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Changes in white cell estimates and plasma chemistry measurements following oral or external dosing of double-crested cormorants, *Phalacocorax auritus*, with artificially weathered MC252 oil

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ABSTRACT

Scoping studies were designed whereby double-crested cormorants (*Phalacocorax auritus*) were dosed with artificially weathered *Deepwater Horizon* (DWH) oil either daily through oil injected feeder fish, or by application of oil directly to feathers every three days. Preening results in oil ingestion, and may be an effective means of orally dosing birds with toxicant to improve our understanding of the full range of physiological effects of oral oil ingestion on birds. Blood samples collected every 5–6 days were analyzed for a number of clinical endpoints including white blood cell (WBC) estimates and differential cell counts. Plasma biochemical evaluations were performed for changes associated with oil toxicity. Oral dosing and application of oil to feathers resulted in clinical signs and statistically significant changes in a number of biochemical endpoints consistent with petroleum exposure. In orally dosed birds there were statistically significant decreases in aspartate amino transferase (AST) and gamma glutamyl transferase (GGT) activities, calcium, chloride, cholesterol, glucose, and total protein concentrations, and increases in plasma urea, uric acid, and phosphorus concentrations. Plasma electrophoresis endpoints (pre-albumin, albumin, alpha-2 globulin, beta globulin, and gamma globulin concentrations and albumin: globulin ratios) were decreased in orally dosed birds. Birds with external oil had increases in urea, creatinine, uric acid, creatine kinase (CK), glutamate dehydrogenase (GLDH), phosphorus, calcium, chloride, potassium, albumin, alpha-1 globulin and alpha-2 globulin. Decreases were observed in AST, beta globulin and glucose. WBC also differed between treatments; however, this was in part driven by monocytosis present in the externally oiled birds prior to oil treatment.

1. Introduction

On April 10, 2010 when the *Deepwater Horizon* (DWH) well MC252 exploded, the temperate waters of the Gulf of Mexico gradually became contaminated with approximately 3.19 million barrels (507 million liters) of South Louisiana sweet crude oil (PDARP, 2016). Some of this oil made its way to beaches, marshes and shallower fishing grounds used by birds. As a result, thousands of the 150 species of birds that occur in the waters and wetlands of the Gulf of Mexico died, and thousands more became oiled (PDARP, 2016). Still, these numbers are unlikely to reflect the true cost of the DWH spill on bird life as they do not account for the sub-lethal health related effects of oil.

At sub-lethal oral doses the physiological effects of oil exposure include anemia, organ dysfunction, decreased nutrient absorption, altered stress response, and decreased immune function (Szaro et al., 1978; Leighton et al., 1985; Leighton, 1985, 1986, 1993; Peakall et al., 1989). Hemolytic anemia is one of the most commonly reported effects of oil ingestion in birds (Hartung and Hunt, 1966; Eastin and Rattner,

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1982; Pattee and Franson, 1982; Lee et al., 1986; Leighton et al., 1985; Leighton, 1986; Hughes et al., 1990; Yamato et al., 1996; Walton et al., 1997; Newman et al., 2000; Seiser et al., 2000; Troisi et al., 2007), but itself a marker of oxidative damage. Detoxification and elimination of polycyclic aromatic hydrocarbons (PAHs) from the body occurs through activation of cytochrome P450 (CYP450) mono-oxygenases in the liver to metabolize lipophilic PAHs into more hydrophilic and reactive oxides and epoxides (Peakall, 1989; Troisi, 2006 }. These reactive oxygen species can cause oxidative damage to red blood cells and organ systems including damage to liver, kidney, gastro-intestine, adrenal glands, muscle tissue and salt glands in birds (Leighton, 1986). Measurement of plasma biochemistry markers of organ damage such as enzyme activities, blood urea nitrogen, urea, uric acid, creatinine, chloride, cholesterol have been reported in a variety of bird species following oil spills (Fleming et al., 1982; Pattee and Franson, 1982). Exposure to oil can also cause an upregulation of immune and inflammatory responses, and cause endocrine disruption (Briggs et al., 1996; Perez et al., 2010). Oiled birds show increases in inflammatory responses, depressions in lymphocyte concentrations and immunosuppression that results in increased susceptibility to secondary infections (Fry and Lowenstine, 1985; Briggs et al., 1997; McOrist and Lenghuas, 1992; Newman et al., 2000). The irritant effects of oil on the gastrointestinal tract, combined with observed decreases in adrenal function (Rattner et al., 1981; Gorsline et al., 1981; Gorsline et al., 1982; Fry and Lowenstine, 1985; Seiser et al., 2000; Newman et al., 2000), make it unsurprising that inflammatory responses are upregulated. Decreases or increases in lymphocyte and eosinophil concentrations could be indicative of direct effects of oil or reactive oxygen species on white blood cell production; however, inflammatory processes are not fully understood in avian responses to oil (Seiser et al., 2000; Newman et al., 2000; Garcia et al., 2010).

Generally, changes in plasma clinical chemistries differ amongst species studied with exposure route, duration, oil type and species sensitivity; however, few studies have been able to combine clinical measurements with mechanistic toxicological approaches to define a suite of endpoints that fully described the adverse effects of crude oil on birds. Measurements of clinically-relevant plasma endpoints are often collected opportunistically following an oil spill, making them reliant on availability of individuals for which no clinical history is available, including dose. Further, elucidation of the toxicological mechanisms responsible for the clinical health effects observed in wild birds is difficult because experimental dose manipulations have been hampered by a paucity of effective dosing techniques for birds. One of the effects of oil is as a gastro-intestinal irritant, and as such birds given a bolus oral dose will either regurgitate oil or the oil will have a rapid transit time through the gastro-intestinal tract resulting in little PAH absorption. External application of oil, while effective for oral dosing due to the natural tendencies of birds to maintain feather integrity by preening, is still not optimal because dose can only be approximated. Hartung (1963) estimated that 25% of a 20 ml moderate density oil applied to the feathers of a black duck would be preened by day 3% and 50% by day 8. This varied slightly depending on the volume applied, but the utility of this study is limited because only applied once, so estimates for studies in which oil is applied multiple times will only roughly approximate ingestion.

Despite issues with dose determination, external oil application has the potential to be an effective means of understanding the adverse effects of oil on avian health and physiology, but the link needs to be made between definitive oral dosing and external oil application to illustrate the effectiveness of the latter technique. As such, oral and external dosing methods were developed for the double-crested cormorant (*Phalacocorax auritus*,) (Cunningham et al., 2017). The potential differences between the two dosing methods are likely to related to the absolute dose rates, as oral delivery in feed can potentially result in a higher dose that what could be consumed from feather preening, and actual absorption of that dose from the gastrointestinal tract. Here we compare and contrast the changes in plasma chemistry measurements often used for diagnostic purposes to determine if there are changes that indicate oil toxicity, and if those changes are of similar scope and magnitude between the two dosing systems.

2. Methods

Full method details for all aspects of this study are available in Cunningham et al. (2017). General methods are summarized below.

2.1. Animal collection and husbandry

Twenty-six double-crested cormorants were collected for the oral dosing study and 31 were collected for external oil application. All birds were collected in Mississippi and Alabama, then transported to the National Wildlife Research Center Mississippi Field Station. Upon arrival birds were given a unique identifier quarantined in individual pens for 2–3 weeks. Quarantine and experimental procedures conformed and were approved by the NWRC Institutional Animal Care and Use Committee (IACUC; protocols QA2326 and QA2107).

Each pen contained a 190 L water tank used for daily feeding with live fingerling channel catfish (*Ictalurus punctatus*) that were maintained under standard feeding, temperature and aeration settings. At least 600 g of fish were provided to each bird daily. Actual food consumption was calculated per bird based on weight of fish remaining the next day. Water was changed manually every other day and dead fish were removed as soon as possible. Birds were monitored daily.

2.2. Toxicant

Artificially weathered MC252 oil (DWH7937, batch# B030112) was prepared from crude oil collected during the DWH oil spill (Forth et al., 2017). When not in use, the oil was stored in a leak-proof container in a flammable storage cabinet.

2.3. Oral dosing

Double-crested cormorants were randomly assigned to one of three treatment groups: control that was administered fish that had been lightly anesthetized and allowed to revive (n = 8); a group dosed daily with the target 5 ml oil/kg body weight (BW) (n = 9); and a group dosed daily with the target 10 ml oil/kg BW (n = 9). Actual doses as calculated by Cunningham et al. (2017) were 5.2 ± 0.3 ml oil/kg BW and 8.4 + 0.9 ml oil/kg BW respectively.

Fingerling channel catfish were lightly anesthetized using tricaine methanesulfonate (MS222) and given an intraperitoneal injection of 2.0 ml of oil. Each fish was injected with the same volume to ensure that per bird oil consumption could be calculated based on number of fish consumed. Injected fish were placed into a holding tank to monitor recovery from anesthesia and ensure oil retention. Oil-injected fish survived for more than 24 h if not killed by foraging birds. Once birds had consumed all oil-injected fish, additional uninjected fish were offered (up to 600 g per bird). Consumption of both dosed and clean fish were monitored daily for each bird.

