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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

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ABSTRACT

Injury assessment of birds following the Deepwater Horizon (DWH) oil spill in 2010 was part of the Natural Resource Damage Assessment. One reported effect was hemolytic anemia with the presence of Heinz bodies (HB) in birds, however, the role of route and magnitude of exposure to oil is unknown. The purpose of the present study was to determine if double-crested cormorants (*Phalacocorax auritis*; DCCO) exposed orally and dermally to artificially weathered crude oil would develop hemolytic anemia including HB and reticulocytosis. In the oral experiment, sub-adult, mixed-sex DCCOs were fed control (n = 8) or oil–injected fish with a daily target dose of 5 (n = 9) or 10 (n = 9) ml oil/kg for 21 days. Then, subadult control (n = 12) and treated (n = 13) cormorant groups of similar sex-ratio were dermally treated with approximately 13 ml of water or weathered MC252 crude oil, respectively, every 3 days for 6 dosages approximating 20% surface coverage. Collected whole blood samples were analyzed by light (new methylene blue) and transmission electron microscopy. Both oral and dermal treatment with weathered DWH MC252 crude oil induced regenerative, but inadequately compensated, anemia due to hemolysis and hematochezia as indicated by decreased packed cell volume, relative increase in reticulocytes with lack of difference in corrected reticulocyte count, and morphologic evidence of oxidant damage at the ultrastructural level. Hemoglobin precipitation, HB formation, degenerate organelles, and systemic oxidant damage were documented. Heinz bodies were typically < 2 μm in length and smaller than in mammals. These oblong cytoplasmic inclusions were difficult to see upon routine blood smear evaluation and lacked the classic button appearance found in mammalian red blood cells. They could be found as light, homogeneous blue inclusions upon new methylene blue staining. Ultrastructurally, HB appeared as homogeneous, electron-dense structures within the cytosol and lacked membranous structure. Oxidant damage in avian red blood cells results in degenerate organelles and precipitated hemoglobin or HB with different morphology than that found in mammalian red blood cells. Ultrastructural evaluation is needed to definitively identify HB and damaged organelles to confirm oxidant damage. The best field technique based on the data in this study is assessment of PCV with storage of blood in glutaraldehyde for possible TEM analysis.

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1. Introduction

Following the Deepwater Horizon (DWH) oil spill in 2010, many oiled and not visibly oiled bird carcasses were recovered from areas along the Gulf of Mexico. In addition, a large number of live birds was observed, but with lower levels of oiling defined as < 40% body coverage (Burger, 1993). The fate of these oiled birds was an important component of the avian injury assessment for the DWH Mississippi Canyon 252 (MC252) Oil Spill Natural Resource Damage Assessment (NRDA) as well as our understanding of the effects of oil on avian health.

Crude oil from different geographical regions varies in chemical composition, and therefore, may have different toxic effects resulting in diverse clinical signs. MC252 is a south Louisiana sweet (low in sulfur) crude oil. Compared with other crude oils, MC252 has relatively high concentrations of alkanes that microorganisms can use as a food source and relatively low concentrations of polycyclic aromatic hydrocarbons (PAHs) (Faksness et al., 2015; Turner et al., 2014). Therefore, it has been purported to be biodegradable and less toxic than other oils (Kimes et al., 2014). However, MC252 did also contain low levels of volatile organic compounds such as benzene, toluene, and xylene. Further, the toxicity of an oil across taxa may vary with the degree of weathering (Bellas et al., 2013; Finch et al., 2011; Rial et al., 2013). The DWH spill was approximately 80 km off the Louisiana coastline and the oil traveled over 1600 m through the water column to reach the surface (Kimes et al., 2014; Trustees, 2015). This resulted in exposure of avian, aquatic, and terrestrial species to weathered crude oil that had undergone loss of volatile organic compounds such as benzene, toluene and xylene. Indeed, the weathered MC252 tested here had a relatively high concentration of PAHs (Forth et al., 2017). Hence, the specific suite of toxic effects on wildlife caused by these variously weathered subtypes of MC252 required further elucidation as these weathered types of oil were more likely to be the cause of avian exposure (Henkel et al., 2012).

When birds are exposed to less than acutely lethal dosages, oil can cause a wide range of adverse effects, including hemolytic anemia, renal, myeloid, and hepatic damage, decreased nutrient absorption, altered stress response, and decreased immune function (Alonso-Alvarez et al., 2007; Leighton, 1985, 1986; Peakall et al., 1981, 1989; Szaro et al., 1978). Hemolytic anemia may be characterized by red blood cell (RBC) regeneration and therefore increased numbers of reticulocytes when the bone marrow is intact. When hemolytic anemia is caused by a toxicant, oxidative damage to hemoglobin results in hemichrome formation, hemoglobin precipitation, and development of Heinz bodies (HB) (Desnoyers, 2010). Heinz bodies are cytoplasmic inclusions composed of tightly associated, oxidized globin molecules generated during the oxidative breakdown of hemoglobin. Red blood cells containing HB have decreased ability to deliver oxygen to cells and are removed from circulation. Heinz bodies are definitive evidence of oxidative damage to hemoglobin, whether due to toxic oxidants (such as oil) or predisposition to genetic abnormalities of hemoglobin or RBC reducing pathways (Desnoyers, 2010). Oil and its oxidized metabolites may damage different stages of RBC maturation including rubriblasts to rubricytes in the bone marrow as well as mature RBCs in peripheral blood (Olsgard, 2007). During the initial DWH MC252 oil spill assessment, increased percentages of HB, reticulocytosis and anemia were reported during field evaluation of live, oiled birds in the Gulf of Mexico (Fallon et al., 2014).

Double-crested cormorants were chosen as a representative animal model because they were impacted by the DWH spill, are common, primarily piscivorous waterbirds that inhabit pelagic, coastal, and inland waterways (Reed et al., 2003), and as such, could be used as surrogates for other piscivorous species such as pelicans (*Pelicanus* sp.), terns (*Sternidae* sp.), and skimmers (*Rynchops* sp.). Additionally, DCCO are listed by the International Union for Conservation of Nature (IUCN) as a species of least concern that is relatively easily managed in captivity.

One objective of the present study was to determine if double-crested cormorants (*Phalacocorax auritis*; DCCOs) orally or dermally exposed to artificially weathered MC252 oil (DWH7937, batch# B030112) would develop clinical signs indicative of hemolytic anemia, including generation of HB, reticulocytosis, and a decrease in packed cell volume (PCV), which were reported by Fallon et al. (2014).

2. Methods

2.1. Toxicant

MC252 (DWH7937, batch# B030112) oil was collected during the 2010 Deepwater Horizon oil spill and artificially weathered prior to use in the studies as previously described (Forth et al., 2017).

2.2. Animals and husbandry

Animal acquisition, maintenance and use were approved by the Institutional Animal Care and Use Committee of the US Department of Agriculture National Wildlife Research Center (NWRC). Complete details of animal collection and husbandry and methodology of the oral and dermal exposure studies are in Cunningham et al. (2017, this issue). Birds were allowed to acclimate to captivity in quarantine for a minimum of 21 days prior to initiation of each study.

2.3. Oral dosing experiment

Briefly, a total of 26 adult, mixed-sex, apparently healthy, wild caught DCCOs were randomly assigned to one of three treatment groups: a control group (n = 8) fed fish lightly anesthetized with MS222; a treatment group dosed daily with up to 5 ml oil/kg BW oil by offering oil-injected fish (n = 9); and a treatment group dosed daily with up to 10 ml oil/kg BW by offering oil-injected fish (n = 9). Daily provision of oiled-injected fish was up to 21 days.

