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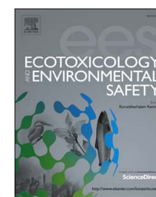

Testing of an oral dosing technique for double-crested cormorants, *Phalacocorax auritus*, laughing gulls, *Leucophaeus atricilla*, homing pigeons, *Columba livia*, and western sandpipers, *Calidris mauri*, with artificially weather MC252 oil

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ABSTRACT

Scoping studies were designed to determine if double-crested cormorants (*Phalacocorax auritus*), laughing gulls (*Leucophaues atricilla*), homing pigeons (*Columba livia*) and western sandpipers (*Calidris mauri*) that were gavaged with a mixture of artificially weathered MC252 oil and food for either a single day or 4–5 consecutive days showed signs of oil toxicity. Where volume allowed, samples were collected for hematology, plasma protein electrophoresis, clinical chemistry and electrolytes, oxidative stress and organ weigh changes. Double-crested cormorants, laughing gulls and western sandpipers all excreted oil within 30 min of dose, while pigeons regurgitated within less than one hour of dosing. There were species differences in the effectiveness of the dosing technique, with double-crested cormorants having the greatest number of responsive endpoints at the completion of the trial. Statistically significant changes in packed cell volume, white cell counts, alkaline phosphatase, alanine aminotransferase, creatine phosphokinase, gamma glutamyl transferase, uric acid, chloride, sodium, potassium, calcium, total glutathione, glutathione disulfide, reduced glutathione, spleen and liver weights were measured in double-crested cormorants. Homing pigeons had statistically significant changes in creatine phosphokinase, total glutathione, glutathione disulfide, reduced glutathione and Trolox equivalents. Laughing gulls exhibited statistically significant decreases in spleen and kidney weight, and no changes were observed in any measurement endpoints tested in western sandpipers.

1. Introduction

During the Deepwater Horizon oil spill that began on April 20, 2010 many important bird habitats in the northern Gulf of Mexico became contaminated and resulted in birds becoming oiled. The Gulf of Mexico is not only an important migratory stopover, but it also represents important life cycle and breeding habitat for a wide range of bird species. The length of time that oil was present in these habitats and the wide area that it covered meant there was the potential for birds to be oiled multiple times and to varying extents.

The acute mortality caused by heavy oiling is well understood. Loss of feather function and thermoregulatory abilities can be lethal within a

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matter of hours in cold waters (Burger, 1993). But the damage to individual and population health caused by sub-lethal exposures are more difficult to quantify. Not only are there a range of conditions that may be observed including, anemia, organ dysfunction, loss of feather function, decreased nutrient absorption, altered stress response, and decreased immune function (Szaro et al., 1978; Leighton et al., 1985; Leighton, 1985, 1986; Peakall et al., 1982, 1989; Leighton, 1993), there are also be functional repercussions caused by the deficits. Reduced take off speed in western sandpipers due to a light oiling of wings (Maggini et al., 2017; this edition) may increase predation risk, although there may be no associated measurable health effects. The *Deepwater Horizon* oil spill is also made more complicated by the spill duration. The spill occurred over a period of months, and oil persisted in the environment for considerably longer (PDARP, 2016). Determining the oral oil exposure that results in adverse outcomes for avian species is of critical importance, but following the DWH spill confounding factors such as determining when the bird was oiled, by how much and how many times, made it virtually impossible to estimate dosage and relate it to health deficits. Rather than try to untangle the complex nature of such events a simpler approach was attempted for determining MC252 oil toxicity to birds. That was to determine if a single oiling event could in a measurable way, affect the health of an individual bird.

These health impacts can be measured through the use of a number of clinically relevant plasma markers, blood cell counts, necropsy findings and hepatic antioxidant enzymes. Hemolytic anemia is often a defining feature of oil toxicity in birds. It is reported in both field and laboratory studies (Hartung and Hunt, 1966; Eastin and Rattner, 1982; Pattee and Franson, 1982; Lee et al., 1986; Leighton et al., 1985; Leighton, 1986; Hughes et al., 1990; Yamato et al., 1996; Walton et al., 1997; Newman et al., 2000; Seiser et al., 2000; Troisi et al., 2007). Hemolytic anemia occurs by oxidative damage to hemoglobin during detoxification and elimination of polycyclic aromatic hydrocarbons (PAHs) from the body (Peakall et al., 1989; Troisi et al., 2007). In young and growing birds such as herring gulls and Atlantic puffins, a single oral dose of oil can result in hemolytic anemia within 4–5 days (Leighton, 1986)