2.4. External oil application

The external oiling study took place after the oral dosing study. Baseline differences in plasma markers were measured in pre-study testing for the external oiling study indicated the presence of pre-existing disease in some individuals. Diseased animals were not excluded so that the population would accurately represent wild bird populations. Birds were assigned to either control or moderate (16–40%) oiling groups based on blood samples collected at the beginning of quarantine. High monocyte counts, greater than 2.0×10^9 cell/L, were considered abnormal so these birds were divided evenly between

control and treatment groups. Additionally, a small oil spill took place in November 8, 2013 near where some birds were collected. As such, these birds were also evenly distributed between groups. Initial sample sizes for control group and moderate externally exposed group were 12 and 13 respectively. One control bird and two treated birds died during the study leaving a sample size of 11 for each group.

Oil exposed birds had a total of approximately 13 g applied to breast (6.5 g) and back (6.5 g) feathers by brush. Templates were used to ensure consistent area of application and provide a total surface area coverage equivalent to "moderate" oil coverage. Sham treatment of the control birds included the same handling procedures and equivalent amount of water applied to the breast and back. Sham and oil applications took place every three days through day 15 of the trial (days 0, 3, 6, 8, 12, and 15). By day 15 the dosed birds had become heavily oiled, were showing clinical signs of oil toxicity such as postural changes and abnormal feces, and had engaged in significant feather plucking. No further oil application occurred at this time as we judged that preening would continue to result in similar rates oil ingestion in the dose birds. Necropsy and sampling occurred on day 21 and 22.

2.5. Blood sampling

For the external oiling study, a single blood sample was collected at the beginning of quarantine. This collection did not take place for the orally dosed birds as there were initial concerns that capture and handling stress may be too great to warrant an additional stressor.

Blood samples began with a sample on Day 0 (prior to dosing) and then every 7 days (oral dosing) or 6 days (external dosing). The slight difference between the two sampling protocols was necessary to ensure that blood sampling was timed to occur on a day when oil application was also being undertaken. Oil was applied every three days (Day 0, 3, 6, 9, 12 and 15) with blood being collected on days 0, 6, 12, 18 and 21.

Approximately 3–4 ml of blood was collected from either the brachial or jugular veins using a 25 G butterfly needle, then transferred to labeled lithium heparin Vacutainer™ tubes and kept on ice for subsequent processing. Plasma was separated by centrifugation of whole blood at 2000 g for 5 min and stored at −80 °C until shipping, which generally occurred within 24–48 h of collection. Samples were scored for hemolysis using a 3-point scale, Samples receiving a score of "3" indicating severe hemolysis were excluded from analysis.

2.6. Sample analysis

All laboratory personnel were blinded to sample origin. Blood smears for WBC estimates were prepared using a standard push technique". The slide was allowed to air dry, fixed in methanol, and sent to a clinical pathology laboratory. Blood smears were stained using Platinum Quik-Dip™ Stain (Mercedes Medical, Inc. 7590 Commerce Court - Sarasota, FL 34243) in an automated slide stainer (Midas III slide stainer, EMD Millipore, 290 Concord Road, Billerica, MA 01821). Samples with $\geq$25% WBC lysis, as assessed on blood smear, were not analyzed further. WBC count was estimated from the blood smear by averaging number of WBC in 10 fields and multiplying by the square of the objective. Manual differentials quantified cell types based on a 200 total cell count. White blood cell morphology including presence of thrombocyte clumping and adequacy were determined. Clumped thrombocytes were not enumerated but clumping subjectively indicated adequate numbers.

Plasma samples were analyzed by a clinical pathology reference laboratory (University of Miami, Miller School of Medicine, Avian and Wildlife Laboratory). Analyses included plasma protein electrophoresis, bile acids, sodium, potassium, chloride, phosphorus, calcium, alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), creatine kinase (CK), glucose, cholesterol, urea, uric acid, total protein, creatinine. Uric acid concentrations were not measured on Day 0 for the orally treated birds due a sampling error. Plasma biochemical analysis was performed using the Roche Cobas Mira Plus chemistry analyzer (Roche Diagnostics, Indianapolis, Indiana 46250, USA). Protein electrophoresis was conducted as per Delk et al. (2014). Protein fractions were determined via the Helena SPIFE 3000 system using Split Beta gels (Helena Laboratories, Inc., Beaumont, Texas 77707, USA). Percentages and absolute values (g/dl) for each fraction were obtained by multiplying the percentage by total protein concentration. The albumin:globulin (A:G) ratio was calculated by dividing albumin by the sum of the globulin fractions.

Bile acid concentrations were determined following validation according to Rayhel et al. (2015), by radioimmunoassay using the Conjugated Bile Acids Component System (MP Biomedicals, Santa Ana, California 92707, USA) according to manufacturer directions. Samples and standardized controls were added to tubes coated in rabbit bile acid antiserum and incubated for 1 h with ^{125}I-labeled bile acid. Afterward, tubes were rinsed and read on a gamma counter (Laboratory Technologies Inc., Maple Park, Illinois 60151, USA). A standard curve was formulated from the controls and compared to sample results for quantification

2.7. Statistical methods

2.7.1. Screening for abnormal subjects

Reference intervals for clinical hematology and chemistry endpoints (Harr et al., 2017b) were verified in accordance with the American Society for Veterinary Clinical Pathology (ASVCP) guidelines using MedCalc (Version 14.12.0 64 bit; MedCalc Software, Ostend, Belgium) and a more stringent setting of the Dixon Test using confidence levels of 0.1 or Tukey's Outlier Test (Geffre et al., 2011; Friedrichs et al., 2012). If outlying values were measured that indicated an abnormality, then data collected from these individuals was deleted from the dataset. As described in Cunningham et al. (2017) only birds assessed as healthy based on physical examination, body weight and appetite were included for the oral study. This screen for abnormal subjects at the beginning of the study removed outliers from analyses so only individuals representative of a healthy population were included in statistical analyses. In the external study, all birds were used regardless of severe monocytosis to maintain animal number and better represent the field setting, though animals with severe disease were separated between control and treated groups.

2.7.2. Mortality and sample sizes

Samples sizes varied amongst the endpoints tested following both testing for abnormal subjects (above) and mortality. As described by Cunningham et al. (2017), all group B dosed birds (10 ml/kg BW/day) in the oral dosing study died or were euthanized by Day 14. Mortality related to the 5 ml/kg BW/day dosing schedule was limited to a single individual, with no mortality of control birds. Mortality during the external dosing study was limited to a single control and two dosed birds. Each of the Figs. 1–5 reported here show individual points for all of the birds from which samples were collected on that day.

2.7.3. Statistical analyses

Hematologic and plasma clinical chemistry values collected across multiple time points (during the experimental period) were compared using linear mixed effects regression models with a repeated measures structure. Regression models included effects for elapsed days, treatment and a treatment*days interaction term. Elapsed days and treatment were modeled as continuous variables, where treatment was defined as the average daily consumption determined from daily observations of actual oil consumption by each individual bird or as the precise amount of externally applied oil. Statistically significant differences (see Supplementary materials) between control and treatment groups were identified by significant main effects of treatment ($p < 0.05$) or by a significant interaction between treatment and elapsed