Blood samples were collected from each bird on day 0 (the day before oral dosing began) as a baseline comparison, then twice weekly until humane euthanasia, which occurred either during the study if warranted by clinical signs or at the conclusion of the study prior to complete necropsy. Blood samples were collected in heparinized syringes via brachial veins while animals were manually restrained.

2.4. Dermal exposure experiment

A total of 31 DCCO's were captured and retained in captivity. A total of 25 subadult DCCOs allocated to a control group (n = 12, 5 male, 7 female) and an exposed group (n = 13, 6 males, 7 females) were used in this trial. DCCOs were assigned to treatment groups based on the results of blood samples collected at the initiation of the three-week quarantine period. Complete blood count (CBC) values were used to ensure equal division of birds with potential health concerns between groups. DCCO's with monocyte counts greater than 2.0×10^9 cells/l were considered abnormal (severe monocytosis); and were divided between control (n = 4) and treatment (n = 3) groups. Additionally, a small oil spill took place one year prior to the study, not far from where 6 of the DCCOs were collected and were evenly distributed between groups. During the course of the trial, one bird from the control group and two birds from the treatment group died and were not replaced. Therefore, the final number of birds in the control and exposed group was 11 birds each to total 22 in the study. Oil on exposed birds (13 ml) and water on control birds (13 ml) was applied every three days through Day 15 of the trial (on Days 0, 3, 6, 9, 12, and 15). Detailed description of application is available in Cunningham et al. (2017). Oil exposure may have been via preening, transdermally, or through inhalation.

Blood samples were collected in heparinized syringes via jugular veins while animals were manually restrained. All birds had a blood sample taken during quarantine to provide baseline data (day −21). During the trial, blood was collected every six days just prior to external application of oil or water (days 0, 6, 12, and 18) and just prior to euthanasia and necropsy (day 21).

Table 1
Effect of oral or dermal weathered MS252 crude oil exposure on hematologic values in double-crested cormorants (*Phalacocorax auritus*). [1]Necopsied at 21 days; [2]Necropsied at 14 days.

	Orally dosed birds			Dermally dosed birds	
Analyte	Control[1]	5 ml oil/kg BW[1]	10 ml oil/kg BW[2]	Control	Exposed
PCV (%)					
Day 0	47 ± 1.2	43 ± 0.9	42 ± 1.1	40 ± 0.3	43 ± 0.3
Day 6	–	–	–	39 ± 0.4	37 ± 0.3
Day 7	40 ± 1.5	33 ± 1.4	34 ± 1.5	–	–
Day 12	–	–	–	40 ± 0.3^{A}	34 ± 0.2^{B}
Day 14	39 ± 1.6^{A}	32 ± 1.4^{A}	24 ± 1.6^{B}	–	–
Day 18	–	–	–	41 ± 0.3^{A}	31 ± 0.2^{B}
Day 21	39 ± 1.8^{A}	28 ± 1.7^{B}	–	40 ± 0.3^{A}	30 ± 0.3^{B}
Aggregate reticulocytes (%)					
Day 0	3 ± 0.4	2 ± 0.5	5 ± 0.5	4 ± 0.3	4 ± 0.4
Day 6	–	–	–	4 ± 0.3	4 ± 0.2
Day 7	2 ± 0.5	2 ± 0.5	3 ± 0.5	–	–
Day 12	–	–	–	5 ± 0.2	6 ± 0.5
Day 14	3 ± 1.1	5 ± 1.2	7 ± 1.4	–	–
Day 18	–	–	–	5 ± 0.4	7 ± 1.0
Day 21	4 ± 1.5	8 ± 1.6	–	4 ± 0.3	5 ± 0.3
Corrected reticulocytes (%)					
Day 0	3 ± 0.5	2 ± 0.5	4 ± 0.6	4 ± 0.2	4 ± 0.3
Day 6	–	–	–	3 ± 0.2	4 ± 0.2
Day 7	2 ± 0.4	1 ± 0.5	2 ± 0.4	–	–
Day 12	–	–	–	4 ± 0.2	5 ± 0.4
Day 14	3 ± 0.7	4 ± 0.7	4 ± 0.9	–	–
Day 18	–	–	–	4 ± 0.3	4 ± 0.6
Day 21	3 ± 1.2	6 ± 1.4	–	3 ± 0.3	3 ± 0.2
Heinz bodies (%)					
Day 0	1 ± 0.3	0 ± 0.3	2 ± 0.4	1 ± 0.1	1 ± 0.2
Day 6	–	–	–	1 ± 0.0^{A}	2 ± 0.2^{B}
Day 7	1 ± 0.3	5 ± 2.1	3 ± 0.3	–	–
Day 12	–	–	–	1 ± 0.1	3 ± 0.6
Day 14	1 ± 0.2^{A}	3 ± 0.7^{A}	7 ± 2.2^{B}	–	–
Day 18	–	–	–	1 ± 0.1	3 ± 0.2
Day 21	1 ± 0.3	2 ± 0.8	–	1 ± 0.1^{A}	5 ± 0.6^{B}

^{AB}Means within study with different superscripts in same row differ significantly ($p < 0.0007$).
[1] Based on 4 time points, 0, 7, 14 and 21 days.
[2] Based on 3 time points, 0, 7 and 14 days.

2.5. Laboratory analysis

Smears for HB evaluation were prepared by mixing heparinized whole blood with new methylene blue (NMB) N stain (Ricca Chemical Co., Arlington, TX) in a 1:2 ratio, respectively, and incubating at room temperature for 20 min. After incubation, standard blood smears were prepared. Percentages of RBCs that contained HB were enumerated in all adequate samples by using 600× (high dry) magnification and counting 200–500 RBCs. Adequate samples contained a fine monolayer of RBCs stained such that the nuclei were dark blue (internal control). Aggregate reticulocytes and ring forms were enumerated using previously described methods (Johns et al., 2008). Relative reticulocyte counts were corrected for anemia with the PCV normalized to a mean PCV of 45% in cormorants (Reference Interval = 34–53%) using the equation reticulocyte count × (measured PCV/normal PCV).

Twenty µl of heparinized whole blood was placed in labeled 1.5 ml polypropylene microcentrifuge tubes containing excess 2.5–3.0% glutaraldehyde buffered to pH 7.2 (Electron Microscopy Services, Hatfield, PA), gently inverted, sealed, and stored in the dark at 4 °C. All whole blood samples from control and treated DCCOs from each experiment were processed and shipped to Michigan State University's Center for Advanced Microscopy (East Lansing, MI) in an identical manner at the same time. Aliquots were post-fixed in 1% osmium tetroxide, dehydrated in a graded acetone series, and infiltrated and embedded in Poly/Bed 812 resin (Polysciences, Warrington, PA). Thin sections (70 nm) cut with a PTXL ultramicrotome (RMC, Boeckeler Instruments, Tucson, AZ) were prepared on copper grids and stained with uranyl acetate and lead citrate. Sections were imaged using a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV. Representative images were taken without knowledge of treatment groups.

Hepatic tissue was excised rapidly following euthanasia and flash frozen in liquid nitrogen as subsamples until transferred to a −70 °C freezer for subsequent assessment of oxidative stress markers following procedures described in Pritsos et al. (2017, this issue).

