2017

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Ecotoxicology and Environmental Safety 146 (2017) 83–90

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Toxic effects of orally ingested oil from the Deepwater Horizon spill on laughing gulls

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ARTICLE INFO

Keywords:
Oil toxicity
Deepwater Horizon
MC252 oil
Laughing gull
Oxidative stress
Gulf of Mexico

ABSTRACT

The explosion of the Deepwater Horizon oil rig released millions of gallons of oil into the environment, subsequently exposing wildlife, including numerous bird species. To determine the effects of MC252 oil to species relevant to the Gulf of Mexico, studies were done examining multiple exposure scenarios and doses. In this study, laughing gulls (*Leucophaeus atricilla*, LAGU) were offered fish injected with MC252 oil at target doses of 5 or 10 mL/kg bw per day. Dosing continued for 27 days. Of the adult, mixed-sex LAGUs used in the present study, 10 of 20 oil exposed LAGUs survived to the end of the study; a total of 10 of the oil exposed LAGUs died or were euthanized within 20 days of initiation of the study. Endpoints associated with oxidative stress, hepatic total glutathione (tGSH), oxidized glutathione (GSSG) and reduced glutathione (rGSH) significantly increased as mean dose of oil increased, while the rGSH:GSSG ratio showed a non-significant negative trend with oil dose. A significant increase in 3-methyl histidine was found in oil exposed birds when compared to controls indicative of muscle wastage and may have been associated with the gross observation of diminished structural integrity in cardiac tissue. Consistent with previous oil dosing studies in birds, significant changes in liver, spleen, and kidney weight when normalized to body weight were observed. These studies indicate that mortality in response to oil dosing is relatively common and the mortality exhibited by the gulls is consistent with previous studies examining oil toxicity. Whether survival effects in the gull study were associated with weight loss, physiologic effects of oil toxicity, or a behavioral response that led the birds to reject the dosed fish is unknown.

1. Introduction

To assess the specific adverse effects of MC252 oil on Gulf of Mexico relevant avian species, we designed a series of avian toxicity studies using different routes of exposure and dosages over time. Background on the oil spill and an overview of the avian toxicity studies are outlined in Bursian et al. (2017). Laughing gulls (*Leucophaeus atricilla*; LAGUs) were chosen specifically for inclusion in these tests because they were impacted by the Deepwater Horizon (DWH) spill, are easily caught and managed in captivity, and are large enough to supply needed blood and tissues for analysis. The LAGU is a small black-headed gull that commonly nests in large groups of up to 50,000. As an omnivore and scavenger, its diet consists of both terrestrial and aquatic invertebrates, fish, seasonal berries, and garbage (Burger, 1988). LAGUs are commonly found on shores, parking lots and landfills, foraging on the ground, and in shallow waters. They are classified by the International Union for Conservation of Nature (IUCN) as a species of least concern following population increases and a halt to the collection of their eggs for food. Their abundance and flexible diet make the species a useful potential model for studying the effects of DWH oil across a broad range of species.

Prior to the study described herein, a pilot study was done to examine multiple dosing methods and doses to determine the effects of DWH oil on LAGU. Initial studies were based on previous work by Leighton (1986). These studies used oral gavage of oil:fish slurries as a method of dosing. This dosing method allowed for very accurate

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http://dx.doi.org/10.1016/j.ecoenv.2017.07.018
Received 18 August 2016; Received in revised form 5 July 2017; Accepted 7 July 2017
Available online 16 August 2017
0147-6513/ Published by Elsevier Inc.

measurements of dose. However, rapid transit time through the gastrointestinal (GI) tract may have limited absorption; therefore a dosing method based on delivery of the DWH oil via food fish was developed to more closely represent natural routes of oil ingestion encountered in the wild and provide a realistic exposure scenario.

2. Methods

All experiments were approved by the Institutional Animal Care and Use Committee of the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services, National Wildlife Research Center (NWRC, Approval 2108), Fort Collins, CO, USA.

2.1. Animal collection and husbandry

Thirty-five resident LAGUs were captured in the Gulf of Mexico in April 2013 and transported to the USDA National Wildlife Research Center animal care facility where they were quarantined and individually housed in pens. Pens were one of two sizes, either 2.4 m × 1.2 m × 1.8 m (length × width × height) or 2.1 m × 2.1 m × 2.4 m. Birds were randomly assigned to cage types so that study groups were evenly distributed in the two cage types. Cages were constructed of coated wire mesh and were set up to face each other to reduce isolation stress.

Upon arrival, each bird was examined for any sign of injury or disease. All pens contained a food bowl and a shallow plastic tank filled with water, which was changed daily. Dri-deck, carpet, or a grate was provided in a portion of each pen for foot relief from concrete floors. In addition, each pen contained a tree stump for perching and loafing. Birds were maintained on a 12L:12D light cycle with a mean room temperature of approximately 22 °C. Bird condition was monitored daily for overt changes in body temperature, behavior and food intake. Twice weekly, body weight (bw) and cloacal body temperature were measured manually.

2.2. Feeding and dosing

LAGUs were fed approximately 75-100 g fresh fish and 25 g Mazuri Fish Analog 50/10 Frozen 5T8L (Purina Mills, St. Louis, Missouri) per bird per day. Food fish were obtained from the Mississippi Field Station (pogies, *Brevooritia patronus*) or purchased from I.F. Anderson Farms (Lonoke, Arkansas, golden shiner minnows, *Notemigonus crysoleucas*o). LAGUs were randomly assigned to one of three treatment groups: 1) a control group (n = 10) that was provided untreated minnows, 2) a group dosed daily with up to 5 mL oil/kg of the bird's body weight (bw) through provision of oil-containing fish as described below (n = 10), and 3) a group dosed daily with up to 10 mL oil/kg bw (n = 10).

