

2017

Development of methods for avian oil toxicity studies using the double crested cormorant (*Phalacrocorax auritus*)

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Cunningham, Fred L.; Dean, Karen; Hanson-Dorr, Katie C.; Harr, Kendal; Healy, Kate; Horak, Katherine E.; Link, Jane; Shriner, Susan A.; Bursian, Steve J.; and Dorr, Brian S., "Development of methods for avian oil toxicity studies using the double crested cormorant (*Phalacrocorax auritus*)" (2017). *USDA National Wildlife Research Center - Staff Publications*. 1926.

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ARTICLE INFO

Keywords:

Methods development
Oil toxicity
Double crested cormorant
Deepwater Horizon
Internal oil exposure
External oil exposure

ABSTRACT

Oral and external dosing methods replicating field exposure were developed using the double crested cormorant (DCCO) to test the toxicity of artificially weathered *Deepwater Horizon* Mississippi Canyon 252 oil. The majority of previous oil dosing studies conducted on wild-caught birds used gavage methods to dose birds with oil and determine toxicity. However, rapid gut transit time of gavaged oil likely reduces oil absorption. In the present studies, dosing relied on injection of oil into live feeder fish for oral dosing of these piscivorous birds, or applying oil to body contour feathers resulting in transdermal oil exposure and oral exposure through preening. Both oral and external oil dosing studies identified oil-related toxicity endpoints associated with oxidative stress such as hemolytic anemia, liver and kidney damage, and immuno-modulation or compromise. External oil application allowed for controlled study of thermoregulatory stress as well. Infrared thermal images indicated significantly greater surface temperatures and heat loss in treated birds following external oil applications; however, measurements collected by coelomically implanted temperature transmitters showed that internal body temperatures were stable over the course of the study period. Birds exposed to oil externally consumed more fish than control birds, indicating metabolic compensation for thermal stress. Conversely, birds orally dosed with oil experienced hyperthermia and consumed less fish compared to control birds.

1. Introduction

The acutely lethal effects of heavy oiling to birds are well known from oil spills worldwide, such as the *Exxon Valdez*, *Nestucca*, *Apex Houston*, and various spills in the North Sea (Burger, 1993). When ingested by birds at less than acutely lethal dosages, oil can cause a wide range of adverse effects, including anemia, 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).

Experimental oral exposure of avian species to oil is generally achieved through either feeding trials or gavage. Feeding trials have most commonly been used for longer duration studies (Holmes et al., 1978; Szaro et al., 1978; Harvey et al., 1981, 1982; Pattee and Franson, 1982; Cavanaugh et al., 1983; Cavanaugh and Holmes, 1987; Alonso-Alvarez et al., 2007b), while gavage is more commonly used to

investigate acute exposure (Hartung and Hunt, 1966; Wootton et al., 1979; McEwan and Whitehead, 1980; Eastin and Rattner, 1982; Leighton et al., 1985; Leighton, 1985, 1986; Lee et al., 1985; Peakall et al., 1989; Brausch et al., 2010).

To assess the specific adverse effects of Mississippi Canyon 252 (MC252) oil that was released during the Deepwater Horizon (DWH) oil spill on Gulf of Mexico-relevant avian species, the double crested cormorant (*Phalacrocorax auritus*; DCCO) was chosen as one of four species for initial oral dosing studies conducted under Phase 2 of the avian toxicity studies for the DWH Natural Resource Damage assessment (NRDA) (Bursian, submitted for publication et al., this issue). Double crested cormorants were chosen specifically for inclusion in these tests because they were affected by the DWH spill. Double crested cormorants are also common, primarily piscivorous waterbirds that inhabit pelagic, coastal, and inland waterways (Glahn et al., 1995; Johnson et al., 2002) and as such can be used as surrogates for other

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piscivorous species, such as pelicans (*Pelicanus* sp.), terns (*Sterna* sp.), and skimmers (*Rynchops* sp.).