Experimental oral exposure to oil in birds is generally achieved through either feeding trials or gavage. Feeding trials have most commonly been used for longer duration studies in ducks (Holmes et al., 1978; Szaro et al., 1978; Harvey et al., 1981, 1982; Cavanaugh and Holmes, 1982; Cavanaugh et al., 1983), but there are a limited number of studies where short-term adapted methods have been used. Alonso-Alvarez et al. (2007b) used bread covered in oil to feed to yellow-legged gulls for a seven day feeding trial. However, gavage is more commonly used to investigate acute exposure (Hartung and Hunt, 1966; Wootton et al., 1979; McEwan and Whitehead, 1978, 1980; Eastin and Rattner, 1982; Leighton et al., 1985; Leighton, 1985, 1986; Lee et al., 1985; Peakall et al., 1989; Brausch et al., 2010). As such, gavage methods were developed for one captive model species, the homing pigeon and three Gulf of Mexico relevant species, present in the gulf for different periods of time. The laughing gull, which is present during the warmer spring and summer months, the double-crested cormorant which overwinters in the area and the western sandpiper which uses the area as a migratory stopover point were also tested to determine if short duration oral gavage or single bolus dose of oil could result in anemia in the 4–5 day duration recorded by Leighton (1986). The homing pigeon, a novel but highly useful laboratory model for flight and toxicity testing was also used for methods comparison. Unlike the other species tested, the pigeon is primarily a seed eating bird with a large crop and muscular gizzard that limit the rate at which food reaches the intestines. Toxicity was monitored not only by development of anemia, but by the use of clinically relevant plasma markers, oxidative stress markers and blood counts.

2. Methods

2.1. Toxicant and general dosing

Artificially weathered MC252 oil (DWH7937, batch# B030112) was prepared from crude oil collected during the DWH oil spill. Oil characterization is described in Forth et al. (2017).

As described in Forth et al. (2017), source oil was artificially weathered by TDI-Brooks using a modification of Carls et al. Approximately 3.5 L of source oil was heated in a glass beaker to 90–105 °C using a digital hot plate (model CMAGHP751, IKA) and stirred using a digital, top-loading mixer (model BDC250, Caframo) to mix but not aerate the oil. The oil was stirred until a mass loss of approximately 33–38% was achieved which generally took about 20 h to complete. This correlated to a BTEX (*i.e.,* sum of benzene, toluene, ethylbenzene, and xylene) depletion of approximately 99.9% and a TPAH50 depletion of approximately 22% relative to hopane. When not in use, the oil was stored in a leaf-proof container in a flammables storage cabinet.

Oil was mixed with species-specific diets for use in each test, aiming for dosing of between 5 and 20 ml/kg body weight/24 h. These doses correspond to literature reports of anemia development in response to gavage with oil in mallards and herring gull chicks (Hartung and Hunt, 1966; Leighton, 1986).

2.2. Species-specific dosing and sampling methods

2.2.1. Double-crested cormorants

2.2.1.1. Animal care. Adult and sub-adult (based on plumage development) double-crested cormorants (*Phalacocorax auritus*) were collected from Little Mossy Lake, MS on November 2, 2012 (33.340, −90.423), according to NWRC approved Institutional Animal Care and Use (IACUC) protocol QA1992. Birds were allowed acclimate for two weeks in individual pens containing 190 L water-filled tanks for feeding and perching. Up to 600 g of fresh live fingerling channel catfish (*Ictalurus punctatus*; 10–20 cm) were supplied and consumption measured daily.

2.2.1.2. Gavage. Birds were divided into three treatment groups (control (1 female, 6 males), single dose of 20 ml oil/kg BW (4 females, 2 males and 1 indeterminate sex) and 5 day dosing of 20 ml oil/kg ((2 females, 5 males)). Human grade catfish fillets were homogenized in a commercial grade blender with an equivalent water volume to form a1:1 slurry. This slurry was then mixed 1:1 with artificially weather MC252 oil. A single batch of oil:food slurry was prepared on the day prior to first dose, then stored as aliquots as −20 °C until use. Slurry was thawed to room temperature each day.

Cormorants were fasted overnight before each gavage. Volume for gavage was adjusted according to body weight, with each bird receiving a total of 40 ml of oil:food slurry per kilogram body weight. That is approximately 80 ml in total, as a single daily bolus. Gavage was achieved by filling a 60 ml BD syringe capped with a 40 cm long, 6 mm inner diameter flexible catheter with the oil:food slurry, then slowly inserting into the esophagus until a moderate level of resistance was felt, indicating tube placement proximal to the stomach (approximately 35 cm). The oil:fish slurry was slowly expelled, then syringe removed and re-filled to reach the 80 ml (adjusted for BW) total. The syringe and feeding tube were weighed before and after dosing to verify the dose administered. Feeder fish were provided immediately in each pen following gavage and birds were monitored for regurgitation and first sign of oil in feces. Dosing continued for 5 days. All birds were handled every day with sham gavage used for controls and single dose birds.

2.2.1.3. Blood collection. Blood was collected from the brachial vein using a 25 G needle and syringe prior to dosing, then on dose days 2 and 4, with additional samples collected at necropsy on day 6. Heparinized blood was used to prepare two blood smears for complete blood count

(CBC) and two blood smears for Heinz bodies. For the latter, 20 µl of heparinized whole blood was incubated for 20 min at room temperature with 40 µl new methylene blue (new methylene blue N [NMB]; Sigma-Aldrich, St. Louis, MO) that was prepared by mixing 0.025 g NMB and 0.08 g potassium oxalate (Sigma-Aldrich, St. Louis, MO) with 5 ml water following the method of Leighton (1985). The blood/NMB mixture was then used to make the Heinz body smears.

EDTA-treated whole blood was sent to the Mississippi State University College of Veterinary Medicine Diagnostic Laboratory Services (MSU CVM DLS; Starkville, MS) for plasma chemistries. Included in the panel were electrolyte concentrations (sodium, potassium, chloride, phosphorus and calcium), enzyme activities (alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), creatine phosphokinase (CK), lactate dehydrogenase (LDH)) and blood analytes (glucose, cholesterol, urea, uric acid, total protein, creatinine). Plasma was obtained by centrifugation of blood at 2000*g* for 5 min. Plasma was removed and stored at −80 °C until shipping. Plasma samples were analyzed by the University of Miami, Miller School of Medicine, Avian and Wildlife Laboratory for plasma protein electrophoresis serum amyloid A and haptoglobin concentrations.