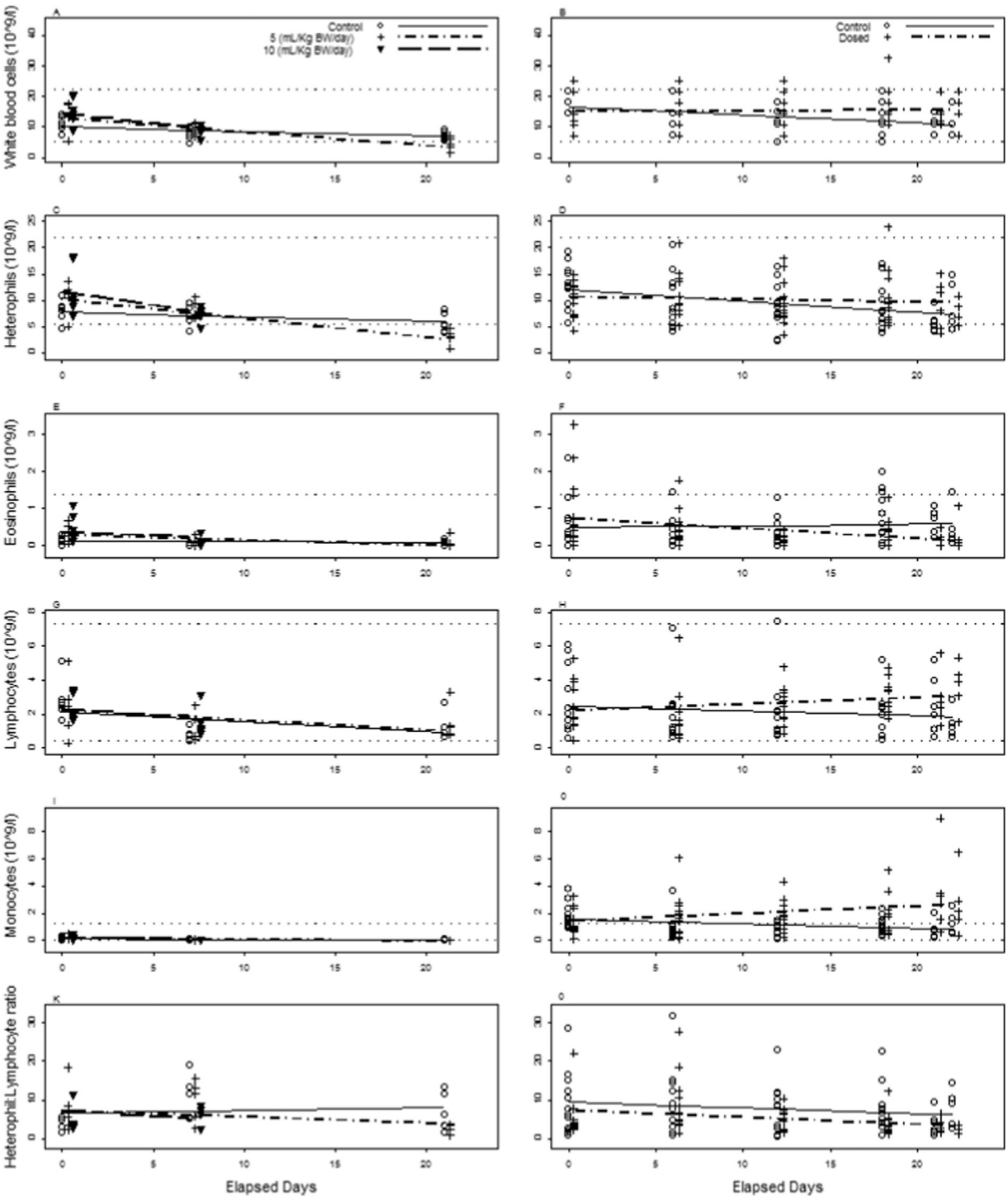

Fig. 1. Regression analyses showing changes in leucocyte counts over time in double-crested cormorants exposed to oil via ingestion of oil-injected fish (LEFT panel) and via application of oil to feathers (RIGHT panel). Solid lines are controls, dashed lines are oil treated, dotted lines are boundaries of reference intervals. White blood cell counts (A & B); heterophils (C & D), eosinophils (E & F), lymphocytes (G & H) and monocytes (I & J) are all shown as cell $\times 10^9$/L. Heterophil:lymphocyte ratio is shown in panels K & L.

days ($p < 0.05$). Data distributions for all endpoints were generally symmetric about their means and did not span more than an order of magnitude, thus data transformations to meet normality assumptions were deemed to be unnecessary. Differences among treatment groups on Day 0 were evaluated using the Kruskal-Wallis test (see Supplementary materials).

3. Results

Raw data for avian toxicity studies conducted as part of the Deepwater Horizon Damage Assessment are publicly available at https://www.diver.orr.noaa.gov/deepwater-horizon-nrda-data, while work plans and reports can be accessed through https://www.doi.gov/deepwaterhorizon/adminrecord.

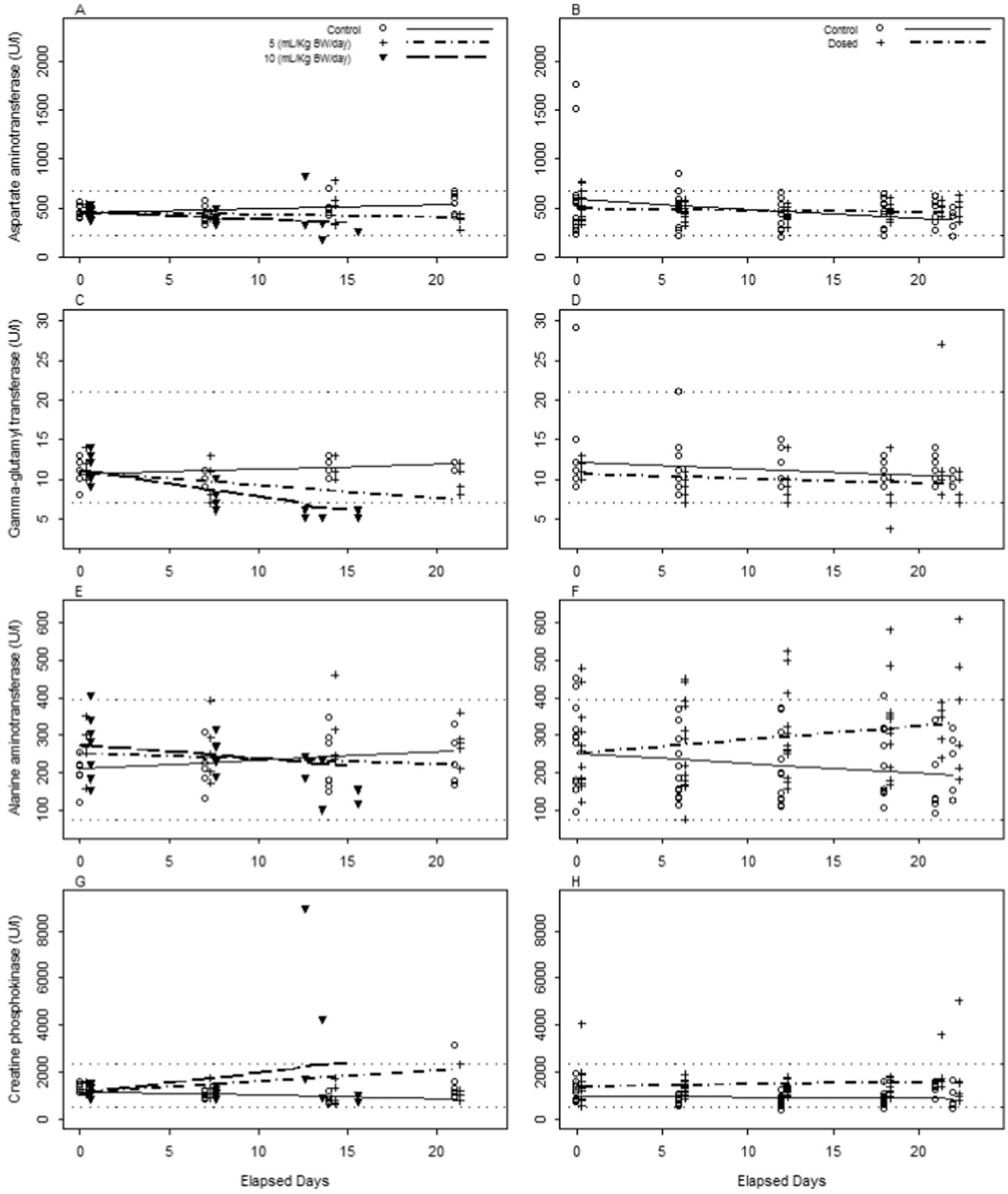

Fig. 2. Regression analyses showing changes in enzyme activities over time in double-crested cormorants exposed to oil via ingestion of oil-injected fish (LEFT panel) and via application of oil to feathers (RIGHT panel). Solid lines are controls, dashed lines are oil treated. Aspartate aminotransferase (A & B), gamma glutamyl transferase (C & D), alanine aminotransferase (E & F) and creatinine phosphokinase (G & H)) are all shown as are all shown as U/l, dotted lines are boundaries of reference intervals.

3.1. Hematology

In the oral treatment study, total WBC, heterophil and eosinophil counts decreased through the course of the trial and the rate of decrease was greater among the dosed birds than control birds ($p < 0.02$, Fig. 1A; $p < 0.001$, Fig. 1C; $p < 0.04$, Fig. 1E, respectively). Lymphocyte and monocyte counts also decreased through time, but the rates of decrease among the control birds and dosed birds were not significantly different (Fig. 1G, Fig. 1I, respectively). The heterophil:lymphocyte ratio among controls birds was relatively constant through time while the ratio among dosed birds declined, although not significantly (Fig. 1K).

In the external oiling study total WBC, heterophil and eosinophil counts, decreased through the course of the trial in a manner similar to the results of the oral dosing study. However, in contrast to the oral treatment study, the rates of decline in WBC and eosinophil counts were slightly greater among control birds than among dosed birds ($p < 0.02$ and $p < 0.05$, respectively) and significant differences in heterophil