Frozen minnows were thawed and each minnow was injected with approximately 200 μL or 400 μL of artificially weathered MC252 oil using a 21- or 22-gauge needle and 1 mL-syringe. Dosing of LAGU was accomplished by providing multiple fish with a total dose of MC252 oil (DWH7937, batch# B030112) equal to approximately 5 or 10 mL/kg bw per day. The oil-containing minnows (and unoiled minnows for control birds) were offered to the LAGUs in the morning and the rest of their daily ration (pogies, minnows, and Mazuri diet) was provided after all oil-containing fish had been consumed (control birds received additional rations at the same time the majority of dosed birds received additional rations). The proportion of minnows, pogies, and Mazuri diet offered was standardized between the three groups. The total food ration was approximately 100-125 g of food/day. Oil consumption was calculated based on the number of fish that were consumed.

At the outset of the experiment, small golden shiner minnows (approximately 5-6 cm long and weighing 3-4 g) were injected with 200 μL of oil and offered to the LAGUs. However, birds frequently rejected these fish. Assuming that larger fish would make the oil less detectable, the golden shiner minnows were replaced by larger (8-10 cm) brooder minnows that were injected with 400 μL oil (in the swim bladder) per fish on day 5. Dosing continued for 27 days.

2.3. Blood sampling

Blood samples were collected during quarantine, on day 0, and twice weekly until the conclusion of the study. Blood smears were prepared for white blood cell and red blood cell (RBC) assessment. A 20 μL aliquot of fresh whole blood was fixed for identification of reticulocytes and Heinz bodies by transmission electron microscopy. All blood samples were collected early in the morning prior to providing morning food rations.

Once a week during the oral dosing study, approximately 300 μL of additional plasma was collected for determination of clinical chemistries. If additional blood was available, larger volumes of blood were centrifuged so that a 40 μL sample of heparinized plasma and a 300 μL sample of packed RBCs could be collected for total antioxidant capacity measurements. The targeted collection volumes were 1.0 mL and 0.40 mL for the first and second weekly draws, respectively, to allow for preparation of blood smears, determination of clinical chemistries, and measurement of antioxidant endpoints. Clinical chemistries and white blood cell types examined are outlined in Dean et al. (2017).

2.4. Necropsy

Moribund birds (Toth, 2000) were euthanized and necropsied before the end of the study to provide fresh tissues adequate for analysis; half of the remaining birds were necropsied on day 27 and half were necropsied on day 28 with half of the birds in each treatment group necropsied on each day. At necropsy, each bird was weighed and blood was collected as described above. Birds were euthanized by cervical dislocation and additional blood was collected by cardiac puncture. Plasma was aliquoted to determine 3-methyl histidine concentration.

The brain, heart, kidneys, liver, GI tract, spleen, thyroid, and adrenal glands were collected and weighed to the nearest 0.1 mg. If a bird appeared to be in breeding condition, the gonads were collected and weighed. In addition, all organs were assessed for gross abnormalities and if present, were documented with digital photographs.

The brain, heart, lungs, spleen, bursa, thymus, gonads, one adrenal gland, and one thyroid gland were placed in 10% neutral buffered formalin for histopathology. The liver was sectioned into six pieces. Sections for assessment of cytochrome P450 (CYP) activity, oxidative damage and PAH concentration were flash frozen in liquid nitrogen, with the remaining portion placed in 10% neutral buffered formalin. The kidney that was not preserved for histopathology was cut into five sections for assessment of oxidative damage. The GI tract was then sectioned into four pieces (esophagus to glandular stomach, section of duodenum containing pancreas, colon from the level of the ceca to the cloaca, and a 2 g portion of the duodenum). The sections were slit vertically and rinsed thoroughly in phosphate buffered saline. The first three sections were placed in neutral buffered formalin.

2.5. Histopathology

All tissue samples obtained at necropsy were preserved in 10% neutral-buffered formalin solution prior to being routinely processed. Paraffin-embedded tissues were sectioned at approximately 5 μm, mounted on glass slides, and stained with hematoxylin and eosin. All slides were examined by a board certified veterinary pathologist (Zoo/Exotic Pathology Service, Carmichael, CA).

2.6. Antioxidant capacity

Samples were sent to Dr. Chris Pritsos at the University of Nevada Agricultural Experiment Station for analysis of markers of antioxidant

Table 1
Target doses, actual mean doses consumed, and the number of days each bird survived the 28 day trial. Percent weight changes for each bird are across the number of days it survived.

Bird	Target dose	Mean dose per kg body weight	Days alive	% Weight change	Proportion of days bird received full food rations
526[a]	5	3.16	9	-25.6	0.33
530[a]	5	1.51	11	-12.7	0.18
518[a]	5	2.50	14	-33.8	0.36
514[a]	5	2.80	18	-14.6	0.50
516[a]	5	3.97	20	-16.1	0.65
517	5	3.64	28	-9.9	0.70
501	5	4.74	28	10.0	0.78
531	5	4.83	28	3.9	0.81
505	5	4.88	28	2.3	0.85
523	5	4.94	28	-2.3	0.78
519[a]	10	2.80	7	-28.1	0.29
532[a]	10	1.15	11	-10.2	0.09
520[a]	10	4.08	14	-8.5	0.21
529[a]	10	6.56	18	2.0	0.50
500	10	7.91	28	-2.1	0.56
513	10	8.61	28	-1.0	0.59
502	10	8.94	28	-3.4	0.81
522	10	9.35	28	-8.0	0.78
533	10	9.49	28	-0.9	0.67
527	10	9.52	28	-7.8	0.93
503	0	0.00	28	8.2	1.00
504	0	0.00	28	3.0	1.00
507	0	0.00	28	6.5	1.00
509	0	0.00	28	1.9	1.00
511	0	0.00	28	-4.9	1.00
512	0	0.00	28	-6.2	1.00
524	0	0.00	28	-2.0	1.00
525	0	0.00	28	-11.1	1.00
528	0	0.00	28	-7.8	1.00
534	0	0.00	28	-8.4	1.00

[a] Individual did not survive through day 28.

capacity (see Pristos et al., 2017, in press, for methods).