2.2.1.4. Necropsy. On day 6 a single brachial vein sample was collected for the above measurements, then birds were euthanized by cervical dislocation and necropsied immediately. Additional blood was collected *via* post-mortem cardiac puncture for analysis of 3-methyl histidine (Metabolic Technologies, Iowa).

Assessment of organ gross abnormalities were undertaken at necropsy. The liver was removed first, weighed to the nearest 0.001 g, then five 1.0 g and one 2.0 g sections were flash frozen in liquid nitrogen for measurement of oxidative damage (Dr. Chris Pritsos, University of Nevada, Reno) and Cytochrome P450 enzyme activity (Dr. Celine Godard-Codding, Texas Tech University), respectively. Samples were sorted on dry ice, then transferred to a low temperature (−70 °C) freezer until they were shipped on excess dry ice. Thyroid gland, spleen, adrenal glands, brain, heart, lungs, remainder of liver, kidneys and gastrointestinal (GI) tract were removed and weighed, then placed in 10% neutral buffered formalin (NBF). If present, the thymus and bursa of Fabricius were noted and removed and placed in 10% neutral buffered formalin. Twenty-four hours later organs were transferred to fresh 10% NBF before being shipped to Zoo Exotic Pathology Services (ZEPS) for standard hematoxylin and eosin staining by Dr. D. Reavill and Dr. R. Schmidt.

2.2.2. Homing pigeons

2.2.2.1. Animal care. Adult homing pigeons (*Columba livia domestica*) aged 2–7 years were purchased from a racing pigeon breeder (Foy's Pigeon Supplies, Beaver Falls, PA) and maintained at the University of Nevada Reno (UNR) Homing Pigeon Research Facility, following a 7 days quarantine. Pigeons were maintained according to the University of Nevada, Reno IACUC guidelines.

Birds were maintained in lofts with outdoor fly pens attached. During quarantine and maintenance, birds were provided Purina Mills Nutriblend Green (5454; St. Louis, MO) and water *ad libitum*. During testing, feed was withheld for approximately 15 h starting in the late afternoon/early evening of the day prior to dosing with MC252 oil. On days when birds were not dosed with MC252 oil, feed was offered *ad libitum*. All experimental birds were transferred daily (control, single dose, and 5 day dosed birds) to portable holding cages to ensure identical handling. Following dosing each bird was placed in an individual cage for observation. At completion of each day of dosing all birds were returned to their lofts overnight.

2.2.2.2. Gavage. Oil and feed were separately aspirated into the gavage syringe because it was not possible to prepare an oil/feed slurry that didn't separate while loading the syringe. One hundred grams of pigeon feed was mixed with 325 ml distilled water in a blender. For dosing, 1.0 ml of feed slurry was drawn up into the syringe followed by oil volume adjusted for bird weight. A 10 cm length of a 14-French catheter tube (Kendall, Mansfield, MA) was attached to the Luer end of the syringe and inserted through the bird's crop, until resistance was met with, indicating the catheter was placed at the entrance to the stomach. When the contents of the syringe were expelled, the dose of oil was followed by the feed slurry, effectively cleaning the oil out of the syringe and gavage tube.

Birds were fasted overnight (approximately 15 h) prior to the first day of oil administration. The intended nominal daily oil dose was to be 20 ml oil/kg body weight (BW) for a single day or for five consecutive days. However, when this dose was administered to the birds (2 female, 5 male) that would receive only a single bolus dose on Day 1 of testing, the majority of birds regurgitated immediately. As a result, the dose was reduced to 5 ml/kg BW (2 female, 5 male) administered twice daily for a total of 10 mg/kg BW per day, with at least 3 h between doses for the five day dose group. Regurgitation was considerably less, but was not entirely eliminated. Control birds (1 female, 5 male, 3 indeterminate) were sham gavaged by inserting the gavage tube to approximately the same length and restraining the birds for an equivalent period of time.

Between oil doses, birds were allowed *ad libitum* food access; however, consumption was variable. Time between gavage and regurgitation or presence of oily excreta was recorded for each bird.

2.2.2.3. Blood collection. One day prior to the start of the study a blood sample (up to 5.0 ml) was collected from the brachial vein using a 25 gauge needle, then decanted into the appropriate size of BD lithium heparin or serum separate tube before centrifugation. Blood also was collected from all birds on Days 2, 4 and 6 in the same way. CBC and Heinz body slides were prepared as per the double-crested cormorants.

Plasma samples were analyzed by the University of Miami, Miller School of Medicine, Avian and Wildlife Laboratory. Included in the panel were plasma protein electrophoresis serum amyloid A and haptoglobin concentrations, electrolyte concentrations (sodium, potassium, chloride, phosphorus and calcium), enzyme activities (alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), creatine phosphokinase (CK)) and blood analytes (glucose, total protein, creatinine). Plasma was obtained by centrifugation of blood at 2000*g* for 5 min. Plasma was removed and stored at −80 °C until shipping.

