2017

# Dietary intake of Deepwater Horizon oil-injected live food fish by doublecrested cormorants resulted in oxidative stress

Karen L. Pritsos
*University of Nevada*

Cristina R. Perez
*University of Nevada*

Thivanka Muthumalage
*University of Nevada*

Karen M. Dean
*Abt Associates*

Dave Cacela
*Abt Associates*

***See next page for additional authors***

**Authors**
Karen L. Pritsos, Cristina R. Perez, Thivanka Muthumalage, Karen M. Dean, Dave Cacela, K. C. Hanson-Dorr, Fred L. Cunningham, S. J. Bursian, Jane E. Link, Susan A. Shriner, Katherine E. Horak, and C. A. Pritsos

Ecotoxicology and Environmental Safety xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

# Dietary intake of Deepwater Horizon oil-injected live food fish by double-crested cormorants resulted in oxidative stress

Karen L. Pritsos[a], Cristina R. Perez[a], Thivanka Muthumalage[a], Karen M. Dean[b], Dave Cacela[b], Katie Hanson-Dorr[c], Fred Cunningham[c], Steven J. Bursian[d], Jane E. Link[d], Susan Shriner[e], Katherine Horak[e], Chris A. Pritsos[a,*]

[a] Department of Agriculture, Nutrition, and Veterinary Sciences, University of Nevada, Reno, United States
[b] Abt Associates, United States
[c] US Department of Agriculture, APHIS/Wildlife Services' National Wildlife Research Center, MS, United States
[d] Department of Animal Science, Michigan State University, East Lansing, MI, United States
[e] US Department of Agriculture, APHIS/Wildlife Services, National Wildlife Research Center, Fort Collins, CO, United States

ARTICLE INFO

*Keywords:*
Double-crested cormorants
Oil spill
Deepwater Horizon
Oxidative stress
Glutathione
Antioxidant enzymes

ABSTRACT

The Deepwater Horizon oil spill released 134 million gallons of crude oil into the Gulf of Mexico making it the largest oil spill in US history and exposing fish, birds, and marine mammals throughout the Gulf of Mexico to its toxicity. Fish eating waterbirds such as the double-crested cormorant (*Phalacrocorax auritus*) were exposed to the oil both by direct contact with the oil and orally through preening and the ingestion of contaminated fish. This study investigated the effects of orally ingestedMC252 oil-contaminated live fish food by double-crested cormorants on oxidative stress. Total, reduced, and oxidized glutathione levels, superoxide dismutase and glutathione peroxidase activities, total antioxidant capacity and lipid peroxidation were assessed in the liver tissues of control and treated cormorants. The results suggest that ingestion of the oil-contaminated fish resulted in significant increase in oxidative stress in the liver tissues of these birds. The oil-induced increase in oxidative stress could have detrimental impacts on the bird's life-history.

## 1. Introduction

The Deepwater Horizon (DWH) oil spill in 2010 released more than 3 million barrels of South Louisiana Sweet Crude oil which was widely dispersed by ocean currents, exposing countless organisms as the oil dispersed throughout the Gulf of Mexico (Chang, 2011). This oil spill resulted in a large number of dead birds as well as a large number of live birds that were exposed to sublethal amounts of oil (USFWS, 2011). Crude oils, including South Louisiana Sweet crude oil, contain toxic compounds that are readily accumulated by organisms, such as polycyclic aromatic hydrocarbons (PAHs) (Leighton, 1993). PAH metabolites are known toxicants that have been found to cause a wide range of adverse effects to oiled birds including liver and kidney damage, immunosuppression, suppressed growth, reduced hormone function, gastrointestinal irritation, failed reproduction, behavioral changes, and hemolytic anemia (Leighton, 1993; Briggs et al., 1997; Hartung and Hunt, 1966).

Oxidative stress is one of the fundamental toxic mechanisms that can occur from exposure to xenobiotics, such as the PAHs found in crude oil. It occurs when the balance between pro-oxidants and antioxidants is perturbed in favor of the former. Oxidative stress is manifested as oxidative damage and leads to the oxidation of key biological constituents such as proteins, lipids, and DNA. PAH compounds are metabolized by cytochrome P-450 oxidases (Nebert and Dalton, 2006). Cytochrome P4501A has been shown to be able to specifically metabolize some of the PAHs found in oil (Sarasquete and Segner, 2000). The metabolism of PAHs can produce toxic secondary metabolites that can react with molecular oxygen to generate many reactive oxygen species (ROS) in a redox cycling reaction. This redox cycling is the basis for chemical-induced oxidative damage. Thus, organisms that ingest these chemicals would be subject to potential oxidative damage. Oxidative stress has been implicated in the etiology of a great number of diseases and conditions (Limón-Pacheco and Gonsebatt, 2009).

One of the most commonly reported clinical indications of oxidative damage in birds is hemolytic anemia, which is damage to erythrocytes mediated by oxidative PAH metabolites (Troisi et al., 2007). These metabolites oxidize hemoglobin resulting in hemichrome formation and hemoglobin precipitation to form dense granular bodies called Heinz

* Corresponding author.
*E-mail address:* pritsos@cabnr.unr.edu (C.A. Pritsos).

http://dx.doi.org/10.1016/j.ecoenv.2017.06.067
Received 12 August 2016; Received in revised form 22 June 2017; Accepted 27 June 2017

bodies (Harr et al., in this issue). Heinz body hemolytic anemia has been reported in several species of oiled birds (Leighton et al., 1983, 1985; Leighton, 1986; Yamato et al., 1996; Troisi et al., 2007). In addition, field studies during the DWH oil spill assessment found increased percentages of Heinz bodies, reticulocytosis and anemia in live oiled birds captured and assessed in the Gulf of Mexico (Fallon et al., 2014). These findings thereby suggest that exposure to oil during the DWH oil spill resulted in oxidative stress that resulted in hemoglobin damage and led to the development of hemolytic anemia in these birds. Anemia is a particularly important oxidative outcome because it causes reduced availability of oxygen to tissues (Fallon et al., 2013). Birds suffering from hemolytic anemia are often fatigued and have reduced energy available for metabolic processes, which could have profound impacts on exercising birds that rely on high energy to sustain energy intensive flights.