2.7. Statistical analyses

Only birds that survived to the end of the study were used for regression analyses because many endpoints were only measured at necropsy. Moreover, many of the birds that did not survive to the end of the trial had reduced food consumption such that confounding between oil consumption and food consumption may have been a factor for those birds. Finally, because many birds did not consistently consume all of the oil-injected fish offered, mean oil exposure did not consistently meet our target doses of 5 or 10 mL/kg bw per day. Therefore, actual mean doses consumed by each individual were calculated (Table 1) and were used in the analyses in place of the target doses.

Hematologic and plasma clinical chemistry endpoints were compared using linear mixed effects regression models, where the mean dose and the sample day were modeled as a fixed effect and individual birds were modeled as a random effect (modeled as a random slope) for endpoints measured multiple times. Models included day, treatment (mean oil dose as mL/kg bw/day), and a treatment*day interaction term. Sample day and treatment were modeled as continuous variables, where treatment was defined as the average daily consumption determined from daily observations of actual oil consumed by each individual bird (Table 1). Oxidative stress endpoints, 3-methyl histidine, and relative organ weights (i.e., organ weight as a percentage of body weight) were compared using linear models where each endpoint was modeled as a function of the mean dose ingested. Statements of significance are on based individual models and p-values < 0.05.

Because the reference laboratory noted issues with plasma sample quality for some endpoints, they provided a quantitation of lipolysis and hemolysis in the samples. As random error has been demonstrated as a potential effect of these characteristics, plasma samples with hemolysis scores of 2 or 3 (moderate or severe) were not included in the statistical analysis and samples with lipemia scores of 2 or 3 (moderate or severe) were excluded in the statistical analysis of selected analytes (CLSI [2012] has a discussion of interference resulting from hemolysis or lipemia).

All analyses were conducted using R, version 3.2.2 (R Development Core Team, 2008). Regressions were run using the lme4 package (Bates et al., 2015) and the survival plot was generated using the survminer package.

3. Results

3.1. Food consumption and oil intake

Daily food consumption for oil-dosed birds was somewhat depressed during the first week of the study prior to the switch to larger minnows (Fig. 1). Food consumption (g/kg bw) was similar among groups for the birds that survived to the end of the trial (day 28, Fig. 1) with regression analysis indicating no significant differences in consumption. For

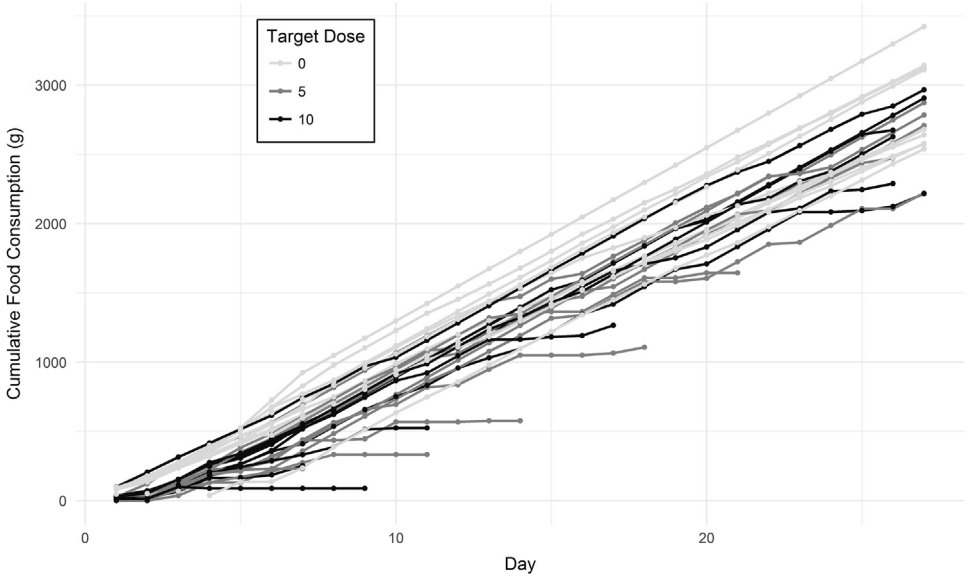

Fig. 1. Daily cumulative food consumption for laughing gulls orally exposed to artificially weathered MC252 crude oil. Note: many of the birds that did not survive through the end of the trial experienced decreased food consumption prior or death or euthanasia.

birds that did not survive until the end of the trial, individuals often refused fish and food consumption was often limited for several days prior to death or euthanasia (Fig. 1).

Birds were only offered their full ration after they consumed their oil-injected fish for their target dose of 5 or 10 mL/kg bw. Therefore, if birds failed to eat all oiled fish they did not get a full ration of food that day. Animals that did not consistently get full rations of food had lower survival (as determined by the number of days alive) compared with the birds that often received full rations (Table 1). For the birds in the 5 mL/kg bw group, 5 of 10 died or were euthanized prior to the end of the study (day 28). All of these birds had reduced food consumption and lost weight (12.7–33.8%) prior to death or euthanasia (Table 1). The mean daily dose of oil consumed by these birds ranged from 1.51 to 3.97 mL/kg bw whereas mean daily dose of oil consumed by birds that survived until the end of the trial ranged from 3.64 to 4.88 mL/kg bw (Table 1).