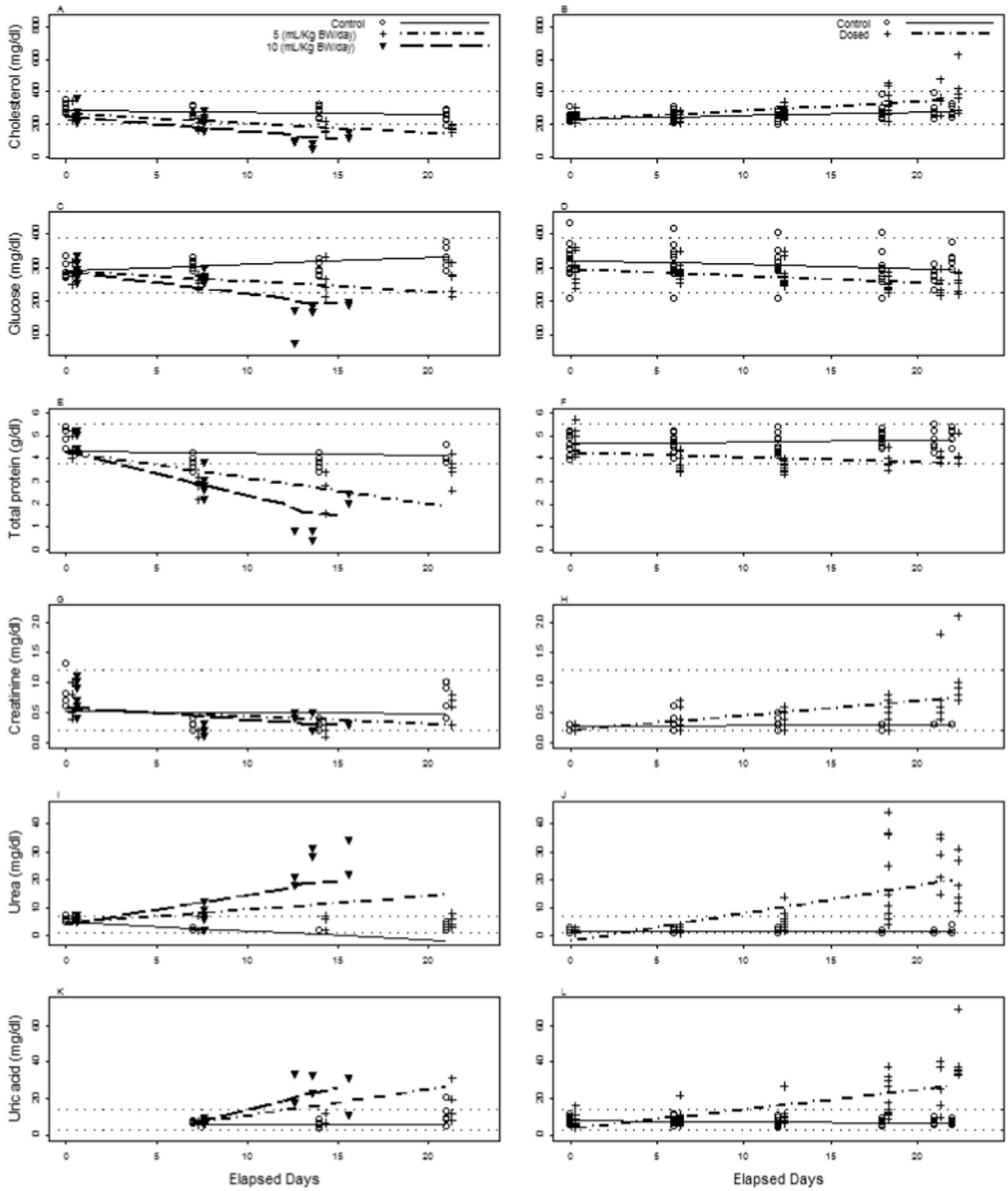

Fig. 3. Regression analyses showing changes in plasma analytes over time in double-crested cormorants exposed to oil via ingestion of oil-injected fish (LEFT panel) and via application of oil to feathers (RIGHT panel). Solid lines are controls, dashed lines are oil treated. Cholesterol (A & B), glucose (C & D), total protein(E & F), creatinine (G & H), urea (I & J) and uric acid (K & L) are all shown as mg/dl; dotted lines are boundaries of reference intervals.

counts were not seen (Fig. 1A, Fig. 1B, Fig. 1C). Of clinical significance was that only treated birds had monocyte counts greater than 3.7×10^9/L. Through the course of the trial lymphocyte counts and monocyte counts increased slightly among dosed birds and decreased slightly among control birds ($p < 0.006$, Fig. 1H and $p < 0.005$, Fig. 1J, respectively). The heterophil:lymphocyte ratio declined through the course of the trial ($p < 0.02$), but the rate of decline was similar among control birds and dosed birds (Fig. 1L).

3.2. Plasma clinical chemistry

3.2.1. Enzymes

In the oral exposure trial, AST, GGT, and ALT activities each decreased at a greater rate among dosed birds than among control birds ($p < 0.02$, Fig. 2A; $p < 0.01$, Fig. 2C; and $p < 0.03$, Fig. 2E, respectively). In contrast, CK activity increased at a greater rate among dosed birds than among control birds, although the difference was not statistically significant (Fig. 2G).

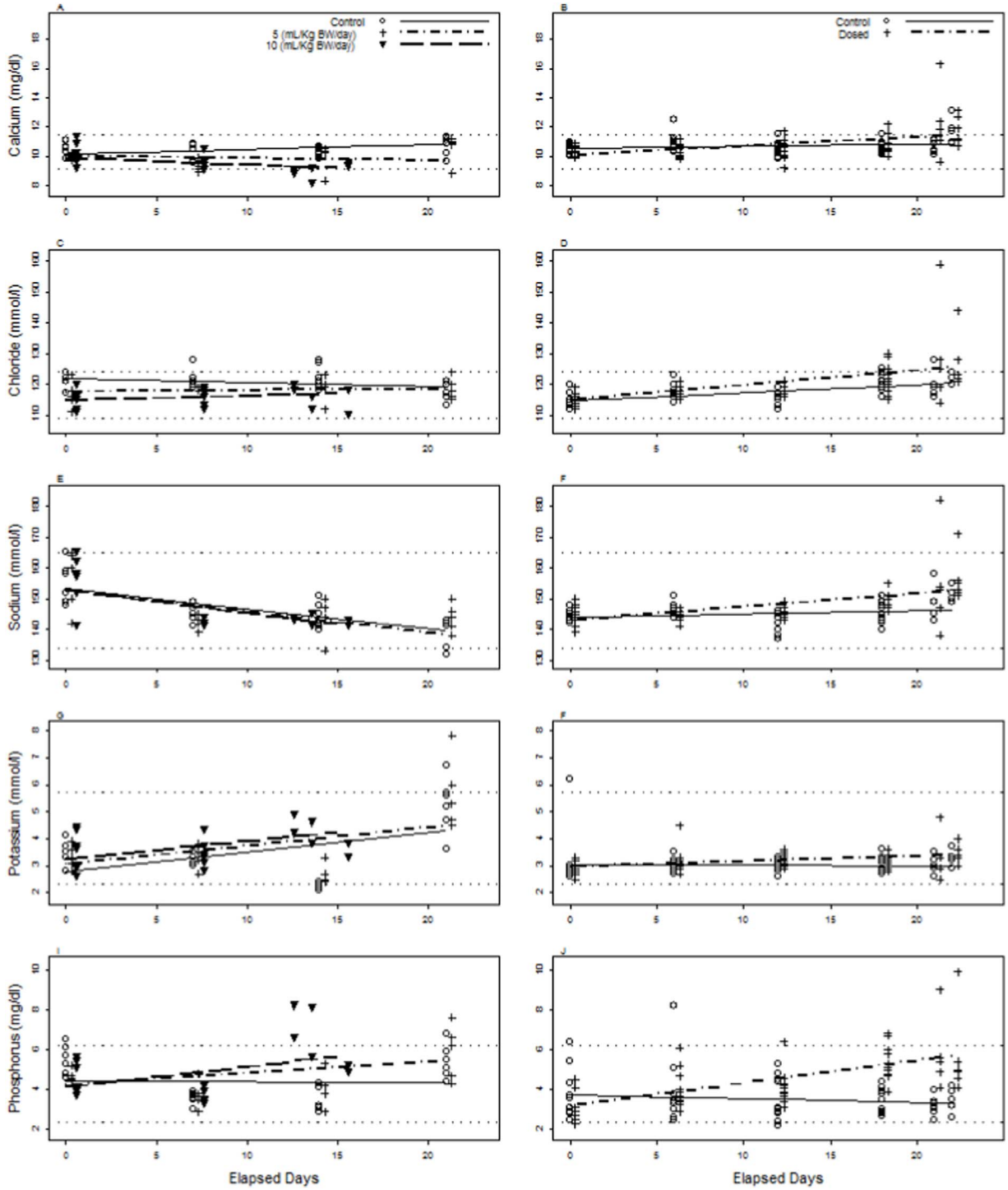

Fig. 4. Regression analyses showing changes in plasma mineral and salt concentrations over time in double-crested cormorants exposed to oil via ingestion of oil-injected fish (LEFT panel) and via application of oil to feathers (RIGHT panel). Solid lines are controls, dashed lines are oil treated. Calcium (A & B), chloride (C & D), sodium (E & F), potassium (G & H) and phosphorus (I & J) are all shown as mg/dl; dotted lines are boundaries of reference intervals.

Externally oiled birds showed a significant decrease in AST activity through the course of the trial ($p < 0.05$, Fig. 2B). There was a slight decrease in GGT activity through time among dosed and control birds but there was no statistically significant difference between groups (Fig. 2D). ALT activity increased through time among dosed birds while ALT decreased among control birds ($p < 0.003$, Fig. 2F). CK activity was significantly greater amongst the dosed birds relative to the control group throughout the study activity ($p < 0.001$; Fig. 2H), and activities in both groups were relatively constant through the course of the study. Although there were some significant changes, enzyme activities for most individual birds were within the reference intervals with the exception of ALT activity which was occasionally markedly increased.