2.6. Statistical methods

Hematologic values, including PCV, relative HB, and relative and corrected reticulocytes, collected across multiple time points were compared using linear mixed effects regression models with a repeated measures structure. Regression models included elapsed days, treatment, and treatment*days interaction as fixed effects and individual birds within treatment as random effects. Elapsed days and treatment (oral or external oil dose as ml/kg/day) were modeled as continuous variables. Within-day contrasts among treatment groups were assessed with the Kruskal-Wallis test. The criterion for statistical significance was $p < 0.05$. Calculations were performed using TIBCO Spotfire S-PLUS 8.2 for Windows. Reference intervals were established in accordance with ASVCP guidelines using Reference Value Advisor (Friedrichs et al., 2012; Geffré et al., 2009).

3. Results

Details on total food consumption, weight loss, other clinical signs, and mortality for both studies can be found in Cunningham et al. (2017), this issue.

3.1. Oral dosing experiment

Orally dosed birds had incidental dermal exposure due to defecation of oil into the tanks. Although not quantified, oil was present on feathers of all dosed birds, especially in the high dose group. Clinical signs included reduced cloacal temperatures, apparent hypothermia based on observations of birds seeking supplemental heat, weight loss, lack of appetite, lethargy, feather damage, moribundity, anemia, abnormal feces and hematochezia, and death. All dosed birds had at least some clinical signs including anemia at a total dose of approximately 80 ml/kg and all were dead prior to 200 ml/kg total dose. All birds with a measured PCV ≤ 24% died. Of the 26 adult, mixed-sex DCCO used in the oral dose study, 16 were euthanized on Day 21. A total of 10 treated DCCOs died or were euthanized within 17 days of the start of the study for humane reasons, including all 9 high dose animals. Control birds exhibited normal behavior, did not seek heat or lose weight and maintained normal cloacal temperatures throughout the study. No control birds died prior to necropsy on day 21.

Packed cell volume was significantly decreased by exposure to oil in a dose-related manner (Fig. 1a and Table 1, $p < 0.002$). Packed cell volume decreased over time in all three groups, but the decrease was more pronounced in oil-dosed birds and was dose dependent. The PCV regression line for control birds was within the reference interval (34–53%), while the regression lines for birds in the 5 and 10 ml oil/kg/day groups were below the reference interval by day 14 and day 7, respectively, indicating anemia. All birds in the high-dose group became moderately (< 31%) to severely (< 20%) anemic by day 14. All birds in the low-dose group became anemic by day 21 at the time of scheduled necropsy. At day 7 of oral oil administration, mean PCV was 40 ± 1.5%, 33 ± 1.4%, and 34 ± 1.5% (mean ± SE) for control, low-dose, and high-dose groups, respectively. At day 14, mean PCV of control, low-dose, and high-dose groups was 39 ± 1.6%, 32 ± 1.4%, and 24 ± 1.6%, respectively. No control animals exhibited anemia at any time during the study. Oil exposure also induced statistically significant changes in plasma clinical chemistries and gross findings at necropsy (Dean et al., 2017; Harr et al., 2017).

Aggregate reticulocyte quantification by light microscopy in DCCO blood indicated an increasing trend in a dose-related manner by day 14 (Table 1 and Figs. 2 and 3a). At day 7 of oral oil administration, mean reticulocyte percentage of all groups was similar at approximately 2%. At day 14, mean reticulocyte percentages were 3 ± 1.1%, 5.0 ± 1.2% and 7 ± 1.4% (mean ± SE) for control, low-dose, and high-dose groups, respectively. When the relative reticulocyte count was corrected for anemia, there was still a mild increase in the reticulocytes at day 14, however, these values did not extend past the reference interval (control, 3 ± 0.7%; low dose, 4 ± 0.7%; high dose, 4 ± 0.9% reticulocytes/PCV; mean ± SE; RI 0–6%, Fig. 4a) and the change was not statistically significant.

3.2. Dermal exposure experiment

Of the 25 adult, mixed sex DCCOs, one bird from the control group died immediately after blood sampling on day 0 and two birds from the treatment group were found dead in their cages on days 14 and 18. The control bird had a severe monocytosis (> 4000 monocytes/μl) and multiple, pulmonary granulomas including one involving the heart apex. Granulomatous pneumonia with intralesional bacteria was diagnosed upon histopathology. One exposed bird died with probable septicemia (underlying etiologic agent not identified). One exposed bird died with no significant lesions that could be assessed as a cause of death.

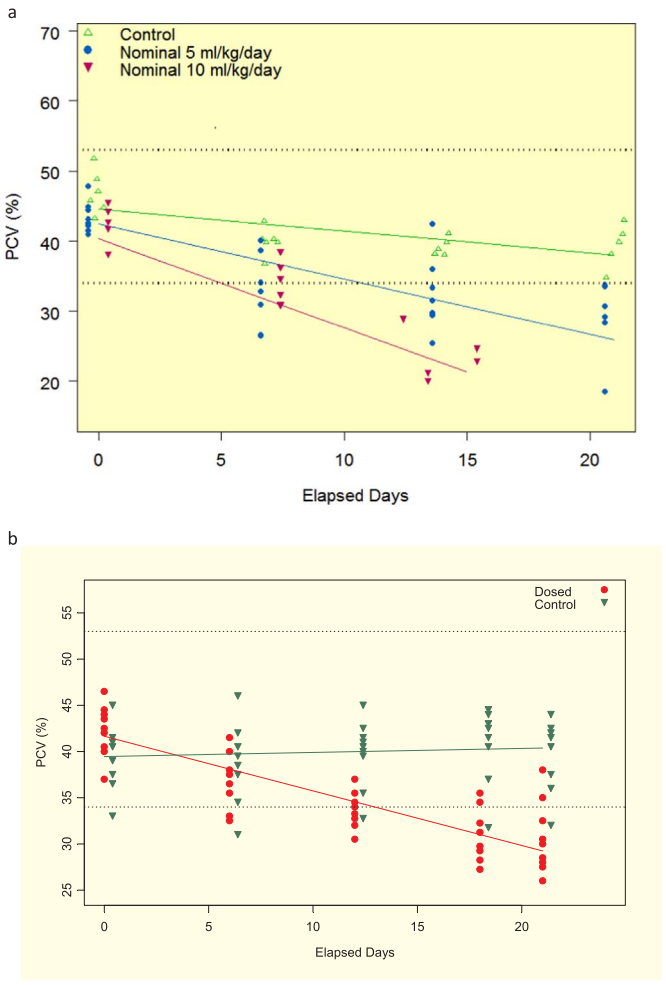

Fig. 1. a. Effect of daily oral dosing with artificially weathered MC252 oil on PCV in sub-adult, mixed-sex DCCO. Regression analysis showed a significant day effect ($p = 0.0017$) and a significant treatment*day interaction ($p < 0.001$). Dotted lines indicate reference intervals for apparently healthy cormorants from this population (34 − 53%). b. Regression analysis of the effect of dermal oiling and elapsed days of treatment on PCV in in sub-adult, mixed-sex DCCO. By day 12 and continuing through the end of the study, % PCV was significantly lower in oiled birds ($p < 0.001$). Dotted lines indicate reference intervals for apparently healthy cormorants from this population (34 − 53%).