An initial oral toxicity study conducted with DCCOs followed similar protocols described in the literature (Leighton, 1986) as well as recommendations from an expert panel. Artificially weathered MC252 oil (DWH7937, batch# B030112) was prepared from crude oil collected during the DWH oil spill as described by Forth et al. (2016). Birds were gavaged with a 1:1 mixture of artificially weathered MC252 oil and fish slurry that provided a dose of 20 mL oil/kg body weight (bw) once or on five consecutive days at daily doses of 20 mL oil/kg bw. Results of this initial oral toxicity trial suggested an apparent treatment-related decrease in packed cell volume (PCV) with mild to moderate anemia by day 3. There were significant changes in complete blood count (CBC), plasma chemistry, plasma protein and acute phase protein endpoints, although values were consistently within reference intervals. Some endpoints indicative of oxidative stress were significantly different in oil-dosed birds compared to controls. Specifically, there were significant changes in white cell counts, activities of alkaline phosphatase, alanine aminotransferase, creatine phosphokinase and gamma glutamyl transferase, concentrations of uric acid, chloride, sodium, potassium, calcium, total glutathione, glutathione disulfide and reduced glutathione. Kidney and liver weights were increased in birds administered oil, although exposure of DCCOs to oil did not result in treatment-related pathology or other observable abnormalities at necropsy (Dean et al., 2017a, this issue). While the results of this initial oral dosing study were inconclusive, they did suggest potential adverse effects after short internal exposure time to polycyclic aromatic hydrocarbons (PAHs) that are principle components of oil.

The decision was made to modify dosing methods to increase the exposure time of birds to oil and to better emulate the field conditions. Birds can be exposed to oil via multiple paths following an oil spill. Exposure routes include consumption of contaminated food, exposure of skin, feathers and mucus membranes to oiled water and inhalation of volatiles as they weather. Externally oiled birds devote long periods of time to removal of oil and maintenance of feather integrity, making consumption of oil via preening an important route of exposure (Leighton, 1993).

The direct application of oil to feathers, while it mimics conditions in the wild whereby birds are able to preen as they deem necessary, and therefore consume oil throughout the day, has the disadvantage for toxicological studies that the dose consumed may vary between individuals. Oral dosing by gavage should allow better control of dose, but the method has the disadvantage that dose can only be delivered over a prescribed period, potentially limiting the absorption of oil by the gastrointestinal tract. As such, modifications were implemented to improve oral and external dosing of DCCOs to determine the utility of each method and whether the two types of dosing provided different insight into the effects of oil on birds. In the first method, the oil dose was delivered to DCCOs in live food catfish, consumed throughout the day, rather than by bolus gavage. The second method involved application of a calculated dose of oil to the DCCO feathers every three days.

2. Materials and methods

2.1. Animal collection and husbandry

National Wildlife Research Center Mississippi Field Station (NWRC-MSFS) staff conducted all bird capture, transport, quarantine, feeding and maintenance according to standard Institutional Animal Care and Use Committee (IACUC) approved procedures. Capture was conducted under the authority of USFWS Migratory Bird Permit # MB019065-3, and Mississippi and Alabama state scientific collection permits. Twenty-seven DCCOs were collected on March 12, 2013 from McIntyre Scatters, Leflore County, MS for the oral dosing study and 31 DCCOs were captured from Mississippi and Alabama from night roosts on December

29 and 30, 2014 and January 11 and 12, 2015 for the external oiling study using a customized capture boat, flood lights, and dip nets (King et al., 1994). Birds were transported from the field to captive facilities in an enclosed trailer.

2.2. Feeding and housing

Upon arrival, DCCOs were weighed to the nearest gram (g) and individually marked with a unique alphanumeric coded plastic leg band. In both dosing studies the DCCOs were held in a 23 m (m) x 10 m mechanically ventilated building. DCCOs were individually housed in cages that measured 3.3 m x 1.5 m x 2.0 m (length x width x height) and contained shallow, 190-liter (L) plastic tanks filled with water containing an air stone to provide adequate dissolved oxygen for maintenance of live channel catfish (*Ictalurus punctatus*). Water was changed daily to limit oil re-exposure. During the external dosing study perches made of 7.6 cm (cm) diameter polyvinyl chloride (PVC) were provided in each pen.

Cormorants were fed live, farm-raised fingerling channel catfish. Each DCCO was offered approximately 600 g catfish/day placed into the water tank within each individual pen. The following day, all uneaten catfish were removed from individual tanks and weighed to assess individual food consumption.