Biomarkers of oxidative stress have been used as valuable indicators of environmental change and the physiological responses to that change. In addition, they may also be used as indicators of individual health, because they provide quantification of tissue damage (Constantini and Dell'Omo, 2015). Oxidative stress biomarkers may be particularly useful as a tool to assess the health status of individuals after an environmental pollution event, such as the DWH oil spill. Animals have multiple mechanisms of defense against oxidative damage, including antioxidants and catalytic enzymes. The concentration of antioxidants and antioxidant enzymes in the body are indicators of the amount of oxidative stress in the body (Koivula and Eeva, 2010). In a polluted environment, antioxidant systems can be induced as an adaptive response to allow organisms to combat oxidative stress (Marasco and Constantini, 2016). In contrast, the antioxidant system may also be inhibited, allowing for reduced protection and a greater susceptibility of cell damage. The activities (induction or inhibition) of antioxidant defense components have been used as biomarkers of oil exposure (Cossu et al., 1997; Cheung et al., 2000; Doyotte et al., 1997). Multiple biomarkers are commonly needed to reliably assess oxidative stress, including both enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are important antioxidant enzymes responsible for the removal of free radicals and reactive species (Limón-Pacheco and Gonsebatt, 2009). Together with the antioxidant enzymes, antioxidant compounds that quench free radicals are also used as endpoints of oxidative stress, specifically glutathione. Additionally, levels of lipid peroxidation products are commonly used as a measure of oxidative cellular damage (Peréz et al., 2010). However, to date, this multiple oxidative stress marker assessment strategy has not been employed in migratory bird studies.

The objective of this study was to determine if double-crested cormorants (*Phalacrocorax auritis*) fed fish injected with artificially weathered MC252 oil would develop signs of oxidative stress, consistent with the red blood cell oxidative stress observed in the wild, as reported by Fallon et al. (2014). Double-crested cormorants are easily managed in captivity, and were chosen as a model species that could be used as a surrogate species for other piscivorous waterbirds that were impacted by the DWH oil spill. To assess oxidative stress in this study, we used the multiple biomarker approach. We measured the effect of sublethal oil exposure on the levels of hepatic enzymatic antioxidants by analysis of superoxide dismutase and glutathione peroxidase activities. We also measured the levels of hepatic antioxidants by analysis of total glutathione, reduced glutathione (GSH), oxidized glutathione (GSSG), and total antioxidant capacity. In addition, we measured levels of hepatic lipid peroxidation products as a marker of oxidative damage in the liver. We predicted that oil-exposed birds would exhibit elevated oxidative stress in a dose-dependent manner as indicated by multiple oxidative stress markers.

## 2. Methods

### 2.1. Animal collection and husbandry

Double-crested cormorants (*Phalacrocorax auritus*; DCCO) were chosen to test forMC252-induced oxidative stress as part of the Deepwater Horizon Natural Resource Damage Assessment avian toxicity studies because they were affected by the DWH spill. All animal procedures were approved by the National Wildlife Research Center (NWRC) Institutional Animal Care and Use Committee (IACUC, approval #2107). The specific details of the capture, transport quarantine feeding and maintenance of these birds are provided in this issue (Cunningham et al., 2017).

### 2.2. Oral dosing study

Cormorants were randomly assigned to one of three treatment groups: a control group (n = 8); a group dosed daily with up to 5 ml oil/kg bw/day (n = 9); a group dosed daily with up to 10 ml oil/kg bw/day (n = 9). Fingerling catfish were given an intraperitoneal injection of 2.0 ml of artificially weatheredMC252 (*DWH7937, batch# B030112*) oil using a 20-gauge needle on a 25 ml stainless steel/glass barrel Hauptner® syringe as described in this issue (Cunningham et al., 2017). The oil-injected catfish were subsequently fed to cormorants in the oil treatment groups in their water-filled foraging tanks at a dosage of either 5 or 10 ml oil/kg body weight (bw) as described in this issue (Cunningham et al., 2017). The birds were necropsied on the last day of the study (day 21). Birds were weighed and euthanized by cervical dislocation according to IACUC-approved protocols. Liver tissues were extracted, cut up for each individual analysis, placed in separate cryovials, flash frozen with liquid nitrogen and shipped on dry ice to the Pritsos laboratory at the University of Nevada, Reno for oxidative stress marker analyses. Upon arrival at the lab in Reno, samples were recorded and stored into a −70 °C freezer until analyzed. Although blood and liver tissue were both collected during this study, liver tissue was chosen as the matrix to assess oxidative stress due to greater confidence in sample viability of the liver tissues and limited quantities of blood tissue for analyses.