Clinical signs reported in oil-exposed birds in this study included deterioration of feather integrity, abnormal feces, hematochezia, excessive preening and feather plucking, shivering, cardiac arrhythmia, dyspnea, and lethargy. Feathers of all oiled birds appeared matted and rough by day 3. Although oil was only applied to feathers, preening resulted in the skin on the breast and back of some oiled birds being noticeably discolored by day 6 and by day 9, oil covered much of the surface area of all birds with subjective compromised feather integrity. Abnormal feces (Fig. 5a, b) were observed in four oiled birds beginning on day 12 and by the end of the trial seven of 11 oiled birds that survived to necropsy had abnormal feces. Only one control bird had abnormal feces that consisted of green diarrhea with no evidence of gelatinous protein or blood as noted in the oiled birds. Plucking of down feathers was first apparent on day 14 in two birds and by day 16 all oiled birds were engaged in this activity. On the day of necropsy, only the treated birds were noted to be positioned by their heat lamps, occasionally shivering. Control birds were typically perched in their enclosures and did not exhibit abnormal clinical signs.

Packed cell volume of oiled DCCOs declined throughout the 21-day trial with the decrease being significantly ($p < 0.001$) different compared to controls by day 12 (Table 1, Fig. 1b). By day 21, all but two treated birds were anemic, based on a lower reference value of 34%. At

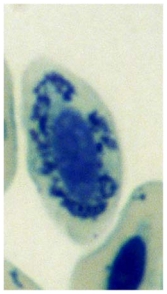

Fig. 2. Aggregate to ring form cormorant reticulocyte stained with supravital New Methylene Blue at 1000×.

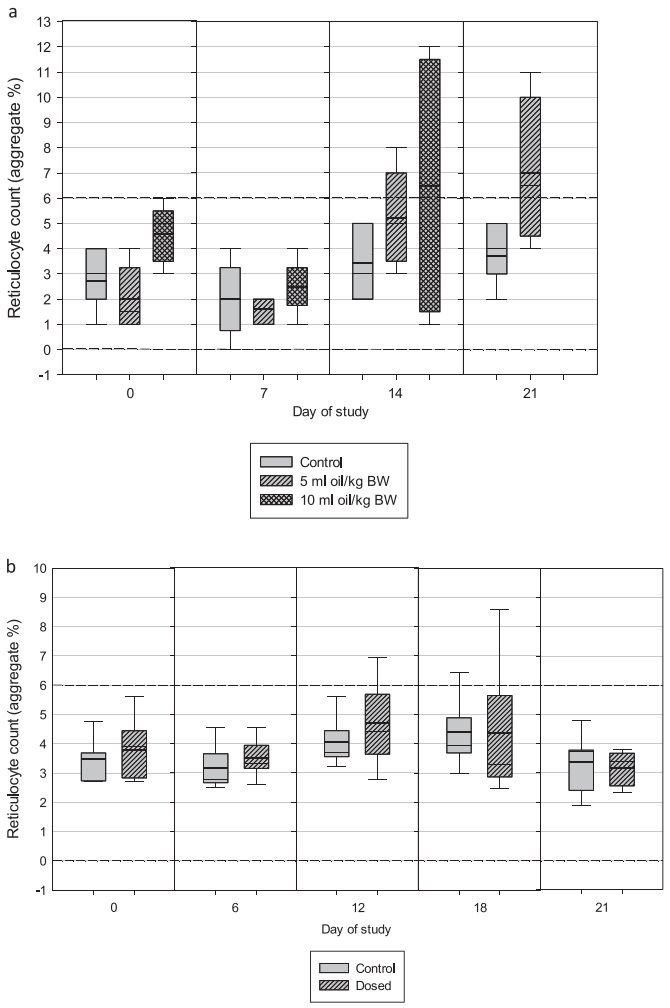

Fig. 3. a. Effect of daily oral dosing with artificially weathered MC252 oil on reticulocytes (aggregat%) in sub-adult, mixed-sex. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the boxes is the median and the heavy black line within the boxes is the mean value. The lower and upper whiskers indicate the 10th and 90th percentiles, respectively. Dotted lines indicate reference intervals for untreated cormorants from this population (0 – 6%). Regression analysis showed no significant main effect of treatment, day, or treatment *day interaction ($p > 0.05$). b. Effect of dermal oiling and elapsed days of treatment on percent reticulocytes in sub-adult, mixed-sex DCCO. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the boxes is the median and the heavy black line within the boxes is the mean value. The lower and upper whiskers indicate the 10th and 90th percentiles, respectively. Dotted lines indicate reference intervals for untreated cormorants from this population (0 – 6%). On day 12, reticulocyte percentage was significantly lower in control than in oiled birds ($p = 0.01$).

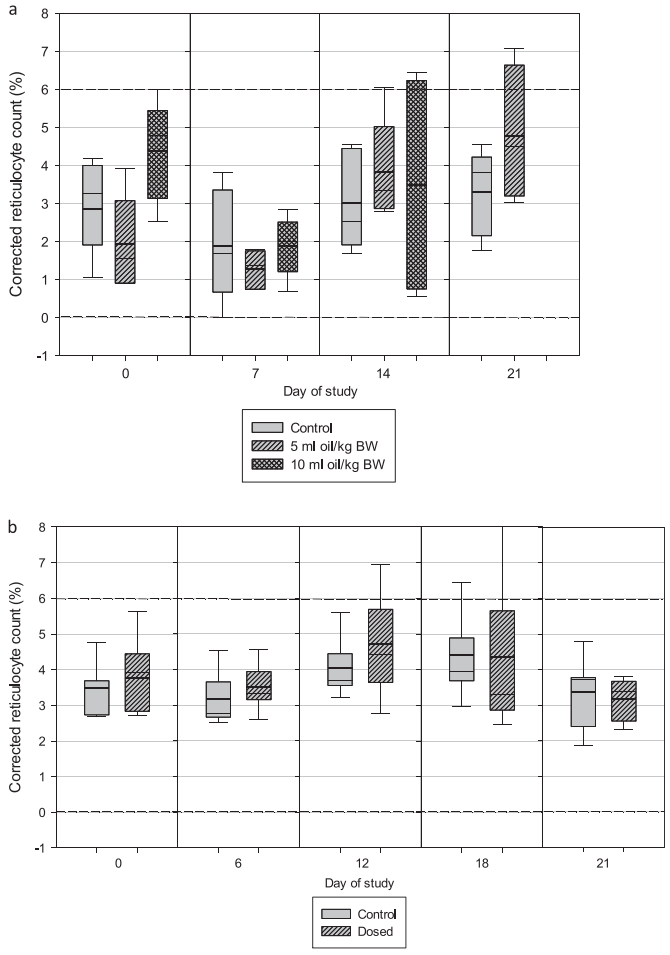

Fig. 4. a. Effect of daily oral dosing with artificially weathered MC252 oil on % reticulocytes normalized to PCV in sub-adult, mixed-sex. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the boxes is the median and the heavy black line within the boxes is the mean value. The lower and upper whiskers indicate the 10th and 90th percentiles, respectively. Dotted lines indicate reference intervals for untreated cormorants from this population (0 – 6%). There was no significant difference between doses for % normalized reticulocytes, however, there was a significant time effect ($p < 0.0004$) and a significant dose*time interaction ($p < 0.0409$). There is no data box for the d 21, 10 ml oil/kg BW treatment group because all cormorants in that group had either died or been euthanized by d 21 of the study. b. Effect of dermal exposure with artificially weathered MC252 oil on % reticulocytes normalized to PCV in sub-adult, mixed-sex. The lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, respectively. The black line within the boxes is the median and the heavy black line within the boxes is the mean value. The lower and upper whiskers indicate the 10th and 90th percentiles, respectively. Dotted lines indicate reference intervals for untreated cormorants from this population (0 – 6%). There was no significant dose effect and no significant dose *time interaction, however, there was a significant effect of time ($p < 0.01$). Percent normalized reticulocytes were significantly greater on days 12 and 18 than on days 0, 6 and 21.

day 6 of dermal oil exposure, mean PCV was 39 ± 0.4% and 37 ± 0.3 (mean ± SE) for control and oiled groups, respectively. At day 12, mean PCV was 40 ± 0.3% and 34 ± 0.2% (mean ± SE) and at day 18, mean PCV of control and oiled groups was 41 ± 4.45% and 31 ± 0.2%, respectively. A single control bird with severe monocytosis exhibited a mild anemia of 32% throughout the study. All other control birds maintained a normal erythron.