Four of the 10 birds in the 10 mL/kg bw group died prior to the end of the study. These birds consumed their full food ration for fewer days (mean = 27.25% of the days alive) than the birds that survived to the end of the study (mean = 74.67%). The mean daily dose of oil consumed by the birds that died prior to the end of the study ranged from 1.15 to 6.56 mL/kg bw whereas the mean daily dose of oil consumed by birds that survived until the end of the trial ranged from 7.91 to 9.52 mL/kg bw (Table 1).

3.2. Adverse health effects

Of the 31 adult, mixed-sex LAGUs used in the study, 21 LAGUs survived the entire 28 days; a total of 10 LAGUs died or were euthanized within 20 days of initiation of the study. LAGUs were euthanized based on veterinary assessment of severe distress or when the animals were moribund to ensure that necropsies could be performed on fresh carcasses and that a complete suite of endpoints could be sampled. All of the birds that died or were euthanized prior to the end of the trial exhibited clinical signs of lethargy and reduced food intake. While cloacal temperature did not change significantly during the trial, two birds in the 5 mL oil/kg bw group showed a large drop in temperature prior to death. One control bird was euthanized on day 4 of the study (due to presumed capture myopathy) and replaced with a bird captured and maintained in the same conditions. Survival across the 28 days of the trial was significantly lower for birds in the oil-dosed groups compared to birds in the control group ($p = 0.051$, Fig. 2).

Mortality in birds that did not survive until the end of the testing period may have been associated with weight loss caused by dosed birds refusing to eat their full ration of oiled fish such that they did not receive additional rations not containing oil (Table 1). For the 5 mL/kg bw birds, birds that died early only received full rations an average of 40.4% of the time compared with 78.4% of the time for birds that survived for the full 28 days. As a consequence, the birds that died early lost an average of 20.6% of their body weight compared to a weight gain of 0.8% in the birds that survived until the end of the testing period. Similarly, for the 10 mL/kg bw birds, the 4 individuals that died early only got full food rations an average of 27.3% of the time and lost 11.2% of their body weight compared to birds that survived receiving full food rations 72.3% of the time and losing an average of 3.9% of their body weight.

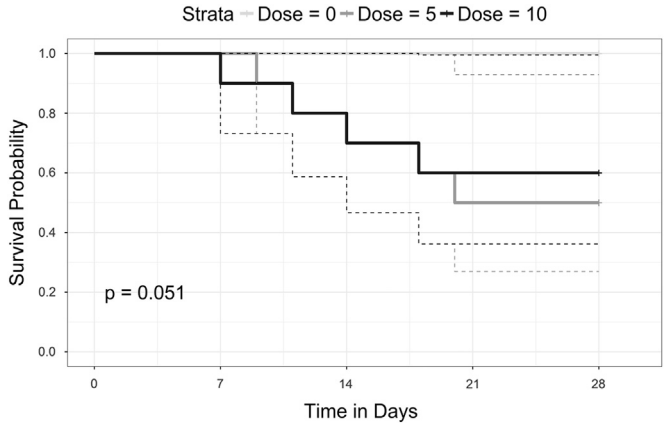

Fig. 2. Survival curves for laughing gulls orally exposed to target doses of 0 mL/kg bw, 5 mL/kg bw, or 10 mL/kg bw of artificially weathered MC252 crude oil. Solid lines show the survival probability over time for the three groups and the dashed lines are the confidence intervals associated with the survival probabilities.

3.3. Gross observation and histopathology

Dose of oil consumed was only correlated with liver, spleen, and kidney organ weights when normalized to body weight (i.e., relative weight). Relative liver and relative kidney weights both significantly increased with oil dose ($p < 0.001$, $p = 0.010$ respectively, Fig. 3) while relative spleen weight decreased as oil dose increased ($p = 0.050$, Fig. 3). Although relative heart weight did not significantly change with oil dose, the hearts of oil-dosed birds were noted to have a loss of structural integrity. This endpoint was not quantified as it was not anticipated; however, the generally more flaccid cardiac tissue of oil-dosed birds resembled dilated cardiomyopathy.

There was a high incidence of parasitism in all groups, including: nematodiasis in the esophagus and small intestine; trematodiasis in the colon, kidney, and proventriculus; coccidiosis in the kidney; and probable *Sarcocystis* sp. in the heart. Periductal inflammation involving large collecting ducts was a frequent renal lesion seen in all three treatment groups, albeit more prominent in the oil-dosed groups which may be a result of oil excretion acting as an irritant.

Inflammatory lesions were common across all three dosing groups. Hepatic inflammation (hepatitis, cholangitis, or cholangiohepatitis) was present in more than half of LAGUs necropsied. Respiratory inflammation (pneumonia or air sacculitis) or cardiac inflammation (myocarditis associated with *Sarcocystis*) was present in nearly a third of LAGUs. Tenosynovitis was confirmed in two LAGUs at necropsy although animal observation records indicated 21 of 30 LAGUs limping at least once. Once limping was observed in multiple animals, additional flooring was added for foot relief and most limping resolved.

3.4. Oxidative stress

The hepatic antioxidant endpoints measured at necropsy were affected by oil in a dose-dependent manner. Hepatic total glutathione (TGSH), oxidized glutathione (GSSG), and reduced glutathione (rGSH) all significantly increased as mean dose of oil increased ($p < 0.001$ for all; Fig. 4). The rGSH:GSSG ratio was not statistically significant, but showed a decreasing trend with increasing oil dose. Interestingly, the data for these parameters were heteroskedastic; i.e., the variance increased with the mean response such that the spread in the data for these endpoints was higher for higher oil doses compared to the controls. Superoxide dismutase activity showed a decreasing trend as mean dose of oil increased ($p = 0.205$; Fig. 4).