3.2.2. Metabolites

Among orally dosed birds, cholesterol, glucose and total protein concentrations each decreased through time while concentrations remained relatively constant in control birds ($p < 0.001$, Fig. 3A; $p < 0.001$, Fig. 3C; and $p < 0.001$, Fig. 3E, respectively). Creatinine

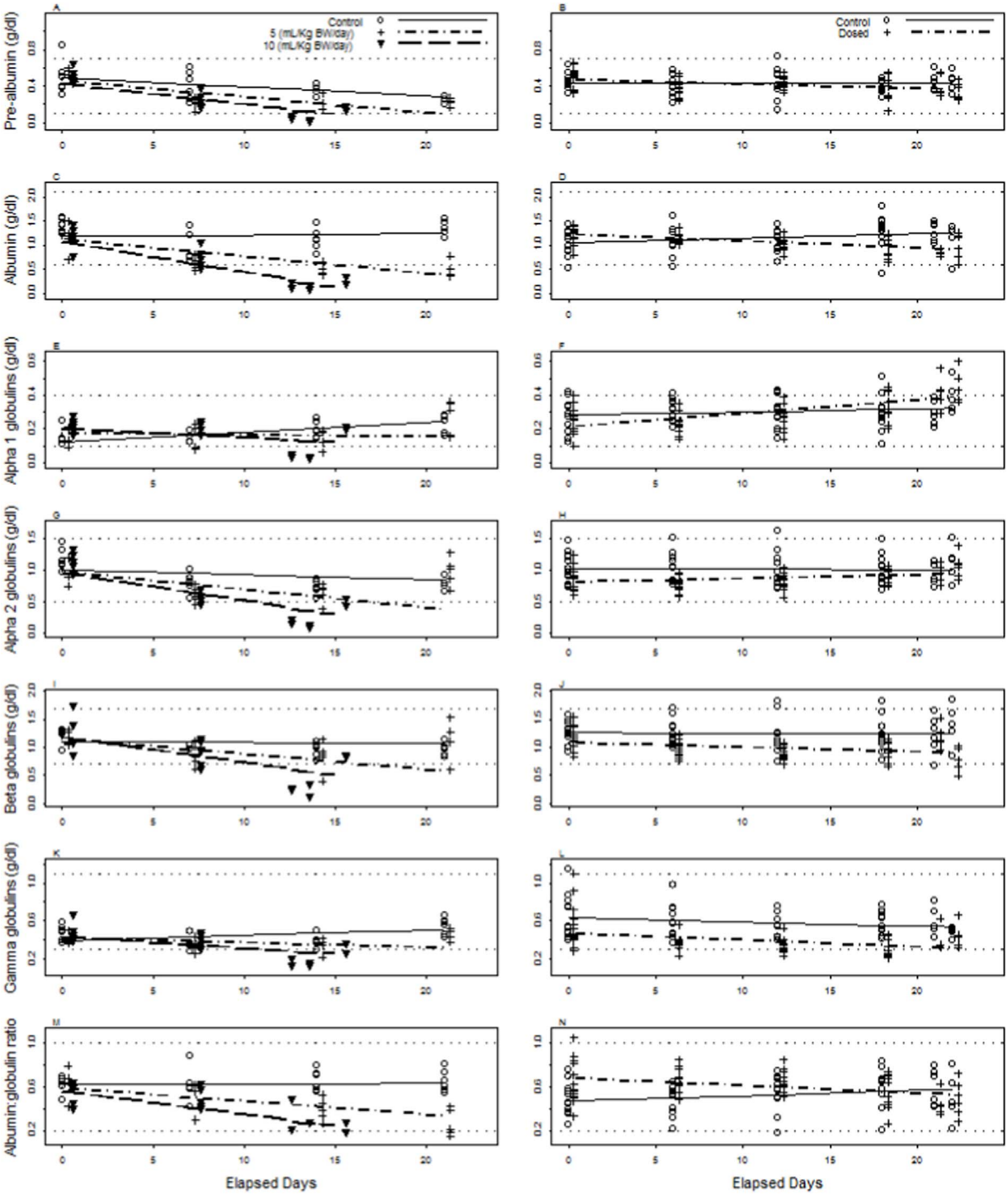

Fig. 5. Regression analyses showing changes in plasma protein concentrations over time in double-crested cormorants exposed to oil via ingestion of oil-injected fish (LEFT panel) and via application of oil to feathers (RIGHT panel). Solid lines are controls, dashed lines are oil treated. Pre-albumin (A & B), albumin (C & D), alpha −1-globulins (E & F), alpha-2-globulins (G & H), beta globulins (I & J) and gamma globulins (K & L) are all shown as g/dl. Albumin: globulin ratio is depicted in M and N; dotted lines are boundaries of reference intervals.

concentration remained approximately constant in all treatment groups (Fig. 3G), while urea concentration increased through time at a significantly faster rate among dosed birds than in control birds ($p < 0.001$, Fig. 3I). Uric acid concentration remained relatively constant among control birds but increased in the dosed groups ($p < 0.002$, Fig. 3K)

Among externally exposed birds, cholesterol concentration increased slightly through the study in both groups, although it increased faster in dosed birds ($p < 0.02$, Fig. 3B). Glucose concentration was significantly lower in dosed birds ($p < 0.01$) and both groups decreased slightly through time ($p < 0.001$, Fig. 3D). Total protein was significantly lower among dosed birds ($p < 0.01$) and concentrations decreased slightly through time among dosed birds ($p < 0.02$, Fig. 3F). Creatinine concentrations among control birds were stable through time but among dosed birds concentrations increased slightly through time ($p < 0.001$, Fig. 3H). Urea and uric acid concentrations each increased significantly among dosed birds, while concentrations among control birds remained relatively constant ($p < 0.001$, Fig. 3J and $p <$

0.001 Fig. 3L, respectively). Measurements for urea and uric acid exceeded reference interval concentrations in dosed birds.

3.2.3. Minerals and electrolytes

In the oral ingestion trial, calcium concentrations in control birds were relatively constant through time, while they decreased through the course of the trial among the dosed birds ($p < 0.04$, Fig. 4A). Chloride concentrations among control birds and dosed birds were both relatively constant, although concentrations were slightly lower among the dosed groups ($p < 0.001$, Fig. 4C). Sodium concentrations decreased significantly through time ($p < 0.001$) in all groups, with no significant differences between treatment groups (Fig. 4E). In contrast to sodium, potassium concentrations increased significantly through time ($p < 0.001$) in all groups, with no significant differences among treatment groups (Fig. 4G). Phosphorus concentrations increased slightly among dosed birds while they were relatively constant among control birds, but the distinction was not statistically significant ($p < 0.06$, Fig. 4I).

Trends were different in the external exposure trial. Calcium concentrations among control birds were relatively constant, while they increased slightly through the course of the trial among the dosed birds ($p < 0.03$, Fig. 4B). Chloride concentrations increased through time among controls and dosed birds ($p < 0.001$), but there were no significant differences between groups (Fig. 4D). Sodium concentrations increased through time among controls and dosed birds ($p < 0.001$), but the rate of increase was significantly greater among dosed birds ($p < 0.03$, Fig. 4F). Potassium concentrations among control and dosed groups were both relatively stable through the course of the trial, but mean concentrations were slightly higher among the dosed birds ($p < 0.03$, Fig. 4F). Phosphorus concentrations increased markedly through time among dosed birds, while they decreased slightly among control birds ($p < 0.001$, Fig. 4J).

3.2.4. Plasma protein electrophoresis

In the oral ingestion trial, consistent with the overall decrease in total protein (Fig. 3E) most of the protein fractions decreased. Pre-albumin concentrations decreased through time in control group and the dosed groups ($p < 0.001$), but the rate of decrease was significantly greater among the dosed groups ($p < 0.03$, Fig. 5A). Concentrations of albumin, alpha-1-globulins, alpha-2-globulins, beta globulins, gamma globulins and the A:G ratio were each relatively constant among control birds, while each of these endpoints in dosed birds decreased significantly through the course of the trial ($p < 0.001$, Fig. 5C; $p < 0.004$, Fig. 5E; $p < 0.02$, Fig. 5G; $p < 0.005$, Fig. 5I; $p < 0.001$, Fig. 5K; $p < 0.001$, Fig. 5M, respectively).

While total protein concentration decreases were both clinically and statistically significant, patterns of change in the plasma protein endpoints in the external exposure trial differed from those in the oral ingestion trial and were not consistent amongst protein fractions. Pre-albumin concentrations decreased slightly among both control birds and dosed birds ($p < 0.04$), with the rate of decrease slightly faster among the dosed group ($p < 0.04$, Fig. 5B). Albumin concentrations decreased faster through the course of the trial among dosed birds than among control birds, although the amount of change in both groups was relatively minor ($p < 0.001$, Fig. 5D). Alpha-1-globulin concentration increased faster through the course of the trial among dosed birds than among control birds ($p < 0.001$, Fig. 5F). No significant treatment effects or trends through time were evident in Alpha-2-globulin concentrations (Fig. 5H). Beta globulin concentration were relatively stable through time, but mean concentrations were significantly lower among the dosed birds ($p < 0.002$, Fig. 5J). Mean gamma globulin concentrations were also significantly lower among the dosed birds ($p < 0.001$) and concentrations among both groups decreased slightly through the course of the trial ($p < 0.007$, Fig. 5K). Trends in A:G ratio differed significantly between the control and dosed groups, where the ratio decreased among dosed birds but increased slightly among control birds ($p < 0.004$, Fig. 5N).