Relative reticulocytosis was apparent by day 12 in oiled birds ($p = 0.01$). (Figs. 2, 3b) Control birds did not exhibit moderate anemia (≤ 31%), reticulocytosis, or Heinz body formation. (Table 1, Fig. 1b). At day 12, mean reticulocyte percentages were 4 ± 0.2% and 5 ± 0.5% for control and treated groups, respectively. At necropsy on day 21, mean

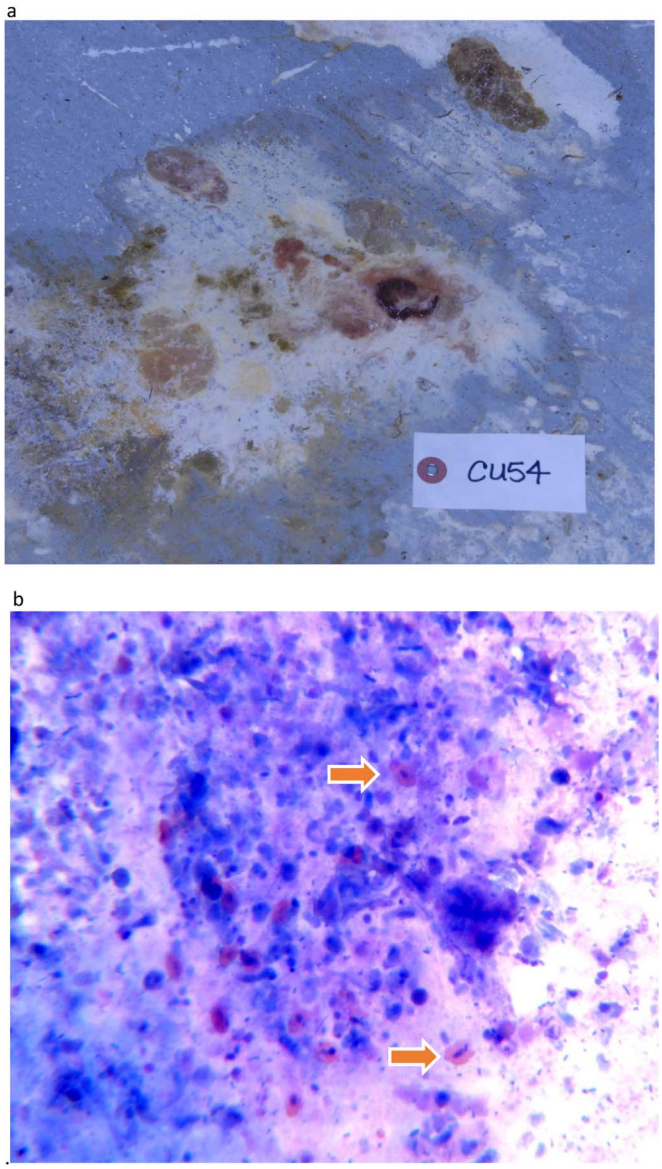

Fig. 5. a. Abnormal feces from a treated cormorant found on day 12 that shows watery diarrhea admixed with yellow urates and clear, tan and red gelatinous components. b. Abnormal feces imprint on slide at 200× magnification showing RBC admixed with degenerate epithelial cells, proteinaceous debris and a mixed population of bacteria, documenting hematochezia. (Platinum Quik-Dip™ Stain, Mercedes Medical, Inc., Sarasota, Florida, USA).

reticulocyte percentages were 4 ± 0.3% and 5 ± 0.3% for control and treated groups, respectively and were not statistically different. When the relative reticulocyte count was corrected for anemia, there was no statistical difference in control and treated birds at any time point (Table 1, Fig. 4b).

3.3. Hemoglobin precipitation and Heinz body formation

Hemoglobin damage was similarly documented in both oral and dermal exposure groups and is described together. Heinz body identification (Fig. 6) by light microscopy in blood from orally-dosed DCCOs revealed a significant treatment*day interaction ($p = 0.01$) as there was a dose-related increase over time (Fig. 7a, Table 1). At day 7 of oral oil administration, there were 1 ± 0.3%, 5 ± 2.1%, and 3 ± 0.3% (mean ± SE) HB in the control, low-dose, and high-dose groups, respectively. At day 14, 1 ± 0.2% RBCs contained HB in the control group, 3 ± 0.7% in the low-dose group, and 7 ± 2.2% in the high-dose

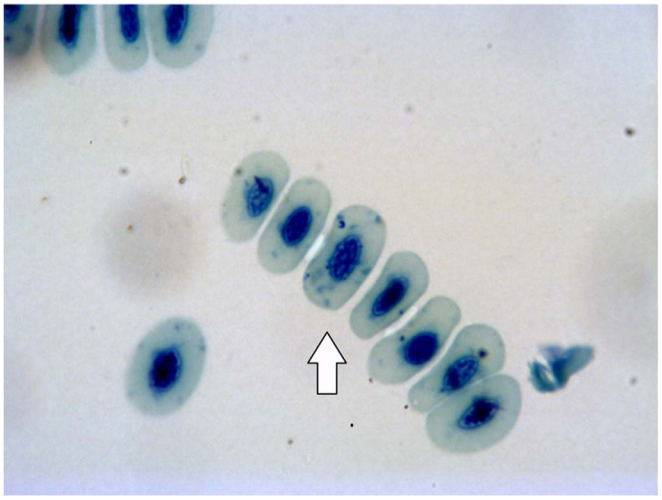

Fig. 6. NMB-stained preparation of cormorant blood at 1000x magnification using light microscopy. The white arrow indicates a cell with eight hemoglobin precipitates or HB that are homogeneous light blue cytoplasmic inclusions consistent in size and shape with those observed on transmission electron microscopy.