3.5. Hematology

Packed cell volume (PCV), and concentrations of white blood cells, heterophils, lymphocytes, monocytes, eosinophils, and basophils were not affected by oil dose. For the limited number of samples that were evaluated, Heinz bodies were rarely (< 1%) identified in RBCs sampled from control birds and frequently (10-40%) found in RBCs from oil-dosed birds as analyzed by transmission electron microscopy (TEM; Fig. 5). Anecdotally, no difference in numbers of Heinz bodies related to increasing oil dose was noted; erythrocytes from both 5 mL oil/kg bw and 10 mL oil/kg bw birds contained similar numbers of dense

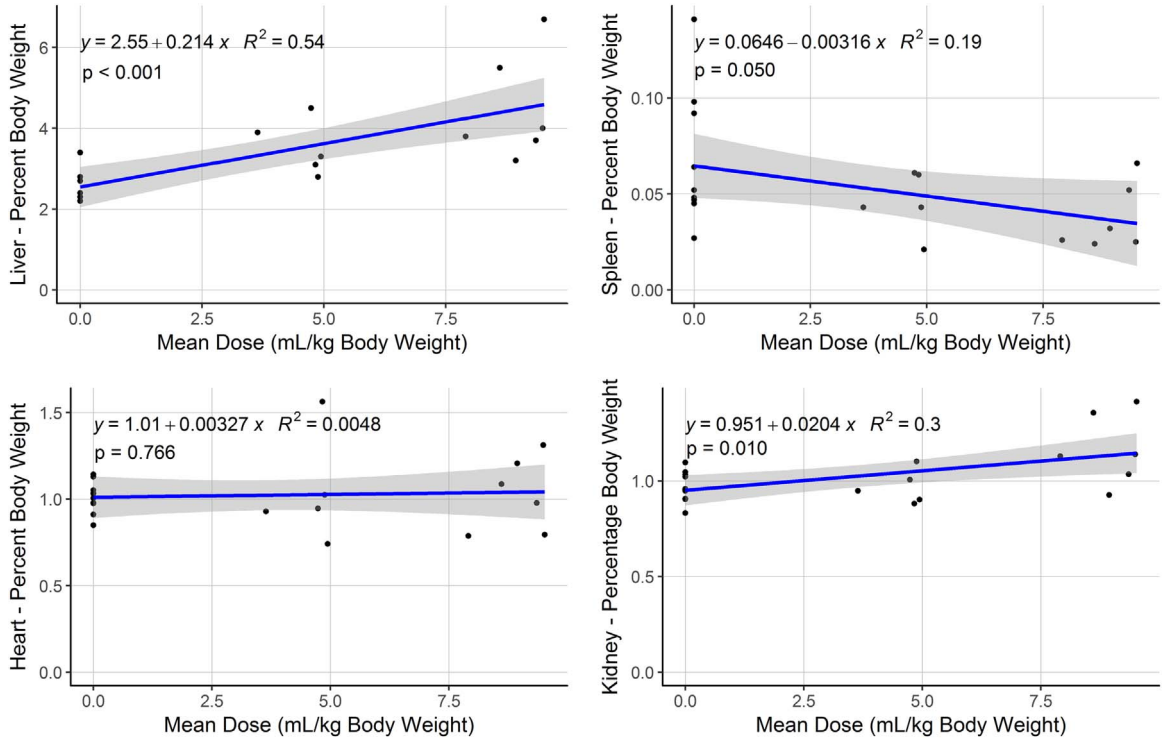

Fig. 3. Relative organ weights (i.e., organ weight as a percentage of body weight) as a function of mean dose of oil ingested for laughing gulls orally exposed to artificially weathered MC252 crude oil.

inclusions that lacked structure, consistent with Heinz bodies. Erythrocytes in oil-dosed birds tended to have smudged nuclei lacking chromatin detail and contained many dark cytoplasmic inclusions with the same homogeneity and electron density as Heinz bodies. Although the dark cytoplasmic inclusions were not membrane-associated as Heinz bodies classically are in mammals, they were consistent in structure and electron density with Heinz bodies. Structures consistent with degenerate organelles were only identified in oil-dosed birds (Fig. 5).