4. Discussion

Measurement of clinically relevant plasma endpoints are important in determining not only the health status of an individual bird during treatment and rehabilitation, but as research tools for determining the full extent of oil toxicity on a species, and assessment of potential population-wide effects of an oil spill. Changes in clinically relevant biochemical endpoints associated with toxicity of artificially weathered MC252 oil were observed using both dosing methods. Changes including increased phosphorus, urea and uric acid concentrations and decreased total protein, albumin, and A:G ratio indicative of organ dysfunction were observed in both experiments and are consistent with previous studies (Hartung and Hunt, 1966; Szaro et al., 1978; Patton and Dieter, 1980; Eastin and Rattner, 1982; Fleming et al., 1982; Pattee and Franson, 1982; Fry and Lowenstein, 1985; Lee et al., 1986; Leighton et al., 1985; Leighton, 1986; Hughes et al., 1990; Yamato et al., 1996; Walton et al., 1997; Newman et al., 2000; Seiser et al., 2000; Troisi et al., 2007). As such it is feasible that there are clinically relevant changes in plasma biochemistries that can be identified for this species, that may be applicable to other oil spills and species. While the volume of oil spilled and the environmental conditions play a major role in avian mortality, we cannot understand the full impact of an oil spill on a species until we can determine how toxic it is compared to other spills. The *Exxon Valdez* killed hundreds of thousands of birds rapidly due to extreme temperatures and large volume of oil within a small area, but the toxicity of the oil itself, particularly to harlequin ducks (*Histrionicus histrionicus*), affected survival rates for up to 14 years following the spill (Iverson and Esler, 2010). It is clear that although there will always be a range of species affected by a single oil spill event, that we need to have a better understanding of how to use standard clinical measurements as a means of assessing damage to a species.

The magnitude and number of changes that were measured in clinical plasma chemistry values indicate that the oral dosing method had a greater degree of toxicity than the external dosing method. This is likely due to relative dose effects. Orally dosed birds received 5.2 ± 0.3 ml oil/kg BW and 8.4 + 0.9 ml oil/kg BW per day totaling 208 and 235 ml respectively, while the externally dosed birds likely only consumed (based on Hartung, 1963) a total of 38–38.5 g over 21 days (Cunningham et al., 2017), or approximately 0.9 ml/kg BW daily. There were additional signs and symptoms of this toxicity reported in other manuscripts describing these studies (Alexander et al., 2017; Cunningham et al., 2017; Harr et a, 2017a, c, d; Pritsos et al., 2017). These include increases in relative organ weight, hypertrophy and histopathological changes at necropsy, development of hemolytic anemia, inflammation and atrophy (Harr et al., 2017a, 2017c), as well as newly documented clotting dysfunction, cardiomyopathy and associated functional losses (Harr et al., 2017d). However, there was a confounding variable for the orally dosed birds in that they developed some food aversion or gastrointestinal irritation that reduced their food intake overall and caused weight loss, while externally dosed birds did not lose weight, and in fact increased food consumption (Cunningham et al., 2017). Reduced food intake and weight loss could exacerbate the problems faced by organ systems as they endeavor to detoxify the ingested oil. While these two studies are useful in that they show similar trends in clinically relevant plasma endpoints, further investigation is required to conduct a direct comparison with similar doses.

4.1. Organ function

Oxidative damage resulting from metabolism of PAHs is also linked to organ damage and dysfunction in juvenile and adult birds. Effects can include liver, kidney, adrenal, salt gland, spleen and gastro-intestinal damage (Hartung and Hunt, 1966; Snyder et al., 1973; Gorman

and Sims, 1978; Szaro, 1977; Holmes et al., 1970; Szaro et al., 1978; Gorsline and Holmes, 1981; Eastin and Murray, 1981; Fleming et al., 1982; Miller et al., 1979, 1982; Pattee and Franson, 1982; Fry and Lowenstein, 1985; Leighton, 1986; Couillard and Leighton, 1990a, 1990b, Couillard and Leighton, 1991; Stubblefield et al., 1995; Newman et al., 2000; Alonso-Alvarez et al., 2007a, 2007b; Duerr, 2013).

Studies examining the effects of oil on birds have suggested that oil exposure can lead to hepatic damage or dysfunction, reporting decreases in plasma cholesterol, glucose, albumin, uric acid and total protein concentrations, and increases in ALP, ALT, AST, GGT, and bile acids (Eastin and Rattner, 1982; Stubblefield et al., 1995; Briggs et al., 1997; Newman et al., 2000; Seiser et al., 2000; Golet et al., 2002; Alonso-Alvarez et al., 2007a, 2007b). Generally, birds with hepatic dysfunction or disease will exhibit declines in albumin, cholesterol, glucose, total protein, uric acid and increases in bile acids, alkaline phosphatase, AST, GGT and LDH (Harr, 2005; Hochleitner et al., 2005). Orally dosed birds had significant declines in cholesterol, glucose, total protein concentrations as well as all protein fractions over the dosing period, while externally oiled birds showed reductions in glucose, albumin, and total protein levels, and an increase in ALT. These functional differences between the two dosing methods likely reflect the extent of damage to the liver caused by differences in daily and absolute dosing. Orally dosed birds actually exhibited declines in GGT and AST that could be indicative of later stages of liver damage, whereby production capabilities are lost. AST is also produced in muscle and other organs. As the body condition and muscle mass also declined in the orally dosed group, this may have also contributed to the decreased AST activity (Cunningham et al., 2017; this edition).

Increases in plasma urea, uric acid, and phosphorus concentrations found in both orally and externally exposed birds are indicative of renal insufficiency at both the glomerular and tubular levels (Braun, 2015) and have also been reported in avian oil exposure studies (Hartung and Hunt, 1966; Eastin and Murray, 1981). Both orally and externally dosed birds had significant increase in both urea and uric acid concentrations that were above the reference intervals. Urea concentration can also increase with water loss, which was supported by increased sodium concentration in this study. Birds were housed with pools of fresh water changed regularly. Thus, dehydration in the face of water ad libitum further supports renal insufficiency. Specific gravity of urates was not possible due to the aquatic nature of the animal and fecal mixing which precluded confirmation of lack of urinary concentration. While increased sodium was measured in the externally oiled birds, control birds in that experiment also had slightly increased sodium concentration, indicating that there may have been some kind of stress-related impact on electrolyte balance, through ACTH release (Olanrewaju et al., 2007). Orally dosed birds, including controls, all showed reductions in plasma sodium concentration over the course of the study, indicating improved hydration when housed with freshwater only, and potential adaptation to captivity.

The gastrointestinal irritation caused by oil can be more difficult to measure, but usually manifests as a deterioration in nutritional state including reduced weight gain in chicks, hyperphagia and weight loss in adults, as well as abnormal excreta (Hartung and Hunt, 1966; Holmes et al.; Snyder et al., 1973; Snyder et al., 1978; Patton and Dieter, 1980; Szaro et al., 1981; Fleming et al., 1982; Pattee and Franson, 1982; Trivelpiece et al., 1984; Hughes et al., 1990; Evans and Keijl, 1993). Malabsorption of nutrients can manifest as low plasma protein and decreased chloride concentration, which were observed in these dosing studies. As stated above, decreased total protein and protein fractions may be due to decreased production by the liver but loss through either the gastrointestinal tract or kidney may contribute to statistically significant cumulative decrease found. Additionally, reduced food intake and/or impaired intestinal transport were also suggested as possible mechanisms for the decreased plasma measurements though this has only been proven in the most cachectic individuals (Eastin and Rattner, 1982; Newman et al., 2000; Alonso-Alvarez et al., 2007a, 2007b). Decreased A:G ratio found in both the oral and external exposure studies supports loss either through the kidney or GI tract as the smaller proteins such as albumin will be lost preferentially to the larger globulin molecules. While malabsorption of nutrients was indicated at necropsy and through anecdotal observation of abnormal excreta (Harr et al., 2017c), there were no specific changes in plasma analytes that specifically assessed the gastrointestinal tract in the panel that was tested. Malabsorption testing with xylose or similar GI functional assays could be used to further assess the gastrointestinal compromise in oral versus external exposure.

There is some indication that oil exposure also results in muscle damage possibly due to direct oxidative damage or secondary to behavioral or physical causes. Newman et al. (2000) suggest that recent net capture may cause elevations in AST and creatine kinase activities as would be expected. Elevated creatine kinase activity was found in orally and externally oiled birds but not in control birds. It increased over time in captivity and did appear to increase with dose in the oral experiment. While this could be related to the direct impacts of increased dosage, other factors such as excessive preening, poorly controlled movements due to poor feather integrity, or muscular injuries may have contributed to the increased activities of these enzymes found within the myocyte cytosol.

4.1.1. Potential immune and adrenal changes

Immunosuppression has been reported in both oil dosing studies and during rehabilitation of oiled birds (Rocke et al., 1984; Leighton, 1986; Khan and Ryan, 1991; Anderson et al., 1996; Newman et al., 2000); however, data interpretation is often confounded by capture/handling stress and presence of pre-existing infections or injuries in wild birds, particularly in the short term assessments (Harvey et al., 1982; Leighton et al., 1986; McOrist and Lenghaus, 1992; Briggs et al., 1997; Newman et al., 2000). In these experiments, immune measurements were limited due to the nature of the study design. There were no increases in gamma globulins in either the oral or external dosing experiments, so we used WBC and differential cell counts as generalized indicators of immune system function. Results were not strongly supportive of oil-induced immunocompromise. Monocyte counts in the external dosing study (controls and treated birds) were an order of magnitude higher than the oral dosing study, but all other leucocyte counts were similar. As stated in the Methods section, a blood sample was collected immediately post-capture for the external dosing study to determine if there were any pre-existing conditions present that could affect data interpretation. This was in fact the case for the monocyte counts, so any counts greater than 2.0×10^9 cell/L were considered to be abnormal and a chronic infection was suspected. Since both these experiments were considered as pilot or scoping studies, birds with high monocyte counts were divided evenly between the control and treated groups as comparisons between the two types of dosing were not an essential component of the study design. Histopathology following necropsy identified parasitic, bacterial or fungal infections in many individuals in the external oiling study (Harr et al., 2017c), including those with high monocyte counts, but there did not appear to be a trend that set apart those with high monocyte counts.

