annual pattern of PAH contamination in top trophic predators feeding in the nGOM, and therefore it is challenging to quantify how much oil (and PAHs) from the DHOS influenced our results. The presence of alkyl PAHs in 2012 and 2013 loon samples indicate that some of the PAHs came from a petroleum source, but we cannot link them directly to the DHOS. There are natural petroleum seeps, petroleum leaks from underwater wells and even small annual spills in the nGOM that may also have contributed to our results (Kannan and Perrotta, 2008). Also, there are potential sources of pyrogenic PAHs from atmospheric deposition or water transport (via the Mississippi River) which may have contributed to the annual fluctuation of our results.

In 2013 (33–35 months post-spill), changes in PAH concentrations and frequencies in loons compared to 2011 and 2012 were striking. There was a significant 17-fold increase in parent PAH concentrations between years (6 ng/g = 2012 vs. 103 ng/g = 2013), and a significant increase in frequency of contamination (13.2% in 2011 and 2012 vs.

Table 3
Type and frequency of parent PAHs in loon samples by year*.

Year	Acenapthene (154.2)**	Phenanthrene (178.2)	Anthracene (178.2)	Fluoranthene (202.3)	Benzo(*a*)pyrene (252.3)	Benzo(−)fluoranthene (252.3)	Chrysene (228.3)
2011	0/0	0/0	0/0	0/0	0/0	0/0	0/0
2012	0/5	0/5	5/5	0/5	0/5	0/5	0/5
2013	0/15	0/15	0/15	15/15	2/15	12/15	2/15
2014	0/0	0/0	0/0	0/0	0/0	0/0	0/0
2015	1/7	3/7	7/7	0/7	0/7	0/7	0/7

* = more than one type of PAH can be detected/sample; ** = Molecular Weight

61.9% in 2013). This high exposure rate in 2013 suggests something atypical may have occurred. One possible explanation for this was hurricane Isaac. For a period of several days in late August 2012, high winds (100–137 km/h) associated with Isaac centered over our study area in southeast Louisiana, stirring up sediments (Brown and Brennan, 2012) and producing a storm surge of 3.4 m (Pasch and Roberts, 2012). Since PAHs can persist in sediments for many years (DeMott et al., 2010; Eisler, 1987; Srogi, 2007), it is possible that Isaac remobilized the settled oil from the DHOS, which then made its way into the food chain and was detected in loons 5–6 months following the hurricane. Some evidence for this hypothesis is that there was a dramatic shift from LMW to HMW parent PAHs between 2012 and 2013 (in 2012, all parent PAHs were anthracene, but in 2013 fluoranthene, benzo(*b*)fluoranthene, benzo(*a*)pyrene, and chrysene were present). HMW PAHs are more likely to settle than LMW (Stout et al., 2001), and the prevalence of heavier molecules in loon blood following the hurricane suggest that settled oil from the DHOS may have been re-mobilized by Isaac. There are likely several factors such as the proportion of oil that settled to the bottom, the likelihood of subsequent resuspension, the rate of biodegradation and photodegradation, and the type and composition of the oil (Gohlke et al., 2011) that need to be considered in understanding the duration and magnification of PAHs in the food chain in the nGOM.

Despite the significant increase in frequency and concentrations of PAHs in loons in 2013, the spike was temporary, as PAHs were undetected in 2014 loons. Since there was no severe storm event to stir up sediments in 2014, we expected PAH concentrations and frequencies would remain low in 2015, but this was not the case (Table 2). Instead, 54% of the loons had PAHs and the average concentration was 89.9 ng/g (Table 1). Also, there was another shift in the PAH signature, with only LMW PAHs being detected, more similar to what was observed in 2011 and 2012. The origin of the PAHs detected in 2015 loons is unclear. We concluded that this region of coastal Louisiana, due to its proximity to the mouth of the Mississippi River, and the prevalence of natural petroleum seeps in the area, is likely being exposed to both pyro- and petrogenic sources of PAHs, irrespective of the DHOS event.

4.2. Sublethal effects

4.2.1. Body mass

In our study, elevated blood PAH concentrations in loons were associated with lower body masses for both adults and subadults. Several studies have shown that gastrointestinal effects of internal oil exposure can lead to wasting and diminished body mass caused by malabsorption of nutrients, hemorrhagic enteritis, and degeneration of intestinal villi (Briggs et al., 1996; Fry and Lowenstine, 1985; Newman et al., 2000; Tseng, 1999). However, some experimental (Boersma et al., 1988) and field (Alonso-Alvarez et al., 2007b; Golet et al., 2002) studies show no effect of PAHs on body mass. More data are needed on this topic, but it may be that species are affected differently by contaminants, making comparisons among studies difficult. Also, loons may be particularly susceptible to the effects of oil contamination during the winter because they experience a number of stressors during that time in their annual cycle.