2.2.2.4. Necropsy. Necropsies were performed as per double-crested cormorants, but organs were weighed to the nearest 0.1 mg.

2.2.3. Laughing gulls

2.2.3.1. Animal care. Adult laughing gulls (*Leucophaeus atricilla*) were collected from Cameron Parrish, LA on August 14, 2012, according to NWRC approved IACUC protocol QA1992. Upon arrival at the NWRC-MS Field Station. For the first three days of quarantine birds were group housed in 3.3 m × 3.3 m × 2.0 m pens (five birds per pen) to improve adaptation to captivity. Then they were transferred to individual stainless steel rabbit cages measuring 61 cm × 46 cm × 38 cm or 71 cm × 46 cm × 38 cm for the remainder of the 14-day quarantine period. Birds remained in this cages for the study. Unique leg bands were applied during the quarantine period. Food [frozen/thawed pogies (*Brevoortia* sp.) and Mazuri Fish Analog 50/10 mix (Purina Mills, St. Louis, Missouri)] and water were provided fresh daily. Birds were randomly assigned to treatment groups

2.2.3.2. Gavage. Preparation of the oil:fish slurry was identical to that used for double-crested cormorants. Human grade catfish fillets were homogenized in a commercial grade blender with an equivalent water volume to form a1:1 slurry. This slurry was then mixed 1:1 with artificially weather MC252 oil. A single batch of oil:food slurry was prepared on the day prior to first dose, then stored as aliquots as

−20 °C until use. Slurry was thawed to room temperature each day.

Birds were divided into three treatment groups (control (1 female, 5 male, 1 indeterminate), single dose of 20 ml oil/kg BW (2 female, 5 male) and 5 day dosing of 20 ml oil/kg (2 female, 5 male)). The total volume for the day was divided into two equivalent doses. Birds were gavaged with an 18-French (approximately 6 mm) polyvinyl chloride feeding tube (20.5 cm long, 6.0 mm outer diameter) attached to a 10 ml glass syringe. Just prior to dosing, birds were weighed. The syringe and feeding tube were loaded with the appropriate volume of oil or oil:fish slurry based on the bird's BW. The feeding tube was inserted into the bird's esophagus until a moderate level of resistance was felt, indicating tube placement proximal to the stomach (approximately 9 cm) and the oil or oil:fish slurry was expelled from the syringe and feeding tube. The syringe and feeding tube were weighed before and after dosing to verify the dose administered. After each bird was dosed, it was placed in its cage and monitored for regurgitation and first appearance of oily excreta. Water and half the daily allowance of food were offered at this time. Approximately 4–6 h later, the birds dosed with an oil:fish slurry dose identical to the first dose of the day, resulting in a total of 20 ml oil/kg BW (40 ml oil: fish slurry/kg BW). Monitoring was continued, and birds were provided with the remainder of their food allotment until approximately one hour after sunset. Control birds were gavaged identically to the treated birds, but with an empty syringe.

2.2.3.3. Blood collection. Blood (up to 1.0 ml) was drawn through the brachial or tarsal vein on Day 1 (just prior to first dose of oil:fish slurry), Day 2, Day 4 and Day 6. The size of these birds would only allow for minimal sampling to be undertaken during the study, so on Days 1, 2 and 4 the only blood collected was for preparation of CBC and Heinz body slides (as above). The brachial vein was punctured with a 25 G needle and blood was drawn with a 1 ml syringe, and decanted into heparinized tubes for processing. Samples were kept at 4 °C prior to processing, which took place within 4 h of collection. Plasma and whole blood were analyzed by Mississippi State University College of Veterinary Medicine Diagnostic Laboratory Services.

On day 6 additional blood was collected from the brachial vein for measurement of packed cell volume, hemoglobin concentration, electrolyte concentrations (sodium, potassium, chloride, phosphorus and calcium), enzyme activities (alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), creatine phosphokinase (CK), lactate hydrogenase (LDH)) and blood analytes (glucose, total protein, creatinine). These samples were taken to the analytical laboratory within 90 min of collection. Additional blood was collected *via* cardiac puncture for plasma protein electrophoresis, haptoglobin and serum amyloid A analyses at the University of Miami following cervical dislocation. Cardiac blood was obtained with a heparinized 22 G needle with a 3 ml syringe. Plasma was obtained by centrifugation of blood at 2000*g* for 5 min. Plasma was removed and stored at −80 °C until shipping.

2.2.3.4. Necropsy. Necropsies were performed as per the double-crested cormorants.

2.2.4. Western sandpipers

2.2.4.1. Animal care. Adult western sandpipers (*Calidris mauri*) that had previously been held at the University of Western Ontario's Advanced Facility for Avian Research (AFAR) were used in this study. These birds were captured in British Columbia under the guidelines of the University of Western Ontario University Council on Animal Care and according to permit CA-0256 from the Canadian Wildlife Service. The birds were captured for a separate study on the effects of immune challenge on flight capacity that took place 11 months prior to this dosing trial. In this previous experiment the birds were treated with lipopolysaccharide to induce an immune response and a blood sample had been collected from each.

Birds were maintained a specialized shorebird room (2.4 m × 3.7 m) under 12 L:12D light conditions at approximately 19 °C. One week prior to dosing, birds were transferred into a large holding room under the same photoperiod and temperature. Four days prior to dosing, dividers were placed in the room to create four 1.8 × 1.8 m corrals. The five or six birds comprising each of the four dose groups (see below) were maintained in their respective corrals throughout the trial.