There were statistically significant decreases in some leucocytes; however, the decreases also occurred in control birds. In the oral dosing study, WBC, heterophil, and eosinophil counts decreased over time, with the greatest decreases being in oil-dosed birds, while lymphocyte counts decreased similarly in all three groups over the course of the study. Control birds in the external oiling experiment showed a greater decline in white cell estimate and lymphocyte counts than treated birds, while heterophil numbers declined similarly between control and treated birds. It is possible that decreases in lymphocyte numbers are indicators of a captivity-induced stress response (Leighton, 1986; Briggs et al., 1996), particularly since they were observed in control birds. As such, we also looked at the heterophil/Lymphocyte ratio, increases in

which are often used as an estimate of activation of the hypothalamic-pituitary-adrenal (HPA) axis, due to decreases in lymphocyte numbers caused by glucocorticoids (Martin, 2009). There were statistically significant decreases in the heterophil/Lymphocyte ratio in the treated birds in both experiments, but only in controls for the oral dosing study. While oil exposure has been linked to adrenal dysfunction and hypertrophy in birds (Peakall et al., 1981; Rattner and Eastin, 1981; Gorsline and Holmes, 1982; Lattin et al., 2014, Lattin and Romero, 2014), due to the limited scope of these studies adrenocorticotrophic hormone stimulation of the HPA axis could not be conducted to determine baseline or HPA activation in response to captivity and oil exposure.

While the effects of captivity-induced stress and pre-existing are likely to contribute to changes in leucocyte counts, the anemia observed in treated birds in both experiments (Harr et al., 2017a) is also likely to have caused a shift by the bone marrow towards erythropoiesis as observed by Leighton (1986) in herring gulls and Atlantic puffins dosed with Prudhoe Bay crude. Thymus and bursa of Fabricius regress in most avian species during development, and whilst involuted bursas were collected from a small number of individuals during necropsy, histopathology did not report anything of note (Harr et al., 2017c). It has been suggested that oil toxicity has more impact on cell-mediated immune responses than antibody-mediated responses (Briggs et al., 1997); however, in this study the only measurements of cell-mediated immune function were WBC and differential counts, so interpretation of the specific effects of oil are somewhat limited. While albumin, pre-albumin and gamma globulin decreased during the study indicating malabsorption and liver disease, alpha-1-globulin increased potentially indicating inflammation.

4.2. Conclusions

Following an oil spill the first measurements made of oil coated birds are naturally used to determine their health status. While sampling oiled birds for health assessment is paramount in ensuring the highest likelihood of survival for each individual, it somewhat limits our understanding of oil toxicity, and ability extrapolate of potential population effects. Additionally, our understanding of the toxicological effects of oil on avian physiology have been somewhat hampered by the difficulties associated with developing reliable and reproducible dosing paradigms. In this study, we tested two oil dosing methods on double-crested cormorants that would result in oral ingestion, and incorporated both clinical health measurements and toxicological assessments to determine the overall impact of artificially weather MC252 on cormorant physiology. Although limited dose ranges were used, both methods resulted in impacts to clinical plasma chemistry measurements that are supported by necropsy findings and the broader avian oil toxicity literature. We concluded that clinically significant increases in urea, uric acid, and phosphorus concentrations combined with decreases in total protein, albumin, and A:G ratio were consistently found in orally and externally exposed double-crested cormorants using artificially weathered MC252 oil. Further experimentation is needed is to more precisely determine the doses and exposure durations that have the potential to cause adverse physiological outcomes and to fine-tune the clinical measurements indicative of organ damage and function loss that can be extrapolated beyond the MC252 oils.

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Appendix A. Supplementary material

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References

Alexander, C.R., Hooper, M.J., Cacela, D., Smelker, K.D., Calvin, C.S., Dean, K.M., Bursian, S.J., Cunningham, F.L., Hanson-Dorr, K.C., Horak, K.E., Isanhart, Link, J., Shriner, S.A., Godard-Codding, C.A.J., 2017. CYP1A protein expression and catalytic activity in double-crested cormorants experimentally exposed to deepwater Horizon Mississippi Canyon 252 oil. Ecotoxicol. Environ. Saf. http://dx.doi.org/10.1016/j.ecoenv.2017.05.015.

Alonso-Alvarez, C., Munilla, I., Lopez-Alonso, M., Velando, A., 2007a. Sublethal toxicity of the *Prestige* oil spill on yellow-legged gulls. Environ. Int. 33, 773–781.

Alonso-Alvarez, C., Perez, C., Velando, A., 2007b. Effects of acute exposure to heavy fuel oil from the Prestige spill on a seabird. Aquat. Toxicol. 84 (1), 103–110. http://dx.doi.org/10.1016/j.aquatox.2007.06.004.

Anderson, D.W., Gress, F., Fry, D.M., 1996. Survival and dispersal of oiled brown pelicans after rehabilitation and release. Mar. Pollut. Bull. 32 (10), 711–718.

Braun, E.J., 2015. Chapter 12: osmoregulatory systems of birds. In: Scanes, C.G. (Ed.), Sturkies AvianPhysiology 6. Academic Press Elsevier, pp. 285–299 (ed).

Briggs, K.T., Yoshida, S.H., Gershwin, M.E., 1996. The influence of petrochemicals and stress on the immune system of seabirds. Regul. Toxicol. Pharmacol. 23, 145–155.

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

Couillard, C.M., Leighton, F.A., 1990a. Sequential study of the pathology of Prudhoe Bay crude oil in chicken embryos. Ecotoxicol. Environ. Saf. 19, 17–23.

Couillard, C.M., Leighton, F.A., 1990b. The toxicopathology of Prudhoe Bay crude oil in chicken embryos. Fundam. Appl. Toxicol. 14, 30–39.

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

Delk, K.W., Wach, F.F., Burgdorf-Moisuk, A., Kass, P.H., Cray, C., 2014. Acute phase protein and electrophoresis protein fraction values for captive American flamingos. J. Zoo. Wildl. Med. 46 (4), 929–933.

Duerr, R.S., 2013. Investigation into nutritional condition and digestive capabilities of seabirds during rehabilitation in California (PhD). University of California Davis.

Eastin, W.C.J., Murray, H.C., 1981. Effects of crude oil ingestion on avian intestinal function. Can. J. Physiol. Pharmacol. 59, 1063–1068.

Eastin, W.C.J., Rattner, B.A., 1982. Effects of dispersant and crude oil ingestion on mallard ducklings (*Anas platyrhynchos*). Bull. Environ. Contam. Toxicol. 29, 273–278.

Evans, M.I., Keijl, G.O., 1993. Impact of Gulf War oil spills on the wader populations of the Saudi Arabian Gulf coast. Sandgrouse 15, 85–105.

Fleming, W.J., Sileo, L., Franson, J.C., 1982. Toxicity of Prudhoe Bay crude oil to sandhill cranes. J. Wildl. Manag. 46 (2), 474–478.

Fry, D.M., Lowenstine, L.J., 1985. Pathology of common murres and Cassin's auklets exposed to oil. Arch. Environ. Contam. Toxicol. 14, 725–737.

Garcia, M.T.A., Hermosa, Y., Aguirre, J.I., 2010. Does breeding status influence haematology and blood biochemistry of yellow-legged gulls? Acta Biol. Hung. 61 (4), 391–400.

Golet, G.H., Seiser, P.E., McGuire, A.D., Roby, D.D., Fischer, J.B., Kuletz, K.J., Newman, S.H., 2002. Long-term direct and indirect effects of the 'Exxon Valdez' oil spill on pigeon guillemots in Prince William Sound, Alaska. Mar. Ecol. Prog. Ser. 241, 287–304.

Gorman, M.L., Simms, C.E., 1978. Lack of effect of ingested Forties Field crude oil on avian growth. Mar. Pollut. Bull. 9 (10), 273–276.

Gorsline, J., 1982. The Effects of South Louisiana Crude Oil on Adrenocortical Function. Aspects of AvianEndocrinology: Practical and Theoretical Implications. Texas Tech Press, Lubbock, TX, pp. 359–364.

Gorsline, J., Holmes, W.N., 1981. Effects of petroleum on adrenocortical activity on hepatic naphthalene-metabolizing activity in mallard ducks. Arch. Environ. Contam. Toxicol. 10, 765–777.

Harr, K.E., 2005. Ch 23: diagnostic values of biochemistry. In: Harrison, G., Lightfoot, T. (Eds.), Clinical AvianMedicine 1. Spix Publishing, pp. 611–630.

Harr, K.E., Cunningham, F.L., Pritsos, C.A., Pritsos, K.L., Muthumalage, T., Dorr, B.S., Horak, K.E., Hanson-Dorr, K.C., Dean, K.M., Cacela, D., Link, J.E., Healy, K., Tuttle, P., Bursian, S.J., 2017a. 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. http://dx.doi.org/10.1016/j.ecoenv.2017.07.030.

Harr, K.E., Dean, K., Hanson-Dorr, K., Healy, K., McFadden, A., Cunningham, F.L., 2017b. Hematologic and biochemical reference intervals in double-crested cormorants (*Phalacrocorax auritis*) from the southeastern United States. Vcp. Prog.