### 2.3. Oxidative stress sample preparation and analyses

Oxidative damage in liver was assessed on liver homogenates prepared from the individual liver subsamples described above. For total, oxidized and reduced glutathione (TGSH, GSSG, and RGSH, respectively) liver tissue was homogenized in ice-cold 50 mM 2-(N morpholino) ethanesulphonic acid (MES), 1 mM EDTA buffer (pH 6)/g of tissue. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C and the supernatant was collected and kept on ice. The supernatant was deproteinated by addition of an equal volume of 0.1% metaphosphoric acid and then vortexed. After allowing the mixture to stand at room temperature for 5 min, it was centrifuged at 5000 × *g* for 5 min at room temperature. The supernatant was collected and used in the assay for TGSH and GSSG (kit #70300, Cayman Chemical). Reduced glutathione was calculated as the difference between TGSH and GSSG. To determine glutathione peroxidase activity, liver tissue was homogenized in ice-cold 50 mM Tris-HCl, 5 mM EDTA, 1 mM DTT buffer (pH 7.5)/g tissue. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C and the supernatant was collected and kept on ice. The assay was performed on appropriately diluted supernatant with GPx assay kit #703102 (Cayman Chemical Co.). To determine total antioxidant capacity (Trolox), liver tissue was homogenized in 5–10 ml of ice-cold phosphate buffered saline (PBS)/g tissue. The homogenate was centrifuged at 3000 × *g* for 12 min at 4 °C and the supernatant was collected for the assay. Trolox was determined using assay kit #TA02 (Oxford Biomedical Research) according to manufacturer's directions. For lipid peroxidation (LPO) assessment, 1 g of liver tissue was

homogenized in 5–10 ml of ice-cold 20 mM Tris buffer, pH 7.4, containing 5 mM BHT (to prevent sample oxidation). The homogenate was centrifuged at 3000 × *g* for 10 min at 4 °C and the resulting supernatant was diluted appropriately and used in assay kit #FR22 (Oxford Biomedical Research) according to manufacturer's directions. Liver peroxidation was chosen as a measure of oxidative damage over other endpoints, such as DNA damage and protein oxidation because of its more accessible assay methods and widespread use in environmental toxicology allowing for more direct comparisons between studies.

### 2.4. Statistical methods

For each oxidative stress parameter, a mean value per treatment group was calculated. Mean values of the two oil treated groups were contrasted with the mean values of the control group for all parameters using Dunnett's test. Data transformation was deemed unnecessary after visual inspection of data distributions. Calculations were performed using TIBCO Spotfire S-PLUS 8.2 for Windows. A *p*-value of less than 0.05 was interpreted as statistically significant.

## 3. Results

Double-Crested Cormorants were treated with artificially weathered MC252 crude oil internally through their food as previously described. Liver tissues were assessed for several markers of oxidative stress including; total glutathione, reduced glutathione, oxidized glutathione, glutathione peroxidase, superoxide dismutase, total antioxidant capacity and lipid peroxidation. The effects of oral exposure to MC252 crude oil in double-crested cormorants on hepatic total glutathione, reduced glutathione and oxidized glutathione are illustrated in Fig. 1a, b and c. Total glutathione levels increase in a dose-dependent manner from 26 nmol GSH/mg protein in the controls to 79.9 nmol GSH/mg protein in the highest dosed group. Similarly, reduced glutathione levels increased dose-dependently from 24 nmol GSH/mg protein in controls to 76.8 nmol GSH/mg protein in the highest dosed group. Hepatic oxidized glutathione levels were also increased in the groups receiving oil, however the increases were not dose-dependent. Overall, hepatic glutathione levels in birds exposed to oil orally were greatly increased.

Superoxide dismutase and glutathione peroxidase are considered front line enzymatic defenses against oxidative stress in biological organisms. Hepatic superoxide dismutase activity decreased in both groups receiving oil treatment compared to the control group (Fig. 2). Glutathione peroxidase activity in hepatic tissues of orally treated birds significantly decreased compared to control birds (Fig. 3).

During an oxidative insult due to a xenobiotic exposure, some antioxidants may be induced while others may be inhibited. Total antioxidant capacity is a measure of the total amount of antioxidant protection, both enzymatic and non-enzymatic, available to a tissue. The total antioxidant capacity of hepatic tissues in these birds increased with increasing exposure to oil (Fig. 4). Statistical significance was observed between the highest dosage group and controls.

Lipid peroxidation is a measure of oxidative damage that results when pro-oxidation events overwhelm the antioxidant defenses of an organism. Polyunsaturated fatty acid decomposition generates both malondialdehyde and 4-hydroxyalkenals. The measurement of both of these breakdown products is considered an excellent assessment method for lipid peroxidation. Lipid peroxidation was determined in these studies by assaying for malondialdehyde + 4-hydroxyalkenals. No statistically significant difference was observed between control and treatment groups (Fig. 5).