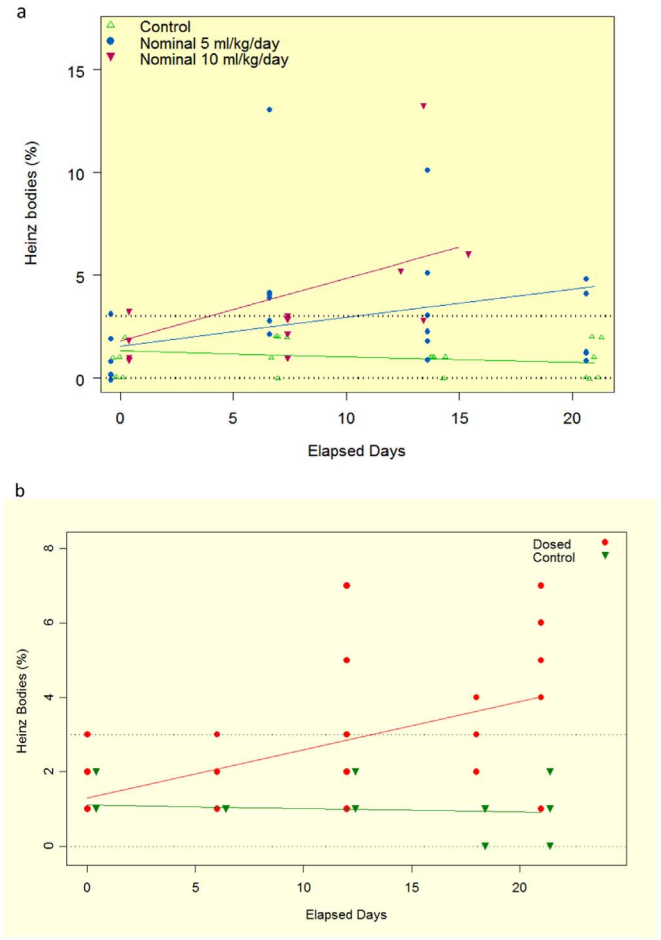

Fig. 7. a and b. Effect of daily oral (a) and dermal (b) dosing with artificially weathered MC252 oil on percent HB in sub-adult, mixed-sex DCCO. Results were similar for both routes of exposure. Regression analysis showed a significant treatment*day interaction ($p < 0.05$). Dotted lines indicate reference intervals for untreated cormorants from this population (0 − 3%).

group. Similarly, in the dermally-exposed birds, there was a mild, but significant ($p < 0.001$), increase in the percentage of HB in oil-treated birds beginning at day 6 that persisted throughout the study indicating oxidant damage to RBCs (Fig. 7b). At day 6 and at necropsy on day 21,

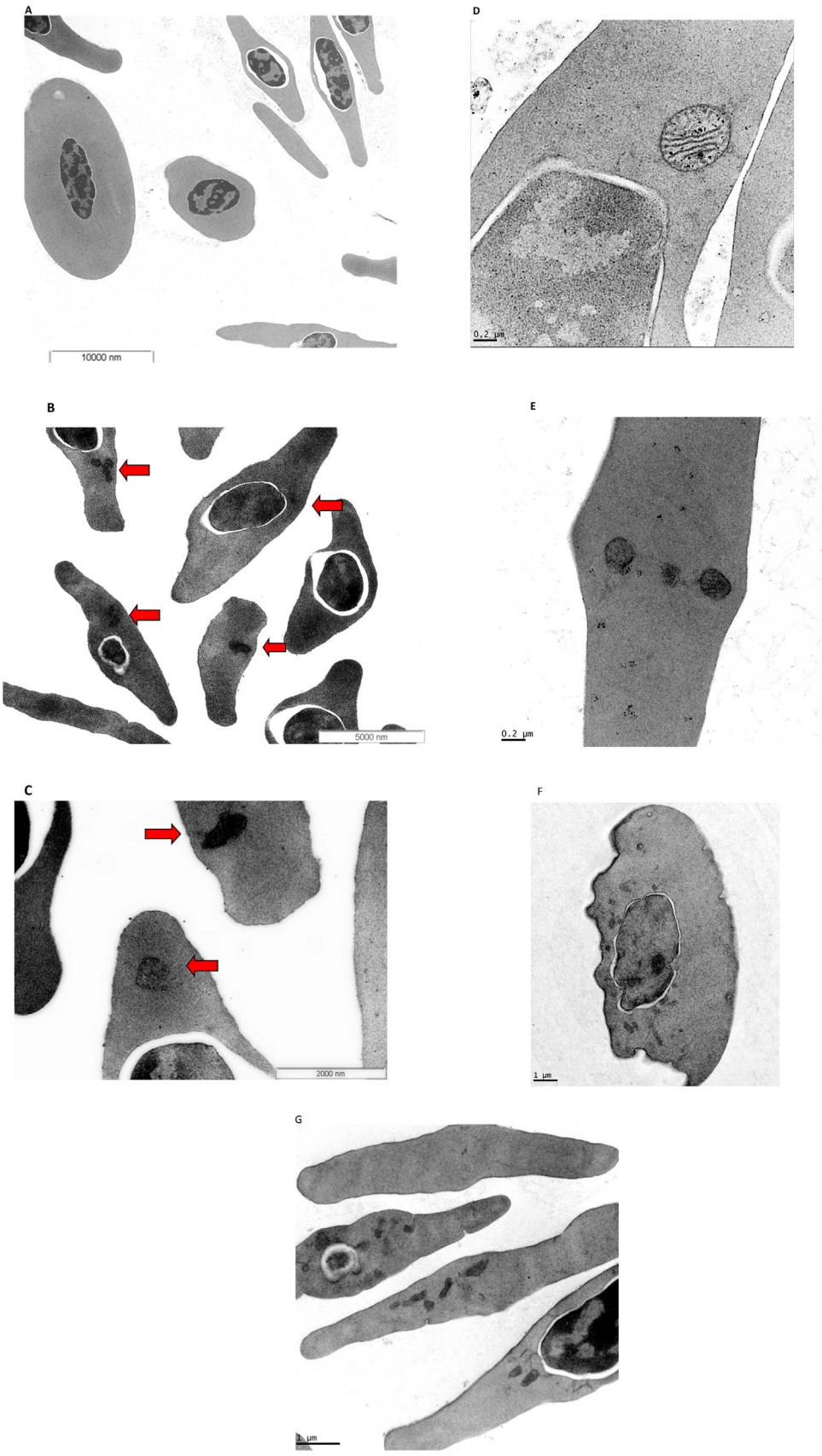

Fig. 8. (a) Representative RBC ultrastructural image from a control cormorant lacking HB. The cytoplasm is uniform gray in all cells indicating a uniform density and protein concentration with almost no evidence of organelles or HB. Depending upon the TEM orientation, cells may appear ovoid or ellipsoid. There is some nuclear membrane separation in many cells due to processing. The mild background stain precipitation does not change the homogeneous gray appearance of the cytoplasm in samples from the normal control birds. (b) Relatively low magnification images of RBCs from exposed DCCO showing the frequency of dark opacities with no typical organelle structure (RED arrows), consistent with HB. (c) Higher magnification image of a cytoplasmic inclusion with lack of organelle structure and density consistent with a Heinz body (top) and a slightly lighter cytoplasmic inclusion. (d) Normal appearing mitochondria with distinct cristae from an untreated cormorant. (e) Probable degenerate mitochondria with cristae still visible in an RBC from an oil treated cormorant. (f) RBC from treated cormorant showing both probable degenerate mitochondria and inclusions which lack any organelle structure consistent with denatured hemoglobin. (g) Low magnification ultrastructural image of RBCs from a treated cormorant of different orientation showing different size, shape, and frequency of degenerate organelles and hemoglobin.

Table 2
Effect of oral and dermal oil exposure on indicators of systemic oxidative damage in double-crested cormorants (*Phalacocorax auritis*) (Pritsos et al., 2017, this issue).