Birds were fed an *ad libitum* diet of 6:1 mix of Aquamax fish diet (SD03 Fingerling Starter 300; Agribrands Purina Canada, Woodstock, Ontario) and Purinature Chick Starter (Agribrands Purina Canada). The diet was supplemented with mealworms every other day.

2.2.4.2. Gavage. Birds were divided into four groups for testing (control (1 female, 4 males), 20 ml/kg BW for one day (1 female, 4 males), 10 ml/kg BW for 4 days (1 female, 4 males) and 20 ml/kg BW for 4 days (2 females, 4 males)). Oil was administered as a 1:1 oil/mealworm slurry. A mealworm homogenate was prepared at the ratio of six mealworms per 2 ml water, placed in a 12 ml polypropylene tube and homogenized with an Omni 2000 (Omni International, Kennesaw, GA) variable speed tissue homogenizer. This mixture was centrifuged at 2000*g* in a Galaxy Mini microcentrifuge (VWR International, St. Catharines, Ontario) to remove cuticle particles that would clog the gavage needle.

Birds were weighed daily to the nearest gram prior to dosing. The total volume for the day was divided into two equivalent doses, the first of which was given after a one hour fast. The appropriate volumes of homogenate and oil for each bird's BW were combined in a microcentrifuge tube and the mixture thoroughly vortexed for 30 s. The appropriate volume was administered to a manually restrained bird through a 5.08 cm, 20 G stainless steel gavage needle attached to a 1 ml Luer lock glass syringe. The second dose was administered approximately one hour after the first dose for a total dose of 10 or 20 ml oil/kg BW.

Birds were observed for the first 30 min after dosing to assess whether oil was regurgitated or excreted. After the first 30 min, birds were observed every 30 min for two hours to assess whether oil was regurgitated or excreted, and the approximate amount. Birds were provided food *ad libitum* approximately 30 min after the second dose of the day until one hour prior to receiving the next day's first dose.

2.2.4.3. Blood collection. The small size of the western sandpipers limited the blood volume collected during the study to preparation of Heinz body slides. Approximately 30 µl of blood was collected on Day 3 from a pricked 27 G, 1.27 cm needle) brachial vein using a heparinized microhematocrit tube. The blood was added to two times its volume of New Methylene Blue N stain (NMB, Sigma-Aldrich, St. Louis, MO; 0.025 g NMB plus 0.08 g potassium oxalate in 5 ml of deionized water mixed fresh just prior to use) and incubated at room temperature for 20 min. Two smears were made from this preparation and allowed to air dry. Following blood collection, birds were gavaged as above.

On day five, birds were bled (26 G, 1.27-cm needle, 1-ml syringe) *via* jugular and/or brachial vein to collect as much blood as possible. The limited blood volume of the birds precluded extensive analyses, however, blood smears for Heinz bodies, complete blood counts (CBCs) and clinical chemistries were prioritized. Collection volumes ranged from 500 to 1150 µl. Heinz body blood slides were prepared as described above. The slides and remaining whole blood were sent immediately to the University of Guelph Animal Health Laboratory (Guelph, ON, Canada) for analysis.

2.2.4.4. Necropsy. Necropsies were performed as per the double-crested cormorants, but without collection of liver for oxidative stress or CYP450 measurements. Organs were weighed to the nearest 0.1 mg.

2.3. Statistical methods

Body weights and hematologic and plasma clinical chemistry values determined over multiple time points were modeled as a repeated measures analysis of covariance (ANCOVA) with interaction, where treatment group is a categorical explanatory variable and elapsed days is a continuous explanatory variable. Differences among treatment groups on Day 0 were evaluated. 3-Methyl histidine, malondialdehyde + 4-hydroxylalkenals, hepatic oxidative stress, and organ weight endpoints were analyzed by one-way analyses of variance (ANOVAs) utilizing the proc mixed procedure of SAS (v. 9.4). Means were compared using the least squares means function and considered significantly different if $p < 0.05$. Relative organ weights were analyzed by one-way analyses of variance (ANOVAs) utilizing the proc mixed procedure of SAS following arc sin transformation.

3. Results/discussion

Of primary interest for each of the studies reported here was whether the gavage method for delivery of oil was appropriate, and second if any of the species tested showed signs of oil toxicity. As such, the endpoint responses for all species are summarized in Table 1. Raw data for avian toxicity studies conducted as part of the Deepwater Horizon Damage Assessment are publicly available at https://www.diver.orr.noaa.gov/deepwater-horizon-nrda-data, while work plans and reports can be accessed through https://www.doi.gov/deepwaterhorizon/adminrecord.

It should be noted that for this study we were unable to accurately count Heinz bodies or reticulocytes in any of the slides prepared, despite consultation with three different veterinarians. This was likely due to a combination of factors including, some the oil exposure methods perhaps not causing Heinz body formation, the interval between dosing and blood collection being too short to detect Heinz bodies, variation in timing of stain exposure, and in some cases fading of the stain due over time. Following consultation with Dr. Kendal Harr (Urika Pathology LLC) it was decided that further experiments would incorporate electron microscopy for detection and light microscopy for quantitation of Heinz bodies.