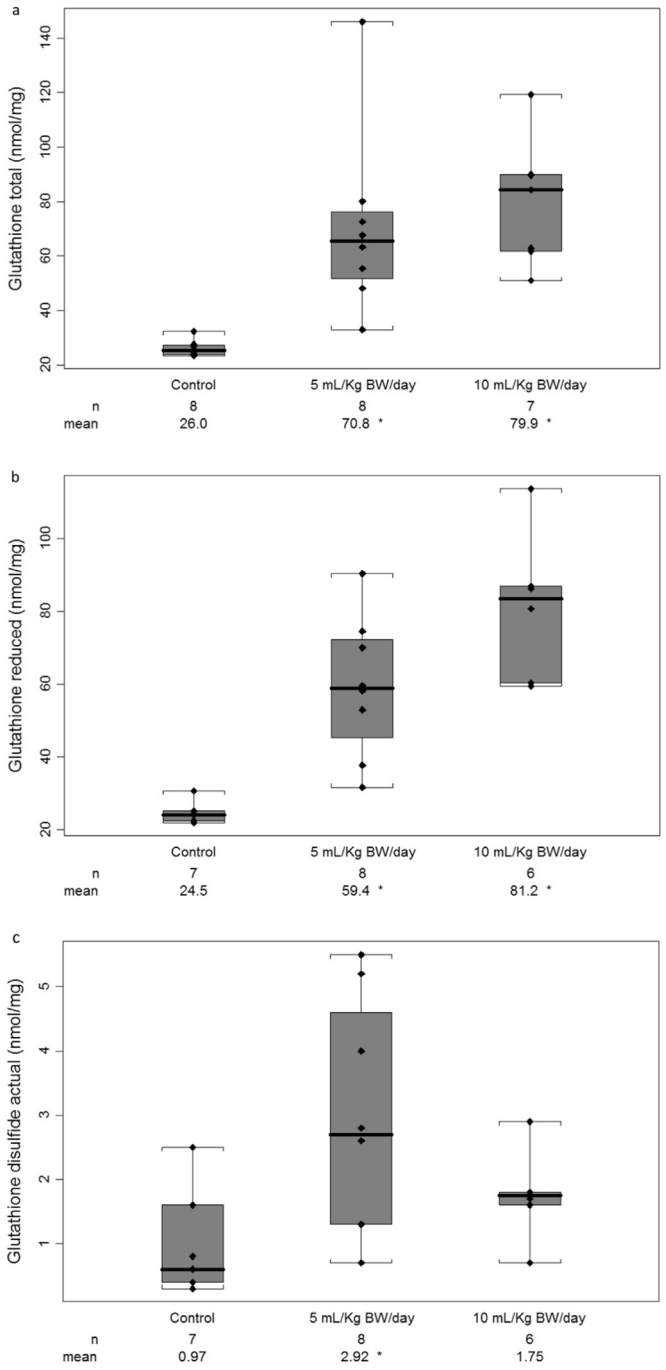


**Fig. 1.** a. Activity levels of hepatic total glutathione in two groups of double-crested cormorants orally exposed to artificially weathered MC252 crude oil compared to control birds. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the box is the median and the dots are individual values. * $p < 0.05$ vs control, Dunnett's test. b. Activity levels of hepatic reduced glutathione in two groups of double-crested cormorants orally exposed to artificially weathered MC252 crude oil compared to control birds. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the box is the median and the dots are individual values. * $p < 0.05$ vs control, Dunnett's test. c. Activity levels of hepatic glutathione disulfide in two dosing groups of double-crested cormorants orally exposed to artificially weathered crude oil compared to control birds. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the box is the median and the dots are individual values. * $p < 0.05$ vs control, Dunnett's test.

## 4. Discussion

In this study, we confirmed that oral exposure of double-crested cormorants to weathered DWH MC252 crude oil-induced hepatic oxidative stress. Birds ingesting oil injected fish exhibited increases in hepatic glutathione disulfide, reduced glutathione, and total glutathione concentrations, as well as decreases in hepatic superoxide dismutase and glutathione peroxidase activities compared to control

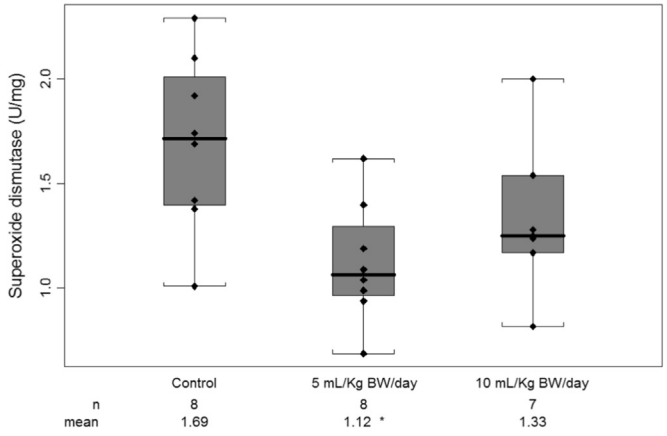


**Fig. 2.** Activity levels of hepatic superoxide dismutase in two dosing groups of double-crested cormorants orally exposed to artificially weathered crude oil compared to control birds. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the box is the median and the dots are individual values. * $p < 0.05$ vs control, Dunnett's test.

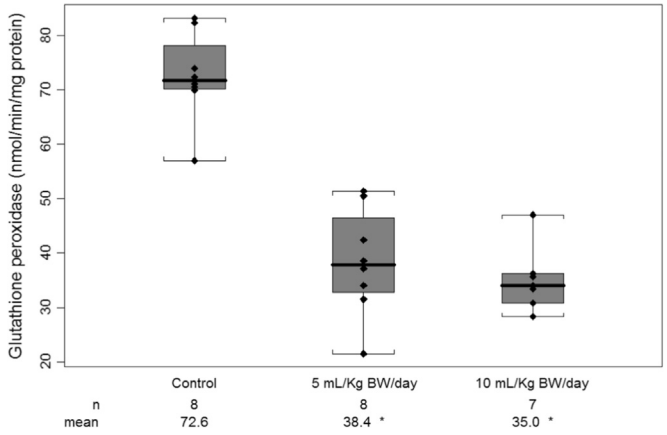


**Fig. 3.** Activity levels of hepatic glutathione peroxidase in two dosing groups of double-crested cormorants orally exposed to artificially weathered crude oil compared to control birds. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the box is the median and the dots are individual values. * $p < 0.05$ vs control, Dunnett's test.