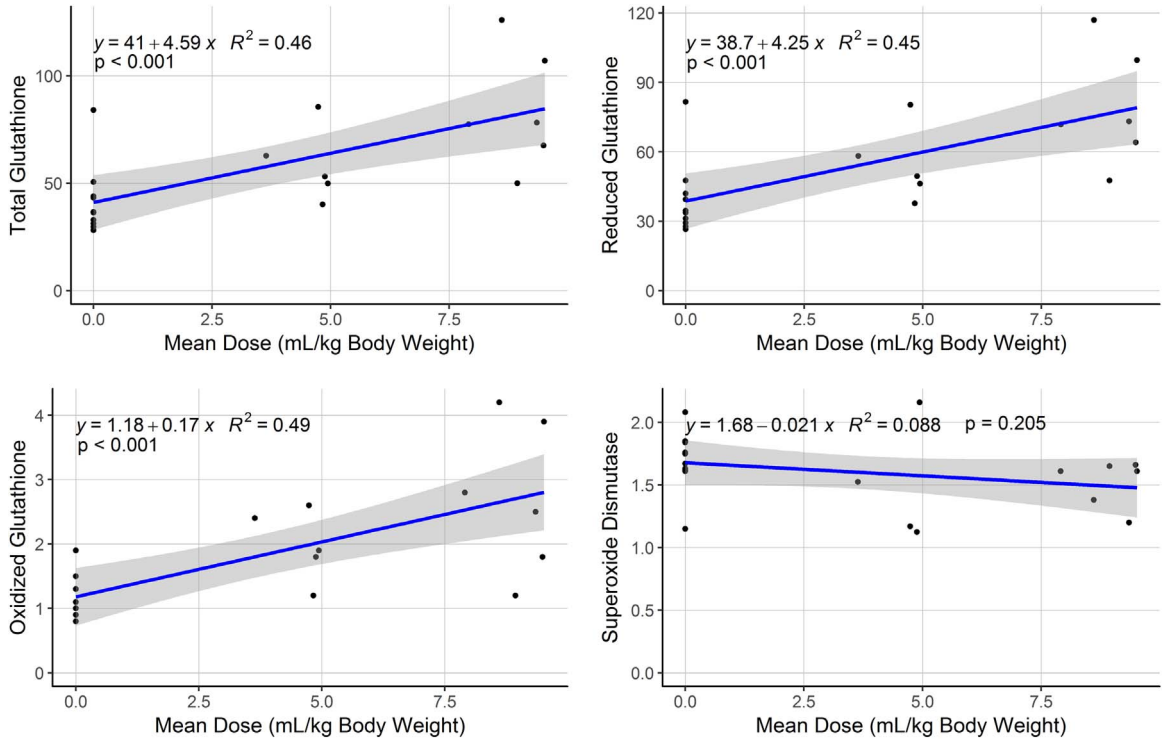

Fig. 4. Activity levels of hepatic total glutathione, reduced glutathione, oxidized glutathione, and superoxide dismutase in laughing gulls orally exposed to artificially weathered MC252 crude oil.

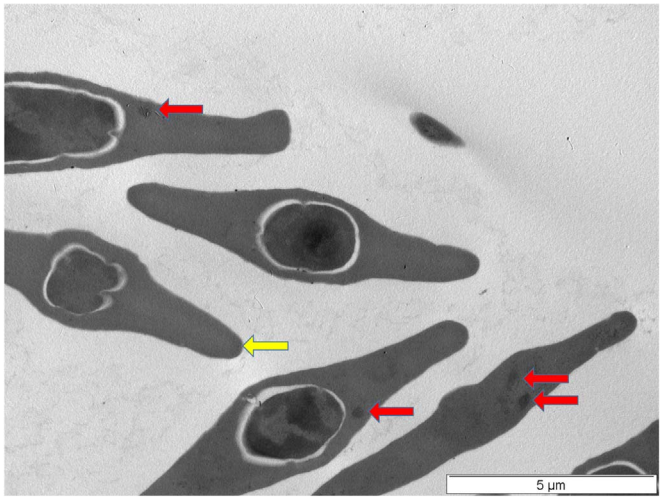

Fig. 5. Dark cytoplasmic inclusions with the same homogeneity and electron density as Heinz bodies and structures consistent with degenerate organelles (red arrows). The yellow arrow indicates a normal cell.

3.6. Clinical chemistry/plasma proteins

Of the endpoints examined from plasma samples, 3-methyl histidine was the only one affected by oil dose. 3-methyl histidine, a marker of muscle wastage, was positively correlated with oil exposure ($R^2 = 0.41$, $p = 0.001$; Fig. 6). There were no significant changes in any of the other clinical chemistry/plasma proteins measured.

4. Discussion

4.1. Food consumption and oil intake

In the current study, much effort was made to ensure that test birds were consuming both oil-injected fish and unoiled fish rations. While food consumption was depressed for the first few days of the study in oil-dosed birds, when larger oil-injected minnows were offered beginning on day 5, consumption in all three treatment groups was similar for birds that survived to the end of the study, presumably because the oil was less detectable by the LAGU compared to the smaller minnows. Conversely, for oil-dosed birds that did not survive through the end of the study, food consumption was often depressed for a number of days prior to death or euthanasia. This refusal of food may have been a behavioral response to the oil-injected minnows or may have had an unidentified physiological basis.

Previous studies have reported variable effects of oil exposure on avian food consumption. Herring gull (*Larus argentatus*) nestlings orally dosed with 10 mL of Prudhoe Bay crude oil/kg bw/day or more consumed less food beginning on the third day of dosing compared to controls and began to lose weight on the fifth day of dosing (Leighton, 1986). Conversely, American kestrels (*Falco sparverius*) administered feed containing 3.0% oil from the Mexican *Ixtoc I* well blowout avoided feed for the first week of the study, but then consumed significantly more feed than birds receiving feed containing 0.3% oil and an equivalent amount of feed as controls (Pattee and Franson, 1982). Despite the increase in food consumption, these birds lost weight (Pattee and Franson, 1982). Hyperphagia with no weight gain was reported for herring gull chicks administered a single oral dose of 0.3 mL Kuwait or South Louisiana crude oil/kg bw (Miller et al., 1978). The lack of weight gain was attributed to reduced transport of essential amino acids and possibly glucose across the GI tract as indicated by in vitro assays and pathological changes in the GI tract (Miller et al., 1978). Oil-induced hyperphagia has also been reported in adult Pekin ducks (*Anas platyrhynchos domesticus*) fed diets that provided approximately 2.9 mL South Louisiana crude oil, 2.5 mL Kuwait crude oil, or 1.3 mL No. 2 fuel oil/kg bw/day (Holmes et al., 1978). There were no changes in food consumption in the LAGU in this study that survived to the end of the study, but animals that succumbed prior to the end of the study showed reduced food consumption.