Migratory organisms in marine environments face both physiological and environmental stressors that they do not face on the breeding grounds (Murphy and King, 1992), including salinity changes, hypoxia, pathogens, and winter storms (Spitzer, 1995; Whitehead, 2013). Winter storms stir up sediments and increase water turbidity; because loons are primarily visual predators (Barr, 1996), they are particularly vulnerable to such disturbances. In addition,

Common Loons undergo a synchronous wing molt during midwinter (Woolfenden, 1967), which requires additional caloric intake and heightened metabolic rates (Howell, 2010). If energy reserves are not available to meet this increased demand, health may be compromised. The leading cause of mortality in loons, especially during the winter, is emaciation syndrome, which occurs when individuals are underweight and malnourished (Spitzer, 1995). It is likely that emaciation syndrome is directly linked to these additional winter stressors (Alexander, 1991; Forrester et al., 1997; Spitzer, 1995), which may include exposure to PAHs.

Interspecific comparisons of the relationship between PAHs and body mass can be challenging because of the variation among orders or families in the tolerance and metabolism of the compounds. Also, differences in broad geographic and ecological conditions, as well as differences in oil types, may render comparisons inconclusive. More importantly, estimates of critical exposure concentrations developed in controlled laboratory conditions may underestimate the real-world risk, because synergistic interactions with natural stressors could exacerbate chemically-induced damage (Sih et al., 2004; Whitehead, 2013). Our field data indicate elevated PAH concentrations affect the body mass of Common Loons, which causes concern since survival and fitness are directly linked to body condition (Weimerskirch, 1992; Schmutz and Ely, 1999).

Table 4
Sublethal Effects of PAHs on adult and subadult Common Loons.

Parameter	Adult		Subadult	
	PAH exposed		PAH exposed	
	Yes (n*)	No (n)	Yes (n)	No (n)
Body Mass (g)	2950 + 142 (11)	3450 + 82 (17)	2491 + 68 (41)	2938 + 94 (24)
Hematocrit (%)	50.8 + 1.0 (10)	55.4 + 1.6 (13)	46.2 + 2.0 (27)	47.1 + 1.0 (17)
Hemoglobin (g/dL)	17.3 + 0.5 (9)	17.2 + 0.6 (11)	14.8 + 0.1 (29)	16.5 + 0.6 (16)
Total Proteins (g/dL)	4.7 + 0.2 (11)	4.1 + 0.2 (13)	4.5 + 0.1 (28)	4.6 + 0.1 (17)

* = sample size.

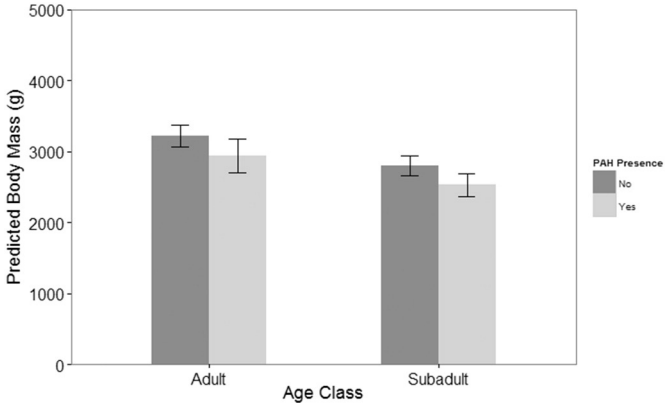

Fig. 3. Predicted mean masses for adult and subadult loons with PAH above 5 ng/g (light gray) or below 5 ng/g (dark gray). Error bars represent the 95% confidence interval of the mean. Overall r^2 for this model was 0.41.

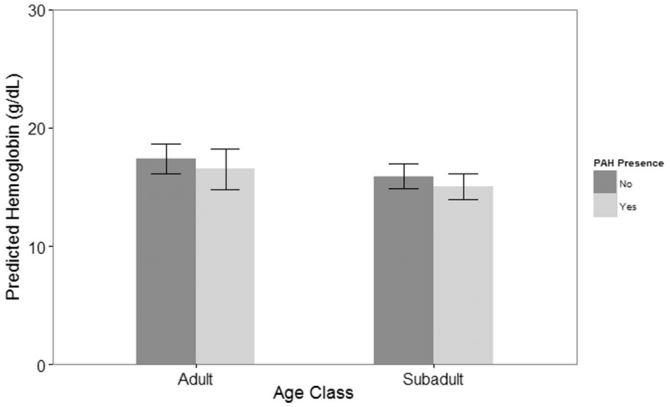

Fig. 5. Predicted hemoglobin concentrations for adult and subadult loons with PAH above 5 ng/g (light gray) or below 5 ng/g (dark gray). Error bars represent the 95% confidence interval of the mean. Overall r^2 for this model was 0.11.