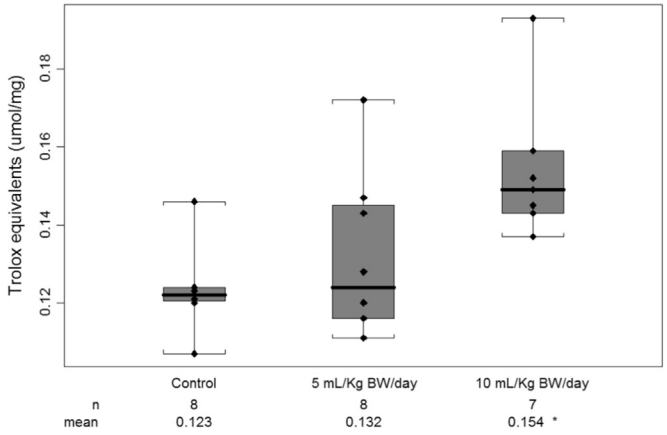


**Fig. 4.** Levels of total antioxidant capacity in two dosing groups of double-crested cormorants orally exposed to artificially weathered crude oil compared to control birds. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the box is the median and the dots are individual values. * $p < 0.05$ vs control, Dunnett's test.

birds. The metabolism of PAHs found in crude oil by cytochrome P450 enzymes produce oxides and reactive oxygen species that create

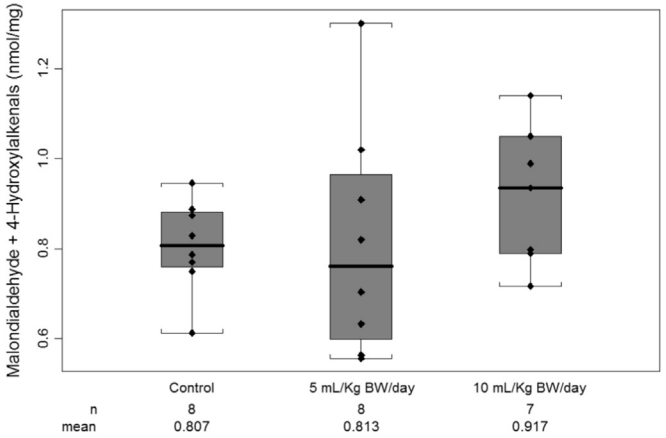


**Fig. 5.** Levels of lipid peroxidation in two dosing groups of double-crested cormorants orally exposed to artificially weathered crude oil compared to control birds. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the box is the median and the dots are individual values.

oxidative conditions in oil exposed birds. Superoxide dismutase and glutathione peroxidase are important antioxidant enzymes that are considered part of the first line of defense against oxidative stress. These enzymes work to catalyze reactions that reduce toxic compounds to nontoxic metabolites. The decreased levels of hepatic SOD and GPx activity in this study likely reflect inhibition by organic electrophilic chemical constituents in oil (Staimer et al., 2012). Inhibition of both of these front line antioxidant enzymes would increase oxidative stress in the oil-exposed double-crested cormorants.

The double-crested cormorants in this study showed evidence of increased hepatic tissue oxidative stress, however, it appears that the birds were able to compensate for this stress primarily by increasing hepatic glutathione levels and thus did not exhibit significantly increased lipid peroxidation levels. Glutathione is the most abundant cellular antioxidant (Limón-Pacheco and Gonsebatt, 2009) and is a primary detoxification pathway for xenobiotics in the liver. Induction of de novo synthesis of GSH occurs as an adaptive response to oxidative stress (Biswas and Rahman, 2009). Increased levels of ROS result in an initial decrease in GSH levels, which can elicit activation of transcription factors and result in increased production of GSH in the tissue (Itoh et al., 1997). GSH becomes oxidized during the reduction of peroxides via glutathione peroxidase. Thus, increased amounts of the oxidized form of glutathione, GSSG, are an indicator of increased oxidative stress, as GSSG normally only represents less than 1% of total cellular GSH (Biswas and Rahman, 2009). GSSG is recycled via glutathione reductase to regenerate GSH. Thus, increased concentrations of hepatic GSH are also consistent with oxidative stress. In this study, we found both total and reduced glutathione levels to be dose-dependent, with the highest levels occurring in the highest dosed group. This indicates that the highest dosed birds suffered greater oxidative stress presumably due to the higher concentration of crude oil constituents and consequently needed greater protection of cells from oxidative damage. The increase in total antioxidant capacity observed in the oil-exposed birds is a reflection of the adaptive increase in glutathione observed in the oil groups. The failure to observe a significant increase in lipid peroxidation in these birds is very likely due to this robust antioxidant response.

The DWH NRDA toxicity testing program used four different oil samples of varying degrees of weathering in all of the studies conducted. The preparation and chemical composition of the DWH artificially weathered MC252 oil used in this study are described in Forth et al. (2016). Weathering of the oil resulted in the loss of the volatile, lighter weight PAH's, including BTEX compounds. Thus, the resulting oil had a compositional shift to the heavier compounds in the oil. Compared to the unweathered source oil, the percentage of PAH

depletion in the artificially-weathered source was 27% (27% weathered). The toxicity of oil samples vary depending on chemical composition. The increased concentration of heavy PAH compounds in the oil sample used in this study, likely contributed to the toxicity observed in the treated cormorants.

Changes in the oxidative endpoints of the double-crested cormorants described here provide supporting evidence of systemic oxide radical damage in the tissues of these birds. Heinz body hemolytic anemia was observed in the birds of this study (Harr et al., in this issue) as well as in birds collected from the field after the DWH oil spill (Fallon et al., 2014). This indicates that both wild and captive DWHMC252 oil-exposed birds are capable of suffering oxidative injury, given that detection of Heinz bodies provides evidence of direct red blood cell damage. Consistent with these findings, Leighton et al. (1985) found that oral exposure of herring gulls (*Larus argentatus*) to Prudhoe Bay oil for two days resulted in oxidant stress on red blood cells as indicated by presence of Heinz bodies and elevated red blood cell GSH. Hemolytic anemia can have significant effects on avian migration and life-history. The destruction of red blood cells through oxidative damage decreases the bird's ability to transport oxygen. Flying birds, particularly migrating birds undergoing long distance flights, require increased oxygen to fuel the increased aerobic demands of the flight muscles. Thus, it would be presumed that birds suffering from oil-induced anemia would exhibit a reduction in the ability to sustain flight. In addition to flight ability, Heinz body anemia also adversely affects reproductive success, and immune function (Henkel et al., 2012; Leighton, 1993; Briggs et al., 1997).