	Orally dosed birds			Dermally dosed birds	
	Control[a]	5 ml oil/kg BW[a]	10 ml oil/kg BW[b]	Control	20% surface area exposure
RBC SOD (U/g hemoglobin)					
Day 0	0.25 ± 0.02	0.30 ± 0.02	0.26 ± 0.02		
Day 6					
Day 7	0.28 ± 0.03	0.29 ± 0.02	0.27 ± 0.02		
Day 12					
Day 14	0.29 ± 0.01	0.31 ± 0.007	0.31 ± 0.02		
Day 18					
Day 21	0.29 ± 0.02	0.31 ± 0.02	–		
Liver					
SOD (U/mg protein)	1.74 ± 0.16^{A}	1.21 ± 0.106^{B}	1.29 ± 0.16^{AB}	0.76	0.64
Actual GSSG (nmol/mg)	0.72 ± 0.19^{B}	3.57 ± 0.67^{A}	1.96 ± 0.24^{AB}	1.27	1.29
Reduced GSH (nmol/mg)	24.90 ± 1.25^{B}	62.20 ± 7.42^{A}	85.38 ± 8.63^{A}	25.4^{A}	33.9^{B}
Total GSH (nmol/mg)	25.94 ± 1.19^{B}	76.75 ± 14.55^{A}	82.88 ± 9.73^{A}	131.00	36.5^{B}
MDA + HAE (nmol/mg)	0.79 ± 0.04	0.78 ± 0.12	0.90 ± 0.06	0.43^{A}	0.32^{B}
Trolox Eq. (μmol/mg)	0.12 ± 0.004^{B}	0.13 ± 0.009^{AB}	0.16 ± 0.008^{A}	0.11^{A}	0.12^{B}
Kidney					
SOD (U/mg protein)	1.13 ± 0.042	1.26 ± 0.12	–	0.53	0.536
Actual GSSG (nmol/mg)	0.06 ± 0.03	0.007 ± 0.007	–	0.22	0.17
Reduced GSH (nmol/mg)	0.62 ± 0.08^{B}	1.12 ± 0.12^{A}	–	0.56	0.95
Total GSH (nmol/mg)	0.69 ± 0.08^{B}	1.13 ± 0.11^{A}	–	0.89	1.29
MDA + HAE (nmol/mg)	1.01 ± 0.05^{A}	0.50 ± 0.04^{B}	–	0.50^{A}	0.34^{B}
Trolox Eq. (μmol/mg)	0.23 ± 0.05	0.17 ± 0.02	–	0.18	0.21

[a] Necropsied at 21 days.
[b] Necopsied by 14 days.

respectively, 1 ± 0.0% and 1 ± 0.1 RBCs contained HB in the control group upon light microscopic examination. Treated birds had 2 ± 0.2% and 3 ± 0.2% at day 6 and day 18, respectively.

Heinz bodies were rarely (< 1% RBCs estimated to contain HB) found in control birds and frequently (10–40% RBCs estimated to contain HB) found in both orally- and dermally-exposed birds on transmission electron microscopy (TEM) evaluation (Fig. 8a, b, c). Erythrocytes in all oil-exposed birds tended to have smudged nuclei lacking chromatin detail, and frequent RBCs contained many dark cytoplasmic inclusions with the same homogeneity and electron density as denatured hemoglobin precipitates and HB, not typically associated with the RBC membrane. Degenerate organelles including mitochondria, ribosomes, and endoplasmic reticulum were also found in the cytoplasm of RBCs from treated DCCOs (Fig. 8d, e). Similar organelles were rarely found in control birds but were more distinct with better ultrastructural detail, and therefore, likely not degenerate (Fig. 8f). Low magnification TEM figures were used to assess the frequency of HB (Fig. 8g). Membrane pitting (not pictured) was found in RBC from both control and treated birds.

All of the antioxidant endpoints measured in hepatic tissue at necropsy of orally-dosed DCCOs were significantly affected by exposure to oil ($p < 0.05$). Reduced and total glutathione concentrations were significantly increased and decreased respectively (Table 2) (Pritsos et al., 2017, in this issue).

4. Discussion

In this study, we demonstrated that both dermal application of and provision of food items containing MC-252 oil to double-crested cormorants resulted in anemia, degenerate organelles, Heinz body formation, extravascular hemorrhage, probable bone marrow damage, and mortality due to oxidative damage. Wildlife mortality has previously been used to assess impact to natural resources caused by oil spills (US Department of the Interior, 2011). Acute avian mortality has been well described for many large scale oil spills including the Exxon Valdez, Prestige, and Deepwater Horizon spills (Alonso-Alvarez et al., 2007; Barron, 2012; Finch et al., 2011; Piatt and Ford, 1996; Zuberogoitia et al., 2006). Although quantifying acute mortality as we saw in this study is clearly important, this is an underestimation of mortality and lack of recruitment induced by chronic, low-level exposure to oil that has ongoing effects impacting individuals, populations and ecosystems (Camphuysen et al., 2002; Iverson and Esler, 2010). This study documents that lasting effects to wildlife also occur through chronic, low-level exposure to oil. Sublethal injury induced by oil intoxication may induce intoxication, lethargy, and decrease feather integrity which impairs the ability to migrate to feeding and breeding grounds, resulting in lack of reproductive success and decreased recruitment of a species that results in decreased total animal numbers, similar to mortality (Iverson and Esler, 2010). It should be noted that especially in the oral dosing study, which resulted in higher mortality, discerning oil on feathers at a distance was difficult in these dark brown birds. Oiling only became noticeable when it was accompanied by moderate to severe disruption of feather integrity and feather plucking. Therefore, birds suffering from oil intoxication may go unnoticed by even the experienced field observer.

Exposure to oil can result in pathologic damage to multiple organ systems due to oxidative injury from oil components and metabolites produced by the cyp1a detoxification pathways. Here we focus on the effects on the erythron that was directly effected by oxidative damage and hemorrhage through the gastrointestinal tract (Pritsos et al., 2017; Harr et al., 2017). Anemia is a generalized term for decreased erythron mass or decreased PCV and may be caused by a myriad of etiologies. Anemia, while a result of other injury or disease, may in turn cause decreased oxygen perfusion to tissues resulting in anaerobic metabolism, altered cell membrane permeability, cell and tissue dysfunction and, if severe, organ failure. These changes result in clinical signs such as lethargy and dyspnea that would contribute to a lack of migratory and reproductive success. Therefore, anemia itself, as a pathologic state, may cause damage to the population.

Oxygen carried by hemoglobin is a strong oxidant because it can generate highly reactive derivatives such as the superoxide free radical and hydrogen peroxide, and because, by reacting with iron, it forms the reactive hydroxyl radical. These oxidants are constantly being produced, and RBCs have several mechanisms to prevent oxidation of hemoglobin through the use of reduced glutathione and enzymes such as superoxide dismutase, glutathione reductase and glutathione

peroxidase. This was measured and proven in the orally dosed population (Table 2). Erythrocytes are subject to oxidative injury when intracellular reducing pathways are insufficient to meet the oxidant challenge. When oxidant injury occurs, denatured hemoglobin forms hemichromes, which may bind to membranes or aggregate to form larger hemoglobin precipitates (Waugh and Low, 1985). Oxidative damage may result in hemolytic anemia due to direct cell membrane damage but may also shorten the life span of RBCs by the formation of HB and eccentrocytes (Desnoyers, 2010). Eccentrocytes are RBCs that have had their membranes partially fused by oxidative damage, resulting in their hemoglobin (and all cell contents) being shifted to one side of the cell. Eccentrocytes have been reported in many mammalian species but have never been documented in birds. In this study, some RBCs with HB appeared to have irregular borders with pits in the cellular membrane, but nothing similar to a classic eccentrocyte was identified upon light or electron microscopy (Caldin et al., 2005).