4.2.2. Hematocrit, hemoglobin and protein levels

Red blood cells are critical for delivering oxygen to vital organs in the body via hemoglobin. Anemia and Heinz bodies (denatured hemoglobin) often result from experimental oil exposure (Balseiro et al., 2005; Fry and Lowenstine, 1985; Leighton et al., 1983; Miller, 1978; Yamato et al., 1996), but results from field studies have been mixed. For example, hematocrit levels decreased in one waterbird species following the Braer Oil Spill (Black-legged Kittiwake, *Rissa tridactyla*), but not in three others (Shag, *Phalacrocorax aristotelis*; Common Murre, *Uria aalge*; and Arctic Tern, *Sterna paradisaea*; Monaghan et al., 1995). We observed marginal decreases with PAH exposure in both hematocrit and hemoglobin concentrations in loons.

Total blood protein is often used to determine the health status of birds. In general, high levels indicate healthy individuals and low levels suggest that birds may have low protein in their diet or are generally malnourished (Harr, 2002). Since adult loons have higher winter survival than subadults (Paruk et al., 2015), we surmised adults would be in better body condition and have higher blood protein levels than subadults. In addition, we predicted subadults would be more affected by internal PAH concentrations than adults. Our results did not support these assumptions; total proteins were higher for adults with PAHs than ones without, but juveniles had similar protein levels regardless of exposure. Total blood proteins may increase in response to infections and inflammation (Alonso-Alvarez and Ferrer, 2001), so this could explain why adult loons had elevated blood protein levels when exposed to PAHs. Also, because adults have more developed organ systems and immune responses compared to subadults, it is possible they were responding to the PAH exposure while subadults were not.

5. Conclusions

Low but chronic levels of PAHs in the environment may lead to a number of sublethal effects that decrease fitness and survival in wildlife. In fact, chronic oil exposure may ultimately have a stronger long-term impact on populations than direct mortality associated with the initial oiling (Day et al., 1997; Esler et al., 2000; Golet et al., 2002; Irons et al., 2000; Iverson and Esler, 2010; Short et al., 2007; see Wiens et al., 2010). Over five years of study, we showed that Common Loons are exposed to both pyrogenic and petrogenic sources of PAHs, and that PAHs in the blood correlated with lower body mass. Loons in this region are long distance migrants (Kenow et al., 2002; Paruk et al., 2014b), and body condition is likely a key component of their winter survival and future reproductive success. As such, the impact of oil pollution on migratory birds may be significant, and it is important to quantify long-term sublethal effects of chronic exposure to determine the impact of oil pollution on wildlife. Oil affects diverse bird species in different ways, and more studies on blood chemistry in oil-exposed wild bird populations would be beneficial in order to better identify and understand the factors, such as type of PAHs, amount ingested, or age and type of birds, which may account for our results.

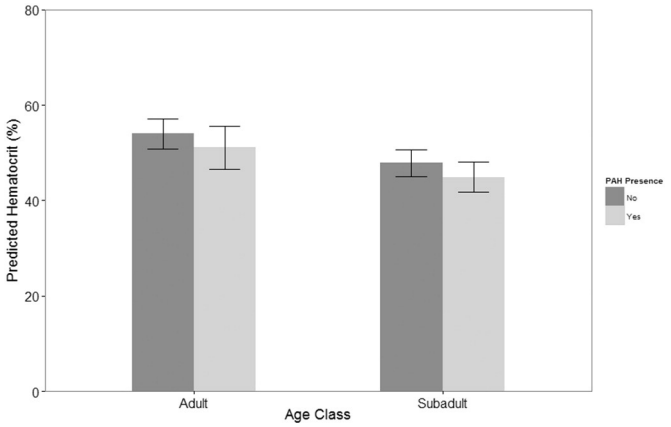

Fig. 4. Predicted hematocrit fractions for adult and subadult loons with PAH above 5 ng/g (light gray) or below 5 ng/g (dark gray). Error bars represent the 95% confidence interval of the mean. Overall r^2 for this model was 0.21.

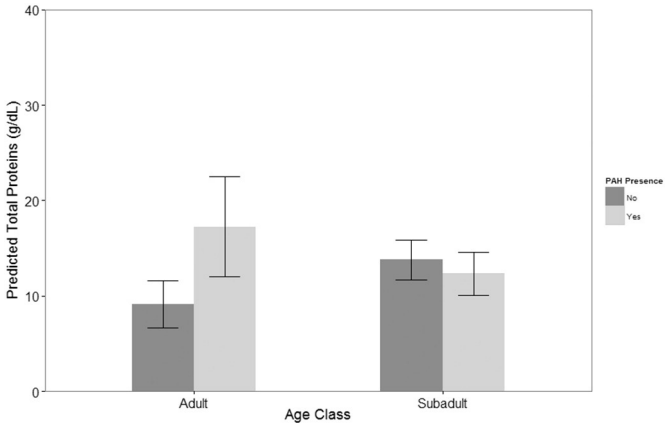

Fig. 6. Fig. 5: predicted total protein concentrations for adult and subadult loons with PAH above 5 ng/g (light gray) or below 5 ng/g (dark gray). Error bars represent the 95% confidence interval of the mean. Overall r^2 for this model was 0.29.

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