Managing oxidative stress is a particularly important physiological function for migrating birds and although the birds in this study were not actively flying, flight is a major component of a bird's life history. Therefore, the combination of flight stress and oil exposure stress should be addressed when considering an individual's long term challenges and probability of survival. Oiled birds are likely to experience great difficulties in trying to combat oxidative stress. In this study, we found that inactive double-crested cormorants fed oil-contaminated fish experienced increased oxidative stress. Although the cormorants in this study were able to induce glutathione levels high enough to avoid oxidative damage (as measured by lipid peroxidation), it would be expected that in combination with ecologically relevant inducers of oxidative stress such as physical activity, immune response, and reproduction (Constantini, 2008), oiled birds would be unable to maintain redox balance and suffer exacerbated levels of oxidative damage. Studies have shown that all flying birds experience oxidative stress (Constantini et al., 2007, 2008; Jenni-Elermann et al., 2014; Eikenaar et al., 2016; Skrip et al., 2015; Skrip and McWilliams, 2016); however, the preparation and repair ability of an individual determines the extent of damage. Individuals in good condition are better able to maintain redox homeostasis, experiencing less oxidative stress than individuals in poor body condition (Constantini et al., 2007). For migratory birds affected by oil spills, the body condition of the individual at the onset of oil exposure is probably a determining factor in its ability to up-regulate defense systems and cope with the oxidative challenge of both oil exposure and flight.

Over time, oil exposure reduces body condition due to changes in foraging behavior, loss of motivation to eat, lethargy, and reduced nutrient absorption from oil ingestion (Perez et al., in this issue; Burger and Tsipoura, 1998; Burger, 1997). Ideally, birds experiencing unexpected oxidative conditions could easily ingest antioxidant-rich food to quickly re-establish redox balance (Catoni et al., 2008). However, birds affected by an oil spill would be unlikely to do this given their immobile, lethargic state and the contaminated nature of their environment. Thus, oiled birds would likely have to rely on the synthesis of de novo antioxidants. Synthesis of endogenous antioxidants is timely and often condition-dependent (Garratt and Brooks, 2012). Birds in poor condition are energy deficient and would be less likely to allocate sufficient energy and resources towards antioxidant production. In addition, it has been well established that oiled birds have difficulty maintaining thermoregulatory processes. Feathers contaminated with crude oil lose their insulating and water-repellant properties, resulting in body heat loss and an increase in metabolic rate in attempts to regulate body temperature, exhausting energy stores (Leighton, 1993). Birds exposed to temperatures outside of their thermoneutral zone experience increases in reactive oxygen species production (Lin et al., 2008; Beaulieu et al., 2014). In this study, oil-dosed birds lost weight over the course of study due to lack of appetite and lethargy and all oil-dosed birds had oil on their plumage as a result of foraging for fish in the feed tanks that contained oil excreted by the birds. Consequently, the oil-dosed birds had reduced cloaca temperatures and were observed to seek supplemental heat under the heat lamps provided (Cunningham et al., 2017), suggesting that the birds were losing substantial body heat as a result of oil contamination. Thus, the adverse effects of oil exposure on the body condition and thermoregulation capabilities of birds would further contribute to their overall oxidative stress load.

The life-histories of birds vary among species and thus so does the complex system underlying oxidative stress. This makes measuring oxidative stress in individuals complicated. Studies suggest that the best way to assess oxidative stress is by use of multiple markers of oxidative stress including the use of at least one antioxidant capacity marker and one marker of oxidative damage (Constantini, 2008; Skrip and McWilliams, 2016). Measuring both aspects of the system are needed to better understand how the bird is coping with the oxidative challenge and to what extent. Marker choice, as well as matrix choice, may produce differing results given that activity levels differ with tissues, and thus should be selected based on research goals. Blood-based markers are often the preferred choice of researchers given the non-invasive nature of blood draw and accessibility in the field. However, the enzyme activity in the blood is relatively low (Constantini, 2008), and suggests that tissue concentrations would provide a better understanding of the changes occurring in the system. In this study, we looked at a total of seven different biomarkers in the liver tissue of double-crested cormorants. We found significant differences in six out of the seven biomarkers measured in the dietary-dosed birds. Given the thorough use of oxidative biomarkers in this study, we can confidently conclude that dietary oil exposure of double-crested cormorants caused increased oxidative stress compared to control birds. However, it should be noted that choosing the appropriate marker for oxidative damage is also important. In this study, we used products of lipid peroxidation as a measure for damage. We did not detect any significant changes in liver lipid peroxidation, suggesting that induction of the defense system was sufficient to prevent cellular damage. Yet, Heinz body formations in red blood cells were detected in birds in both of the oil-exposed groups in a dose-dependent manner (Harr et al., in this issue). This indicates that the oiled cormorants in this study suffered cellular oxidative damage. Red bloods cells may be more susceptible to oxidation due to the oxygen-hemoglobin interactions and low levels of antioxidant protection circulating in the blood compared to tissue concentrations (Pandey and Rizvi, 2011).