Western sandpipers did not show any measurable changes in blood endpoints or organ weights following gavage with the oil:food slurry. However, these birds excreted oil within 10 min of gavage. Rapid oil excretion was also observed for laughing gulls and double-crested cormorants, but was substantially longer in homing pigeons. The minimum duration for detection of oily excreta in pigeons was 90 min; however, there were some observations of oily excreta the following morning before the next gavage.

Pigeons regurgitated more frequently than the other species, and the number of treated birds that regurgitated increased throughout the study. Regurgitation was observed within a 10–120 min window following gavage, but no attempt was made to determine specific volumes, as the oil was often ejected with additional materials from the crop, including some bedding materials consumed by the birds. The pigeon is the avian emesis model for testing of pharmaceutical agents such as cisplatin (Tanihata et al., 2000), exhibiting both early and delayed emetic responses. The immediate regurgitation observed for these pigeons indicates there may have been some element of gastro-intestinal irritation caused by the oil, or through stimulation of central or peripheral nervous system in the pigeons; however, this was beyond the scope of these studies. Regurgitation was not observed in the control birds or the single dose birds after day 1, so it is unlikely that stress or the gavage process itself was responsible. The pigeon was the only species for which food intake began to decrease, but given the short duration of the study there were no changes in body weight.

The only responsive endpoints measured in the laughing gulls were the kidney and spleen weights, both of which were lower in the five day (20 ml/kg BW/day) dosed birds. Generally, oil would be expected, at least following a short duration study, to result in initial increases in organ weight as part of detoxification processes. Alterations in kidney and spleen weights are likely be an incidental finding in this study due to the small sample sizes used, particularly since there were no accompanying changes in clinical chemistry markers. Homing pigeons also showed very little response to the gavage method for oil dosing. Creatine phosphokinase (CK) was higher in the five day (10 ml/kg BW/day) dosed birds. While an increase in CK alone in the five day dosed birds could indicate some liver involvement, it could also be part of a stress response to handling and dosing as the birds may be more prone to slight muscle injury. Although all birds were handled the same way, since the five day dosed group were regurgitating, that the procedures were more stressful to them as a whole. At no point during the study did there to appear to be any differences in the way any of the pigeons responded to handling; however, over the course of the study the five day dosed birds did become more lethargic following dosing. One important difference in homing pigeon relative to western sandpiper and laughing gull was that there were also changes in oxidative stress markers for both the single dose (20 ml/kg BW) and the five day (10 ml/kg BW/day). Glutathione and superoxide dismutase (SOD) both showed decreases, which could be construed as counter-intuitive following polycyclic aromatic hydrocarbon (PAH) exposure; however, the declines are likely just indicative of the early stages of exposure, prior to enzyme activity upregulation within the liver. Nonetheless, these changes are indications that PAHs were absorbed by the homing pigeon following the gavage procedure.

Double-crested cormorants were the only species to show at least a partial response to oil exposure in the gavage trial; however, there were also a number of inconclusive results that will require further study to elucidate. Both the single dose (20 ml/kg BW) and the five day dose (20 ml/kg BW/day) groups had drops in packed cell volume that typify avian response to oil (Hartung and Hunt, 1966; Eastin and Rattner, 1982; Pattee and Franson, 1982; Lee et al., 1986; Leighton et al., 1985; Leighton, 1986; Hughes et al., 1990; Yamato et al., 1996; Walton et al., 1997; Newman et al., 2000; Seiser et al., 2000; Troisi et al., 2007). White blood cells counts also declined for the five day dose birds but not the single dose birds (df;2,51F = 5.00; p = 0.022), while monocyte counts increased more for single dosed birds than controls or five day dosed birds (df = 2,15; F = 5.95; p = 0.013). Heterophils decreased over the course of the study, but this was not dose-related (df = 1,51; F = 4.24; f = 0.045). Changes in white cells counts related to dose would be expected to begin during the study as inflammatory processes are stimulated; however, the complex nature of these responses would not only be driven by oil exposure, but by a generalized stress response to captivity and handling, as well as any pre-existing health conditions, such as the presence of injuries, parasites or diseases. It is clear from even our very basic white cell counts that more complex analyses would be required to understand how oil toxicity influences and itself affects immune function in free-living birds.

A subset of the plasma clinical chemistry markers also had statistically significant dose-related changes. Potential endpoints indicative of liver damage that could be affected by oil exposure are alkaline phosphatase, ALT, AST, cholesterol, glucose, GGT, LDH and total protein (Szaro et al., 1981; Eastin and Rattner, 1982; Newman et al., 2000; Seiser et al., 2000; Golet et al., 2002; Alonso-Alvarez et al., 2007a, 2007b). Total protein (df = 2,51; F = 10.90; p = 0.0001), glucose (df = 2,51; F = 5.48; p = 0.007) and cholesterol (df = 1,51; F = 4.08; p = 0.049) concentrations decreased with treatment for the group treated with oil for five days by day 6, indicative of liver dysfunction. Albumin (df = 2, 51; F = 0.79; p = 0.4576), ALT, alkaline phosphatase, GGT and LDH activities were inconclusive. ALT decreased in controls and 5 day dosed birds, but not in the single dosed birds (df = 2,15; F = 7.61; p = 0.005) while alkaline phosphatase increased in the five day dosed birds, but did not change in controls or single dosed birds (df = 2,51; F = 5.20; p = 0.02). GGT activity decreased in the five day treated birds (df = 2,51; F = 8.70; p < 0.0005), but not in the

Table 1
Summary of results from Deepwater Horizon Natural Resources Damage Assessment avian toxicity scoping studies.