Heinz bodies are named for Robert Heinz, who first described aggregations of protoplasm in cells exposed to oxidizing agents in 1890 (Heinz, 1890). Denatured hemoglobin, documented by the gold standard of electron microscopy in the present study, is indicative of oxidative damage to hemoglobin in RBCs and consistent with oil-induced hemolytic anemia. The changes in hepatic oxidative stress endpoints provide further evidence of systemic oxide radical damage in the body of DCCOs (Table 2). Oxidants are used by the body in immune response to combat pathogens and may also be end products of ongoing physiologic processes (Waugh and Low, 1985). Therefore, occasional hemoglobin precipitates and HB would be expected in RBCs from control animals as documented in this study. There were few HB (< 1% RBCs contained HB) identified in the control population as confirmed using electron microscopy. Control birds also had significantly lower oxidative stress endpoints, indicating that these statistically significant variables were independent of disease that was found in both the control and oil-exposed groups of wild caught DCCOs. Reviewers of the ultrastructural samples could easily distinguish control from treated samples based on the numbers of inclusions (HB and denatured organelles) in RBCs. The HB relative count produced using light microscopsy was significantly lower than those counted in the same samples using electron microscopy. This is likely due to conservative counting of samples where the small blue inclusions could not be discerned from methylene blue stain precipitate. In comparison, stain precipitate versus damaged organelles or HB could be easily differentiated in electron micrographs (Fig. 8).

Heinz bodies found in avian RBCs exhibit some morphologic differences compared to those found in mammals including decreased total numbers, decreased size and a lack of membrane association. The lower numbers and smaller size of HB in cormorant erythrocytes upon examination by electron microscopy in the present study is consistent with previous findings in turkeys (Simpson, 1971). The classic cytoplasmic button typical of the mammalian Heinz body was rarely found in NMB preparations from cormorants, but rather small, oval to irregularly shaped, light blue inclusions were found throughout the cytoplasm (Fig. 6). Although the dark cytoplasmic inclusions found on TEM were not typically membrane-associated as HB classically are in mammals, they are consistent in structure and electron density with HB. Binding of hemichromes and hemoglobin precipitates has been found to be biphasic and exhibit low and high affinity sites of binding in mammals. High affinity sites of hemoglobin precipitate binding have been shown to be on the cytoplasmic domain of band 3 transmembrane protein in rodents (Waugh and Low, 1985; Zhang et al., 2003). It is unknown if band 3 exists in bird membranes and a lack of band 3 would result in decreased Heinz body binding to the membrane. Further cytochemical investigation of the avian erythrocyte is warranted to better understand why avian HB appear to be morphologically different than mammalian HB.

Heinz bodies are removed from RBCs by the reticuloendothelial system, especially in the spleen by histiocytic cells in mammals and birds (Olah et al., 2014; Simpson, 1971; Sugawara et al., 2010). When HB are removed by histiocytic cells the entire RBC may be removed from circulation, resulting in an extravascular hemolytic anemia. Additionally, in this study we documented hematochezia in oil-treated birds only. The amount of blood noted in feces likely also contributed to the documented anemia in oil-treated birds. The body's response to anemia is production of RBCs in hematopoietic tissue. This will result in both mature and immature RBCs (reticulocytes and rubricytes) release into the blood. Hence, increased numbers of reticulocytes (reticulocytosis), as found in the oil-dosed birds in this study, are used to document RBC regeneration. When reticulocyte counts from both dermally- and orally-exposed cormorants were corrected for the severity of the anemia, they were not above the reference interval, indicating a lack of compensatory regeneration. Additionally, there was a reduction in reticulocytosis over time that suggests the absence of production of the erythroid line by the bone marrow repeatedly exposed to crude oil. This is consistent with oil-induced bone marrow damage previously documented in mammals (Meyne and Deaven, 1982).

Hemolytic anemia has been demonstrated in several species of birds exposed to crude oil (Fry and Lowenstine, 1985; Leighton, 1985; Troisi et al., 2006, 2007). It is believed that oxidative damage is mediated by metabolites of polycyclic aromatic hydrocarbons (PAH) generated from metabolic actions of cytochrome P450 enzymes in birds as well as mammals (Troisi et al., 2006, 2007). Controlled dosing studies of Atlantic puffins (*Fratercula arctica*) and herring gull chicks (*Larus argentatus*) demonstrated a dramatic reduction in the number of circulating RBCs in birds orally dosed with large volumes of crude oil (Leighton, 1985). Further, the presence of damaged hemoglobin in the erythrocytes from these birds, as evidenced by Heinz body inclusions, points to oxidative damage as a mechanism of anemia. In heavily oiled birds admitted to rehabilitation facilities, Troisi et al. (2007) further demonstrated a correlation between the percentage of HB and circulating PAH concentrations in plasma from heavily-oiled Common Guillemots (*Uria aalge*), suggesting a dose-response relationship. In the current study, PAH concentrations in cormorant tissues were not measured and so comparisons are not possible. Newman et al. (2000) found a mild to moderate, regenerative hemolytic anemia induced by administration of 2.5 and 10 ml/kg Prudhoe Bay crude oil. This study documented reticulocytosis but not HB detected by light microscopy of RBCs stained supravitally with new methylene blue (Newman et al., 2000). However, electron microscopy was never performed on those samples, so the absence of electron dense inclusions consistent with HB and damaged mitochondria, i.e. evidence of oxidant damage, was never confirmed. In a field study conducted as part of the Deepwater Horizon NRDA, Fallon et al. (2014) reported that American oystercatchers, black skimmers, brown pelicans and great egrets with small amounts of visible oil present on their feathers suffered from oxidative injury to RBCs as indicated by the presence of HBs, had PCVs that were 4–19% less compared to birds from reference sites and had 27–40% more reticulocytes compared to birds from reference sites. Additionally, birds with no visible oiling sampled in areas potentially affected by the oil spill had evidence of HBs, a decrease in PCV and an increase in reticulocytes compared to birds from reference sites. The findings in this study support previous literature and further determine that significant hemolytic anemia may be induced in birds receiving as little as 5 ml/kg body weight dermally applied chronically (to total 65 ml dermal dose and estimated 22 ml ingested).

While HB may be visualized using numerous supravital staining techniques including new methylene blue (NMB), methyl violet, Nile blue sulfate, brilliant cresyl blue, Janus green, neutral red, Victoria blue, Bismark brown, or gentian violet, they are most commonly identified using new methylene blue. The supravital technique must be used, as opposed to NMB staining of air-dried blood smears because blood smears dipped in new methylene blue create a substandard preparation and HB are frequently not visualized as they appear as unstained refractile bodies (Jain, 1973). Due to the lack of distinct button

morphology found in oxidant damaged mammalian erythrocytes, identification of HB by light microscopy is difficult and not definitive. Based on findings in this study, it also underestimates the number of damaged RBC in the sample. Transmission electron microscopy can be very helpful in characterizing or identifying RBC cytoplasmic inclusions, and is recommended by these authors as a confirmatory test for the presence of HB in avian RBCs (Desnoyers, 2010).

5. Summary

Oral and dermal exposure of double-crested cormorants to weathered DWH MC 252 crude oil induced hemolytic anemia as indicated by decreased PCV, relative reticulocytosis with an inadequate regenerative response, and presence of HB and degenerate organelles, which is consistent with other reports of oil-exposed birds. Additionally, this study documents extravascular blood loss through hematochezia contributing to the severity of anemia, potentially due to coagulopathy. Avian HB differ from those found in mammalian RBC in that they are a relatively consistent small size and are located within the cytoplasm, possibly due to the nucleated cell and different cell cytoskeleton. Hematologists should be aware of these differences in Heinz body ultrastructure when attempting to identify them by light or electron microscopy. Ultrastructural assessment of suspected HB in birds is recommended to confirm identification by light microscopy which is challenging and underestimates the damage of the erythron. The best field technique based on the data in this study is assessment of PCV with storage of blood in glutaraldehyde for possible TEM analysis.

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