4.2. Adverse health effects

Five of the 10 birds that died or were euthanized were in the 5 mL oil/kg bw dose group and 4 of the 10 birds assigned to the 10 mL oil/kg bw group died or were euthanized compared to none in the control group, suggesting there was a dose-related response to oil exposure in the present study. However, since many of these birds refused the oil-injected fish (and in some cases did not consume additional rations when they were offered), the potential impact of oil on these birds is confounded with food consumption, and the effects of oil and food consumption cannot be disentangled. Nonetheless, the mortalities in the 5 mL oil/kg bw group may indicate that even low doses of oil could

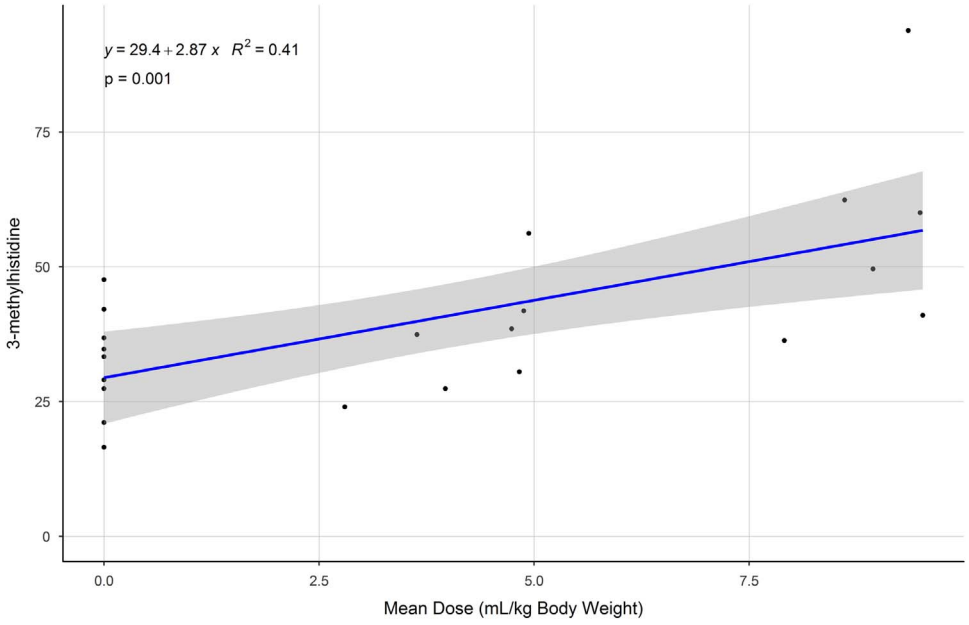

Fig. 6. Levels of 3-methylhistidine, a marker of muscle wastage, in laughing gulls orally exposed to artificially weathered MC252 crude oil.

induce mortality in wild populations of birds if oil-free food sources are not available and birds have limited opportunities to find uncontaminated resources.

Previous studies using oil doses consistent with the present study have shown mortality, loss of body weight, and lethargy in Herring gull (*Larus argentatus*) chicks, Atlantic puffin (*Fratercula arctica*) nestlings, and American kestrels (*Falco sparverius*). Some of these studies point to cold stress as a possible cofactor increasing the toxic effects of oil. Although LAGU in this study did not experience cold stress, they were reluctant to consume oiled food and therefore were calorically restricted (Miller et al., 1978; Pattee and Franson, 1982; Leighton, 1986). Lafferty and Holt (2003) have suggested that additional stressors caused by the presence of oil in the environment, such as increased competition for oil-free food resources or reduced mobility, may cause increased mortality at lower doses. While additional stressors such as temperature or competition were not addressed in the present study, the parasitism and inflammation noted across study groups may indicate that additional stressors might affect the impact of oil exposure in wild populations. Collectively, these studies indicate that mortality in response to oil dosing is relatively common and the mortality exhibited by the gulls is consistent with previous studies examining oil toxicity. Whether these effects in the gull study were associated with weight loss, physiologic effects of oil toxicity, or a behavioral response that led the birds to reject the dosed fish is unknown.

4.3. Gross observation and histopathology

The increase in relative kidney weights in oil-dosed birds in the present study may indicate renal dysfunction and is consistent with previous studies. Cassin's auklets (*Ptychoramphus aleuticus*) exposed to oil via external application and common murres (*Uria aalge*) recovered from a spill of bunker C fuel oil had renal tubular necrosis (Fry and Lowenstine, 1985). Mallard (*Anas platyrhynchos*) ducklings fed a diet containing 5.0% South Louisiana crude had tubular inflammation and degeneration in the kidney (Szaro et al., 1978). On the other hand, Leighton et al. (1986) did not define renal lesions in herring gull chicks and Atlantic puffin nestlings dosed with Prudhoe Bay crude oil. Although renal dysfunction did not impact survivorship in the present study, it is an interesting finding that may have impacts on wild populations.

In this study, relative liver weights were significantly increased in oil-dosed birds, but histological lesions were minimal and generally occurred in birds of all three groups. Increases in liver weight have been reported in previous avian oil exposure studies. Holmes et al. (1978) reported that adult Pekin ducks consuming approximately 6 mL of South Louisiana crude per day had increased relative liver weights compared to controls, but relative liver weights of ducks consuming 6 mL of Kuwait crude were comparable to control weights. American kestrels fed a diet containing 0.3% or 3.0% crude from the *Ixtoc I* well blowout for one, two, or four weeks did not show absolute or relative liver weights that were significantly different compared to controls (Pattee and Franson, 1982). Herring gull chicks administered a single oral dose of 0.3 mL Kuwait or South Louisiana crude oil/kg bw had increased liver weights when necropsied nine days later (Miller et al., 1978). An increase in liver weights of herring gull chicks receiving five daily oral doses of 10 mL of Prudhoe Bay crude oil/kg bw was reported by Peakall et al. (1989), and mallard ducklings fed diets containing 2.5% and 5.0% South Louisiana crude oil for eight weeks had significant increases in liver weights (Szaro et al., 1978).