	WESA Scoping[a]			LAGU Scoping		DCCO Scoping		ROPI Scoping	
	Single dose	Daily dose		Single dose	Daily dose	Single dose	Daily dose	Single dose	Daily dose
Analyte	20 ml oil/kg BW	10 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	10 ml oil/kg BW
Hematology									
Packed cell volume (%)	NA	NA	NA	NC	NC	↓	↓	NC	NC
Hemoglobin	NA	NA	NA	NC	NC	NC	NC	NC	NC
Reticulocyte count	IC	IC	IC	NC	NC	NA	NA	NC	NC
Heinz bodies	IC	IC	IC	IC	IC	IC	IC	IC	IC
White blood cell count	NA	NA	NA	NA	NA	IC	↓	NC	NC
Basophil count	NA	NA	NA	NA	NA	NA	NA	NA	NA
Eosinophil count	NA	NA	NA	NA	NA	NC	NC	NC	NC
Heterophil count	NA	NA	NA	NA	NA	NC	NC	NC	NC
Lymphocyte count	NA	NA	NA	NA	NA	NC	NC	NC	NC
Monocyte count	NA	NA	NA	NA	NA	↑	↑	NC	NC
Plasma proteins									
Pre-albumin	NA	NA	NA	NC	NC	NC	NC	NC	NC
Albumin	NA	NA	NA	NC	NC	NC	NC	NC	NC
α – 1-globulins	NA	NA	NA	NC	NC	NC	NC	NC	NC
α – 2-globulins	NA	NA	NA	NC	NC	NC	↓	NC	NC
β-globulins	NA	NA	NA	NC	NC	NC	NC	NC	NC
γ-globulins	NA	NA	NA	NC	NC	NC	IC	NC	NC
Albumin:globulin ratio	NA	NA	NA	NC	NC	NC	NC	NC	NC
Plasma clinical chemistries									
Alanine aminotransferase	NA	NA	NA	NC	NC	IC	↓	NC	NC
Alkaline phosphatase	NA	NA	NA	NC	NC	IC	↑	NC	NC
Aspartate aminotransferase	NA	NA	NA	NC	NC	NC	NC	NC	NC
Creatine phosphokinase	NA	NA	NA	NC	NC	↑	↑	NC	↑
Creatinine	NA	NA	NA	NC	NC	IC	IC	NC	NC
Gamma glutamyl transferase	NA	NA	NA	NC	NC	↓	↓	NC	NC
Haptoglobin	NA	NA	NA	NC	NC	IC	IC	NC	NC
Lactate dehydrogenase	NA	NA	NA	NA	NA	IC	IC	NA	NA
3-methyl-histidine	NA	NA	NA	NA	NA	NC	↑	NA	NA
Serum amyloid A	NA	NA	NA	NC	NC	NC	NC	NC	NC
Total protein	NA	NA	NA	NC	NC	↓	↓	NC	NC
Cholesterol	NA	NA	NA	NC	NC	IC	IC	NA	NA
Glucose	NA	NA	NA	NC	NC	↓	↓	NC	NC
Urea	NA	NA	NA	NA	NA	NC	NC	↓	NA
Uric acid	NA	NA	NA	NA	NA	NC	↑	NA	NA
Plasma minerals									
Calcium	NA	NA	NA	NC	NC	↓	↓	NC	NC
Chloride	NA	NA	NA	NC	NC	NA	NA	NC	NC
Phosphorus	NA	NA	NA	NC	NC	IC	IC	NC	↓
Potassium	NA	NA	NA	NC	NC	IC	IC	NC	NC
Sodium	NA	NA	NA	NC	NC	IC	↓	NC	NC
Hepatic antioxidant enzymes									
Total glutathione	NC	NC	NC	NC	NC	NC	↑	↓	↓
Glutathione disulfide	NC	NC	NC	NC	NC	↑	↑	NC	↓
Reduced glutathione	NC	NC	NC	NC	NC	NC	↑	↓	NC
Lipid peroxidation	NA	NA	NA	NC	NC	NC	NC	NC	NC
Superoxide dismutase	NC	NC	NC	NC	NC	NC	NC	NC	↓
Trolox	NC	NC	NC	NA	NA	NA	NA	NA	NA
Organ weights[b]									
Adrenals	NC	NC	NC	NC	NC	NC	NC	NC	NC
Brain	NA	NA	NA	NC	NC	NC	NC	NC	NC
Heart	NC	NC	NC	NC	NC	NC	NC	NC	NC
Kidneys	NC	NC	NC	NC	↓	NC	NC	NC	NC
Liver	NC	NC	NC	NC	NC	NC	↑	NC	NC
Spleen	NA	NA	NA	NC	↓	NC	NC	NC	NC
Thyroids	NA	NA	NA	NC	NC	NC	NC	NC	NC
Relative organ weights									
Adrenal	NC	NC	NC	NC	NC	NC	NC	NC	NC
Brain	NA	NA	NA	NC	NC	NC	NC	NC	NC
Heart	NC	NC	NC	NC	NC	NC	NC	NC	NC
Kidneys	NC	NC	NC	NC	↓	NC	↑	NC	NC
Liver	NC	NC	NC	NC	NC	↑	↑	NC	NC
Spleen	NA	NA	NA	NC	↓	NC	NC	NC	NC
Thyroid	NA	NA	NA	NC	NC	NC	NC	NC	NC
Body weight	NC	NC	NC	NC	NC	NC	NC	NC	↓
Feed intake	NA	NA	NA	NC	NC	NC	NC	NA	NA