Acute mortality has proven to be an important endpoint for avian species after oil spill events (Burger, 1993). This study helps demonstrate that sub-chronic effects due to low-level exposure to oil are also important endpoints for avian species after oil spill events and should not be overlooked when estimating mortality. Among other clinical effects, double-crested cormorants fed oil-injected fish at target doses of 5 ml oil/kg/day and 10 ml oil/kg/day for up to 21 days showed elevated oxidative stress in a dose-dependent manner compared to control birds. Birds face oxidative challenges throughout their life, evolving adaptive mechanisms to maintain health status. However, unexpected oxidative challenges such as high PAH exposure may push their adaptive mechanisms past their limit and individuals may not be able to compensate for the stress, leading to unrepairable damage. The ability of an individual to withstand toxicant-induced oxidative stress may depend on its early life experiences, body condition, geographic

location of wintering and breeding sites, reproductive effort, health status, age, migratory status, food choice, sex, and genetics (Constantini et al., 2012; Metcalfe and Alonso-Alvarez, 2010; Constantini et al., 2010); and thus it would be expected to observe substantial individual differential responsiveness to low-levels of oil exposure. The inability to cope with all sources of oxidative stress may force trade-offs in avian life-histories. While it is important to accurately estimate the number of deaths that result from oil spills, it is also important to understand how non-lethal oil exposure affects life-history decisions that impact overall fitness.

## Author contributions

K.M.D., F.C., S.S., K.H., C.A.P, S.J.B., J.E.L. study design; K.H-D., F.C. animal care and experimentation; K.L.P. T.M. oxidative stress analyses; K.L.P., D.C. data analyses; C.R.P., C.A.P. manuscript preparation

## Competing financial interests

The authors declare that they have no competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

## Acknowledgements

This project was funded in part by the Deepwater Horizon Natural Resource Damage Assessment Trustees and the Nevada Agricultural Experiment Station.

## References

Beaulieu, M., Haas, A., Schaefer, H.M., 2014. Self-supplementation and effects of dietary antioxidants during acute thermal stress. J. Exp. Biol. 217, 370–375. http://dx.doi.org/10.1242/jeb.092825.

Biswas, S.K., Rahman, I., 2009. Environmental toxicity, redox signaling and lung inflammation: the role of glutathione. Mol. Aspects of Med. 20, 60–76. https://doi.org/10.1016/j.mam.2008.07.001.

Briggs, K.T., Gershwin, M.E., Anderson, D.W., 1997. Consequences of petrochemical ingestion and stress on the immune system of seabirds. J. Mar. Sci. 54, 718–725.

Burger, A.E., 1993. Estimating the mortality of seabirds following oil spills; effects of spill volume. Mar. Pollut. Bull. 26, 140–143.

Burger, J., 1997. Effects of oiling feeding behavior of sanderlings and semipalmated plovers in New Jersey. Condor 99, 290–298.

Burger, J., Tsipoura, N., 1998. Experimental oiling of sanderlings (*Calidris alba*): behavior and weight changes. Environ. Toxicol. Chem. 17, 1154–1158.

Catoni, C., Peters, A., Schaefer, H.M., 2008. Life history trade-offs are influenced by diversity, availability, and interactions of dietary antioxidants. Anim. Behav. 76, 1107–1119.

Chang, Y.-L., Oey, L., Xu, F.-H., Lu, H.-F., Fujisaki, A., 2011. 2010 oil spill: trajectory projections based on ensemble drifter analyses. Ocean Dyn. 61, 829–839.

Cheung, C.C.C., Zheng, G.J., Li, A.M.Y., Richardson, B.J., Lam, P.K.S., 2000. Relationship between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquat. Toxicol. 52, 189–203.

Constantini, D., 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol. Lett. 11, 1238–1251.

Constantini, D., Dell'Omo, G., 2015. Oxidative stress predicts long-term resight probability and reproductive success in Scopoli's shearwater (*Calonectris diomedea*). Conserv. Physiol. 3 (1), cov024. http://dx.doi.org/10.1093/conphys/con024.

Constantini, D., Cardinale, M., Carere, C., 2007. Oxidative damage and anti-oxidant capacity in two migratory bird species at a stop-over site. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 144, 363–371.

Constantini, D., Dell'Ariccia, G., Lipp, H.-P., 2008. Long flights and age affect oxidative status of homing pigeons (*Columba livia*). J. Exp. Biol. 211, 377–381.

Constantini, D., Rowe, M., Butler, M.W., McGraw, K.J., 2010. From molecules to living systems; historical and contemporary issues in oxidative stress and antioxidant ecology. Funct. Ecol. 24, 950–959.

Constantini, D., Monaghan, P., Metcalfe, N.B., 2012. Early life experience primes resistance to oxidative stress. J. Exp. Biol. 215, 2820–2826.

Cossu, C., Doyotte, A., Jacquin, M.C., Babut, M., Exinger, A., Vasseur, P., 1997. Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves, *Unio tumidus*, as biomarkers of aquatic contamination in field studies. Ecotoxicol. Environ. Saf. 38, 122–131.

Cunningham, F., Dean, K., Hanson-Dorr, K., Harr, K., Healy, K., Horak, K., Shriner, S., Rupp, T., Bursian, S., Dorr, B., 2017. Development of methods for avian oil toxicity studies using the double crested cormorant (*Phalacrocorax auritus*). Ecotoxicol. Environ. Saf. 141, 199–208. http://dx.doi.org/10.1016/j.ecoenv.2017.03.025.

Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M., Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and digestive gland of the freshwater bivalve *Unio tumidus*. Aquat. Toxicol. 39, 93–110.

Eikenaar, C., Jönsson, J., Fritzsch, A., Wang, H.-L., Isaksson, C., 2016. Migratory refueling affects non-enzymatic antioxidant capacity, but does not increase lipid peroxidation. Physiol. Behav. 158, 26–32.

Fallon, J.A., Hopkins, W.A., Fox, L., 2013. A practical quantification method for Heinz bodies in birds applicable to rapid response field scenario. Environ. Toxicol. Chem. 12, 401–405.