Harr, K.E., Reavill, D.R., Bursian, S.J., Cacela, D., Cunningham, F.L., Dean, K.M., Dorr, B.S., Hanson-Dorr, K.C., Healy, K.A., Horak, K.E., Link, J.E., Shriner, S.A., Schmidt, R.E., 2017c. Organ weights and histopathology of double-crested cormorants (*Phalacrocorax auritus*) dosed orally or dermally with artificially weathered Mississippi Canyon 252 crude oil. Ecotoxicol. Environ. Saf. http://dx.doi.org/10.1016/j.ecoenv.2017.07.011.

Harr, K.E., Rishniw, M., Rupp, T.L., Cacela, D., Dean, K.M., Dorr, B.S., Hanson-Dorr, K.C., Healy, K., Horak, K., Link, J.E., Reavill, D., Bursian, S.J., Cunningham, F.L., 2017d. Dermal exposure to weathered MC252 crude oil results in echocardigraphically identifiable systolic myocardial dysfunction in double crested cormorants (*Phalacrocorax auritus*). Ecotoxicol. Environ. Saf. Accept. http://dx.doi.org/10.1016/j.ecoenv.2017.04.010.

Hartung, R., 1963. Ingestion of oil by waterfowl. Pap. Mich. Acad. Sci. Arts Lett. 48, 49–55.

Hartung, R., Hunt, G.S., 1966. Toxicity of some oils to waterfowl. J. Wildl. Manag. 30 (3), 564–570.

Harvey, S., Phillips, J.G., Sharp, P.J., 1982. Reproductive Performance and Endocrine Responses to Ingested North Sea Oil. Aspects of AvianEndocrinology: Practical and Theoretical Implications. Texas Tech Press, Lubbock, TX, pp. 379–395.

Hochleitner, M., Hochleitner, C., Harrison, L.D., 2005. Evaluating and treating the liver. In: Harrison, G., Lightfoot, T. (Eds.), Clinical Avian Medicine 1. Spix Publishing, pp. 441–450.

Holmes, W.N., Gorsline, J., Cronshaw, J., 1970. Effects of mild cold stress on the survival of seawater-adapted mallard ducks (Anas platyrhynchus) maintained on food contaminated with petroleum. Environ. Res. 20, 425–444.

Hughes, M.R., Kasserra, C., Thomas, B.R., 1990. Effect of externally applied bunker fuel on body mass and temperature, plasma concentration, and water flux of glaucous-winged gulls, *Larus glaucescens*. Can. J. Zool. 68, 716–721.

Iverson, S.A., Esler, D., 2010. Harlequin duck population injury and recovery dynamics following the 1989 *Exxon Valdez* oil spill. Ecol. Appl. 20 (7), 1993–2006.

Lattin, C.R., Romero, L.M., 2014. Chronic exposure to a low dose of ingested petroleum disrupts corticosterone receptor signalling in a tissue-specific manner in the house sparrow (Passer domesticus). Conserv Physiol. 2 (1), cou058. http://dx.doi.org/10.1093/conphys/cou058.

Lattin, C.R., Ngai, H.M., Romero, L.M., 2014. Evaluating the stress response as a bioindicator of sub-lethal effects of crude oil exposure in wild house sparrows (Passer domesticus). PLoS ONE 9 (7), e102106. http://dx.doi.org/10.1371/journal.pone.0102106.

Lee, Y.-Z., O'Brien, P.J., Payne, J.F., Rahimtula, A.D., 1986. Toxicity of petroleum crude oils and their effect on xenobiotic metabolizing enzyme activities in the chicken embryo *in Ovo*. Environ. Res. 39 (1), 153–163.

Leighton, F.A., 1986. Clinical, gross, and histological findings in herring gulls and Atlantic puffins that ingested Prudhoe Bay crude 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., 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. Pharmacol. 81 (1), 25–31.

Martin, L.B., 2009. Stress and immunity in wild vertebrates: timing is everything. Gen. Comp. Endocrinol. 163 (1–2), 70–76. http://dx.doi.org/10.1016/j.ygcen.2009.03.008.

McOrist, S., Lenghuas, C., 1992. Mortalities of little penguins (*Eudyptula minor*) following exposure to crude oil. Vet. Rec. 130, 161–162.

Miller, D.S., Kinter, W.B., Peakall, D.B., 1979. Effects of Crude Oil Ingestion on Immature Pekin Ducks (*Anas platyrhynchos*) and Herring Gulls (Larus argentatus) Animals as Monitors of Environmental Pollutants. National Academy of Sciences, Washington, D.C., pp. 27–40.

Miller, D.S., Hallett, D.J., Peakall, D.B., 1982. Which components of crude oil are toxic to young seabirds? Environ. Toxicol. Chem. 1 (1), 39–44.

Newman, S.H., Anderson, D.W., Ziccardi, M.H., Trupkiewicz, J.G., Tseng, F.S., Christopher, M.M., Zinkl, J.G., 2000. An experimental soft-release of oil-spill rehabilitated American coots (*Fulica americana*): II. Effects on health and blood parameters. Environ. Pollut. 107 (3), 295–304.

Olanrewaju, H.A., Thaxton, J.P., Dozier 3rd, W.A., Branton, S.L., 2007. Electrolyte diets, stress and acid-base balance in broiler chickens. Poult. Sci. 86 (7), 1363–1371.

Pattee, O.H., Franson, J.C., 1982. Short-term effects of oil ingestion on American kestrels (*Falco sparverius*). J. Wildl. Dis. 18 (2), 235–241.

Patton, J.F., Dieter, M.P., 1980. Effects of petroleum hydrocarbons on hepatic function in the duck. Comp. Biochem. Physiol. 65C, 33–36.

PDARP, 2016. Deepwater Horizon Oil Spill: Final programmatic damage assessment and restoration plan and final programmatic environmental impact statement. http://www.gulfspillrestoration.noaa.gov/sites/default/files/wp-content/uploads/Front-Matter-and-Chapter-1_Introduction-and-Executive-Summary_508.pdf.

Peakall, D.B., Tremblay, J., Kinter, W.B., Miller, D.S., 1981. Endocrine dysfunction in seabirds caused by ingested oil. Environ. Res. 24 (1), 6–14.

Peakall, D.P., Norstrom, R.J., Jeffrey, D.A., Leighton, F.A., 1989. Induction of hepatic mixed function oxidases in the herringg gull (*Larus agrentatus*) by Pruhoe Bay crude oil and its fractions. Comp. Biochem. Physiol. 94C (2), 461–463.

Perez, C., Lores, M., Velando, A., 2010. Oil pollution increases plasma antioxidants but reduces coloration in a seabird. Oecologia 163, 875–884.

Pritsos, K.L., Perez, C.R., Muthumalage, T., Dean, K.M., Cacela, D., Hanson-Dorr, K.C., Cunningham, F.L., Bursian, S.J., Link, J.E., Shriner, S.A., Horak, K.E., Pritsos, C.A., 2017. Dietary intake of Deepwater Horizon oil-injected live food fish by double-crested cormorants resulted in oxidative stress. Ecotoxicol. Environ. Saf. http://dx.doi.org/10.1016/j.ecoenv.2017.06.067.

Rayhel, L., Aitken-Palmer, C., Joyner, P., Cray, C., Lizarraga, C.A., Ackermans, B., Crowe, C., 2015. Hematology and biochemistry in captive white-naped cranes (*Grus vipio*). J. Zoo. Wildl. Med. 46 (4), 747–754.

Rocke, T.E., Yuill, T.M., Hinsdill, R.D., 1984. Oil and related toxicant effects on mallard immune defenses. Environ. Res. 33 (2), 343–352.

Seiser, P.E., Duffy, L.K., McGuire, A.D., Roby, D.D., Golet, G.H., Litzow, M.A., 2000. Comparison of pigeon guillemot, *Cepphus columba*, blood parameters from oiled and unoiled areas of Alaska eight years after the *Exxon Valdez* oil spill. Mar. Pollut. Bull. 40 (2), 152–164.

Snyder, S.B., Fox, J.G., Soave, O.A., 1973. Mortalities in Waterfowl Following a Bunker C Fuel Exposure. Retrieved from.

Stubblefield, W.A., Hancock, G.A., Prince, H.H., Ringer, R.K., 1995. Effects of naturally weathered Exxon Valdez crude oil on mallard reproduction. Environ. Toxicol. Chem. 14 (11), 1951–1960.

Szaro, R.C., Dieter, M.P., Heinz, G.H., 1978. Effects of chronic ingestion of south Louisiana crude oil on mallard ducklings. Environ. Res. 17 (3), 426–436.

Szaro, R.C., Hensler, G., Heinz, G.H., 1981. Effects of chronic ingestion of No. 2 fuel oil on mallard ducklings. J. Toxicol. Environ. Health 7, 789–799.

Troisi, G., Borjesson, L., Bexton, S., Robinson, I., 2007. Biomarkers of polycyclic aromatic hydrocarbon (PAH)-associated hemolytic anemia in oiled wildlife. Environ. Res. 105 (3), 324–329.

Walton, P., Turner, C.M.R., Austin, G., Burns, M.D., Monaghan, P., 1997. Sub-lethal effects of an oil pollution incident on breeding kittiwakes *Rissa tridactyla*. Mar. Ecol. Prog. Ser. 155, 261–268.

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