(continued on next page)

Table 1 *(continued)*

Analyte	WESA Scoping[a]			LAGU Scoping		DCCO Scoping		ROPI Scoping	
	Single dose	Daily dose		Single dose	Daily dose	Single dose	Daily dose	Single dose	Daily dose
	20 ml oil/kg BW	10 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	20 ml oil/kg BW	10 ml oil/kg BW
Regurgitation	NO	NO	NO	NO	OC	NO	NO	YES	YES
Approximate time to oil excretion	< 10 min	< 10 min	< 10 min	10–20 min	10–20 min	20–30 min	20–30 min	> 90 min	> 90 min

Abbreviations: NA = not analyzed; NC = no change, relative to control; IC = inconclusive; OC = occasional. Only significant ($p < 0.05$) changes are indicated by up or down arrows.
[a] Due to marked hemolysis of the plasma samples, the analyzing lab deemed the samples unusable for intended analyte analysis.
[b] Only absolute and relative weights and body weights were analyzed for this study.

control or single dose birds. LDH decreased in controls and single dose birds, but remained constant in five day dosed birds (df = 2,51; F = 7.72; p = 0.001). The measured changes in GGT, LDH and alkaline phosphatase activities are somewhat confounded by statistically significant differences among controls and dosed birds prior to the start of dosing, so interpretation is limited. AST did not change with treatment or over the course of the study.

Changes in albumin, glucose, cholesterol, total protein, ALT, GGT, LDH, alkaline phosphatase and AST, are likely indicative of damage to the liver and biliary tree, but changes in some of these endpoints such as alkaline phosphatase, AST and LDH are non-specific birds (Harr, 2005; Hochleitner et al., 2005). Further experimentation with larger sample sizes, longer dosing and/or sampling are needed for further clarification. Though increases relative liver weight ($p < 0.05$) and liver oxidative stress markers such as total glutathione ($p < 0.0001$), reduced glutathione ($p < 0.0001$) and oxidized glutathione ($p = 0.0001$) are all indications that the liver is responding to PAH exposure through upregulation of detoxification processes. So while oxidative damage to the liver has not progressed sufficiently for manifestation of clinical disease state to be apparent, initial processes have begun even after only a short exposure time, indicating that there is potential for double-crested cormorants to be a useful species for understanding the effects of *Deepwater Horizon* oil on adult Gulf of Mexico birds. Changes in concentrations and activities of plasma clinical chemistry markers in control birds and differences among groups prior to dosing for some clinical markers make it very difficult to draw conclusions as to which are the most important for interpretation of the effects of oil on organ function in double-crested cormorants.

Additionally there was some evidence in the double-crested cormorants the kidney, gastro-intestinal and muscle damage may also be occurring. Uric acid, a marker of kidney function, was increased significantly during the study in the five day dosed birds, but did not change in single dose birds (df = 2,51; F = 8.05; p = 0.0009). Decreases in plasma calcium in both single and five day dosed birds (df = 2,51; F = 14.37; $p < 0.0001$) and a decrease in sodium in the five day dosed birds (df = 2,15; F = 5.74; p = 0.014) may be indicative of kidney dysfunction, or gastro-intestinal irritation/inflammation preventing absorption across microvilli. Decreases in food intake and/or impaired intestinal transport of oil have been suggested as possible mechanisms for the decrease in the plasma concentrations of cholesterol, glucose and total protein (Eastin and Rattner, 1982; Newman et al., 2000; Alonso-Alvarez et al., 2007a, 2007b), and this may also be the case for minerals. CK activity was increased in dosed birds relative to controls, over the course of the study (df = 2,50; F = 4.83; p = 0.012), suggesting that as suggested by Newman et al. (2000), the muscular damage inducing this increased activity was more likely the result of the stress of captivity and handling rather than oil ingestion. 3-methyl histidine, a marker of muscle wastage, was also higher in the five day dosed birds (p = 0.049). The larger blood volume required for this measurement meant that it could only be measured at necropsy, somewhat hampering interpretation. However, further information would be required before a conclusion could be made as to whether the muscle wastage was due to captivity/handling stress, general lack of activity due to captivity or oil-induced wastage from lack of nutrient consumption or ingestion.

The use of an acute gavage study for oral dosing of birds was largely unsuccessful for adding to our understanding of the effects of oil on avian physiology. The most commonly observed consequence of ingestion of sublethal volumes of oil is the development of hemolytic anemia. However, over the course of the five day studies, the double-crested cormorant was the only species for which anemia was observed. Due to issues with rapid excretion of oil, it was impossible to determine if the lack of response in the other species was due to the dose provided, lack of absorption of the dose or a short exposure time. While it was possible, at least in the double-crested cormorant, to gain some insight into the types of responsive endpoints that should be measured, such as clinical markers of liver, kidney, gastro-intestinal and muscle damage, it is apparent that further study and method development is required in order to fully elucidate the functional impacts.

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