Both Miller et al. (1978) and Peakall et al. (1989) reported hepatic activity of mixed function oxidase enzymes were significantly increased in the absence of hepatic pathology, suggesting that the increase in liver weight could be attributed to a compensatory metabolic response. Szaro et al. (1978) reported that liver pathology in ducklings fed oil-containing feed was subtle, consisting of generally minimal hypertrophy and vacuolation of hepatocytes and bile duct proliferation. Leighton (1986) reported that the most predominant lesion in the livers of herring gull chicks and Atlantic puffin nestlings dosed daily with 10 mL of Prudhoe Bay crude oil/kg bw consisted of enlarged Kupffer cells that were filled with gold-brown pigment indicative of hemosiderin and phagocytized erythrocytes. Necrosis of individual hepatocytes and apoptosis were prevalent in the gulls. Hepatic hemosiderosis in oil-exposed birds has been reported in numerous previous studies (Fry and Lowenstine, 1985; Pattee and Franson, 1982; Yamato et al., 1996). In the present study, hemosiderosis was minimal regardless of the severity of the anemia and no difference between oil-dosed birds and control birds with normal PCVs was identified upon standard hematoxylin and eosin (H & E) staining or Prussian blue staining (Khan and Nag, 1993). Anemia with minimal hemosiderosis indicates that a lack of cellular regeneration in the bone marrow may also be a component of the anemia. The damaging effects of components of crude oil on bone marrow have been documented in mammals (Linet et al., 1996). Therefore, it follows that MC252 oil could have induced hypoplastic marrow contributing to lack of regeneration of erythrocytes.

4.4. Hematology

The presence of Heinz bodies in oil exposed LAGUs is consistent with the finding of changes in hepatic glutathione corresponding to systemic oxidative stress. While Heinz bodies are indicative of hemolytic anemia, no significant change in PCVs was detected. As many of the oil exposed birds were inappetent, especially prior to death, it is possible that dehydration resulted in hemoconcentration and increased PCVs, masking hemolytic anemia due to oil exposure. The presence of Heinz bodies and degenerate organelles in the RBCs in both groups of oil-dosed LAGUs (but not in the control group) indicates that a component of oil-induced anemia could be the result of oxide radical damage of RBCs and intravascular hemolysis as previously documented (Lee et al., 1985; Leighton, 1985, 1993; Troisi et al., 2007). This response is consistent with that reported in other avian species dosed with oil (Fry and Lowenstine, 1985; Hartung and Hunt, 1966; Lee et al., 1985; Leighton, 1985, 1993; Pattee and Franson, 1982; Szaro et al., 1978) or exposed to oil as the result of a spill (Yamato et al., 1996).

4.5. Oxidative stress

There was evidence of an increase in hepatic tissue oxidative stress in the LAGUs based on increases in liver GSH and GSSG and a negative trend in the rGSH:GSSG ratio. Rodríguez-Estival et al. (2016) have reported the utility of assessing oxidative stress as an early indicator of diminished health. GSH is the predominant non-protein thiol in cells and plays a key role in maintaining the redox homeostasis within the cell. Induction of *de novo* synthesis of GSH occurs as an adaptive response to oxidative stress (Biswas and Rahman, 2009). GSSG is the oxidized form of GSH and increases during oxidative stress, primarily by the reactions of the antioxidant enzyme GSH peroxidase. The ratio of GSH to GSSG can be used as a marker of oxidative stress (Zitka et al., 2012) which was demonstrated in this study by a decreasing trend with increasing oil dose. The process of inactivation of PAHs found in crude oil results in the formation of oxides and reactive oxygen radicals that are part of the oxidative stress insult to the birds. Consistent with the present study, Leighton et al. (1985) reported an increase in GSH/PCV in herring gull nestlings dosed with comparable amounts of crude oil from the Prudhoe Bay oil spill.

4.6. Clinical chemistry/plasma proteins

3-methyl histidine was the only parameter in the clinical chemistry and plasma protein panels to have been affected by oil consumption. 3-methyl histidine concentration increased as oil dose increased indicating the possibility of damage to the musculature and general muscle wastage. This result may be correlated with the general

observation of potential dilated cardiomyopathy. This endpoint was more thoroughly examined in double crested cormorants that had been externally oiled (Harr et al., 2017, in press). Although they may not be detectable in visual scans of oiled environments, these changes could alter a bird's ability to successfully complete long flights, migrate, or flee from predators. While changes in 3-methyl histidine were noted in the oil exposed birds, a corresponding change in creatine phosphokinase was not detected.

5. Conclusion

Overall, the birds tested in this study exhibited similar responses to oil exposure (e.g., change in relative organ weights, signs of potential hemolytic anemia, and oxidative damage) compared to many other species studied previously. While the underlying causes of mortality were not attributable to specific physiological or behavioral effects, the significant mortality in dosed groups compared to the control group indicates that exposure to MC252 DWH oil had a negative impact on laughing gulls.

Funding

This work was supported by the US Fish and Wildlife Service as part of the Deepwater Horizon Natural Resource Damage Assessment.

Acknowledgements

This study was supported by many and we are grateful for their excellent assistance: Nicole Mooers, Jeremy Ellis, Rachael Moulton, Benjamin Robey, Dr. Michael Scott, and Alicia Withrow.

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