Fallon, J.A., Smith, E.P., Hopkins W., 2014. Determining physiological injury to birds exposed to oil from The Deepwater Horizon (Mississippi Canyon 252) Spill. USFWS Rep., pp. 1–127.

Forth, H.P., Mitchelmore, C.L., Morris, J.M., Lipton, J., 2016. Characterization of oil and water accommodated fractions used to conduct aquatic toxicity testing in support of the Deepwater Horizon oil spill natural resource damage assessment. Environ. Toxicol. Chem. 999, 1–10. http://dx.doi.org/10.1002/etc.3672.

Garratt, M., Brooks, R.C., 2012. Oxidative stress and condition-dependent sexual signals: more than just seeing red. Proc. Biol. Sci. 279, 3121–3130.

Harr, K.E., et al., 2017. Weathered MC252 crude oil-induced anemia and abnormal erythroid morphology in double-crested cormorants (Phalacrocorax auritus) with light microscopic and ultrastructural description of Heinz bodies. Ecotoxicol. Environ. Saf (in this issue).

Hartung, R., Hunt, G.S., 1966. Toxicity of some oils to waterfowl. J. Wildl. Manag. 30, 564–570.

Henkel, J.R., Sigel, B.J., Taylor, C.M., 2012. Large-scale impacts of the *Deepwater Horizon* oil spill: can local disturbance affect distant ecosystems through migratory shorebirds. BioScience 62, 676–685.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., Nabeshima, Y., 1997. An Nrf2/Small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun. 236, 313–322. https://doi.org/10.1006/bbrc.1997.6943.

Jenni-Elermann, S., Jenni, L., Smith, S., Constantini, D., 2014. Oxidative stress in endurance flight: an unconsidered factor in bird migration. PLoS One 9 (5), e97650. http://dx.doi.org/10.1371/journal.pone.0097650.

Koivula, M.J., Eeva, T., 2010. Metal-related oxidative stress in birds. Environ. Pollut. 158 (7), 2359–2370.

Leighton, F.A., 1986. Clinical gross and histological findings in Herring Gulls and Atlantic Puffins that ingested prudhoe bay oil. Vet. Pathol. 23, 254–263.

Leighton, F.A., 1993. The toxicity of petroleum oils to birds. Environ. Rev. 1, 92–103.

Leighton, F.A., Peakall, D.B., Ronald, G., Butler, R.G., 1983. Heinz- body from the ingestion of crude oil: primary toxic effect in marine birds. Science 220, 871–873.

Leighton, F.A., Lee, Y.Z., Rahimtula, A.D., O'Brien, P.J., Peakall, D.B., 1985. Biochemical and functional disturbances in red blood cells of herring gulls ingesting Prudhoe Bay crude oil. Toxicol. Appl. Pharm. 81, 25–31.

Limón-Pacheco, J., Gonsebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat. Res. 674, 137–147.

Lin, H., De Vos, D., Decuypere, E., Buyse, J., 2008. Dynamic changes in parameters of redox balance after mild heat stress in aged laying hens (*Gallus gallus domesticus*). Comp. Biochem. Physiol. 147, 30–35. http://dx.doi.org/10.1016/j.cbpc.2007.07.005.

Marasco, V., Constantini, D., 2016. Signaling in a polluted world: oxidative stress as an overlooked mechanism linking contaminants to animal communication. Front. Ecol. Evol. 4, 95. http://dx.doi.org/10.3389/fevo.2016.00095.

Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct. Ecol. 24, 984–996.

Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signaling pathways and environmental carcinogenesis. Nat. Rev. Cancer 6 (12), 947–960. http://dx.doi.org/10.1038/nrc2015.

Pandey, K.B., Rizvi, S.I., 2011. Biomarkers of oxidative stress in red blood cells. Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc Czech Repub. 155 (Suppl 2), 131–136.

Perez, C., Moye, J., Cacela, D., Dean, K., Pritsos, C., 2017. Body mass change in flying homing pigeons externally exposed to Deepwater Horizon crude oil. Ecotoxicol. Environ. Saf (in this issue).

Peréz, C., Lores, M., Veland, A., 2010. Oil pollution increases plasma antioxidants but reduces coloration in a seabird. Oecologia 163, 875–884. http://dx.doi.org/10.1007/s00442-010-1677-2.

Sarasquete, C., Segner, H., 2000. Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. Sci. Total Environ. 247, 313–332.

Skrip, M.M., McWilliams, S.R., 2016. Oxidative balance in birds: an atoms-to-organisms-to-ecology primer for ornithologists. J. Field Ornithol. 87 (1), 1–20. http://dx.doi.org/10.1111/jofo.12135.

Skrip, M.M., Bauchinger, U., Goymann, W., Fusani, L., Cardinale, M., Alan, R.R., McWilliams, S.R., 2015. Migrating songbirds on stopover prepare for, and recover form, oxidative challenges posed by long-distance flights. Ecol. Evol. 5 (15), 3198–3209. http://dx.doi.org/10.1002/ece3.1601.

Staimer, N., Nguyen, T.B., Nizkorodov, S.A., Delfino, R.J., 2012. Glutathione peroxidase inhibitory assay for electrophilic pollutants in diesel exhaust and tobacco smoke. Anal. Bioanal. Chem. 403 (2), 431–441.

Troisi, G., Borjesson, L., Bexton, S., Robinson, I., 2007. Biomarkers of polycyclic aromatic hydrocarbon (PAH)-associated hemolytic anemia in oiled wildlife. Environ. Res. 105, 324–329.

USFWS, 2011. Deepwater Horizon Bird Impact Data from the DOI-ERDC NRDA Database 12 May 2011.

Yamato, O., Koto, I., Maede, Y., 1996. Hemolytic anemia in wild seaducks caused by marine oil pollution. J. Wildl. Dis. 32 (2), 381–384.