2.3. Quarantine and daily monitoring

Birds were allowed to acclimate to captivity for a minimum of 21 days prior to initiation of the study. All individuals were inspected once daily during quarantine and twice daily during the trial for signs of pain or distress. Distressed animals (e.g., those exhibiting lethargy, emaciation, persistent recumbency) were evaluated and either treated and retained in the study or euthanized. If an individual bird exhibited signs of cold stress such as hunched posture and “fluffed” feathers, then heat lamps were provided to all birds at that time. Use of heat lamps was recorded on health and daily monitoring sheets. All animal care and monitoring procedures were approved by the IACUC under NWRC protocol QA-2107 for the oral dosing study and QA-2326 for the external dosing study. Health assessment checklists were completed daily.

2.4. Screening for abnormal subjects

A wild population of any species contains a cross-section of age classes and individuals of variable health status. To avoid miscalculating or overestimating oil toxicity effects, only birds assessed as healthy based on physical exam, body weight and appetite were included in the internal dosing study. This study was also used to establish reference intervals for hematological and biochemical analysis values which were used in the external dosing study to further screen study candidates. To determine which birds were healthy and which were abnormal, proposed reference intervals were established in accordance with American Society for Veterinary Clinical Pathology (ASVCP) guidelines, using the Reference Value Advisor 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 found that indicated an abnormality according to these proposed reference intervals, all blood values for that bird were deleted from the dataset. In the oral dosing study, there were six birds in each of the three treatment groups that were considered to be representative of a healthy population at the beginning of the study, based on hematology and clinical chemistry endpoints. In the external dosing study, 25 birds were randomly assigned to the treatment or control groups (12 birds and 13 birds respectively).

2.5. Oil source

The oil used in both the oral dosing and external dosing studies was artificially weathered MC252 oil (DWH7937, batch# B030112) prepared from crude oil collected during the DWH oil spill (Forth et al., 2016).

2.6. Dosing

2.6.1. Oral

DCCOs were randomly assigned to one of three treatment groups: a control group (n=8) that was fed catfish that had been lightly anesthetized and allowed to revive; a group dosed daily with up to 5 mL oil/kg bw/day through provision of oil-containing catfish as described below (n=9); a group dosed daily with up to 10 mL oil/kg bw/day through provision of oil-containing catfish as described below (n=9).

Fingerling catfish (approximately 7.5–10 cm) were lightly anesthetized using MS222 (tricaine methanesulfonate), given in an intraperitoneal (IP) injection of 2.0 mL of oil using a 20-gauge needle on a 25-mL stainless steel/glass barrel syringe. Each catfish was injected with the same volume of oil to ensure that oil consumption per bird could be calculated easily based on the number of catfish that were consumed. Injected catfish were placed into a separate holding tank to ensure revival from anesthesia and retention of oil.

Oil-injected catfish were fed to cormorants in their water-filled foraging tanks at a rate that offered DCCOs either 5 or 10 mL oil/kg bw. Oil-injected catfish were observed to survive for more than 24 h if not consumed by birds while foraging. The method by which DCCOs consume catfish usually involves capture and handling of the catfish by hooking the end of the bill into the catfish gills. This often results in death of the catfish due to damage to the gills. If the handled catfish was dropped by the bird, it usually died and was not consumed. Thus, cages were checked once or twice each afternoon to replace any uneaten dead catfish with an equal number of oil-injected live catfish. This method provided the greatest opportunity for DCCOs to have live catfish available to them and encourage maximum consumption of oil-injected catfish.

Once a cormorant had consumed its allotment of oil-injected catfish, a subsequent feeding of non-oil injected catfish was offered to each bird to achieve a possible daily intake of greater than 600 g catfish per cormorant. If birds did not consume the total quantity of injected catfish, they were not offered additional non-oiled injected catfish. If an individual bird was observed to eat all of the food provided on a given day, its food ration was increased the next day to ensure that ingestion volume was not restricted up to the 600 g whole catfish maximum. The amount of catfish consumed daily by each bird was recorded.

In addition to internal dosing with oil, there were instances of inadvertent external oiling. Although all tanks were cleaned daily with 100% water replacement, oil was present in the tanks of all oil-treated birds due to defecation by the birds during normal daily activities. No oil was purposefully placed on the integument of the DCCOs during this study. The inadvertent presence of oil in the water and the subsequent external oiling of the birds is somewhat representative of the multiple exposure routes expected to occur in the field. Data related to the degree of external oiling (light or moderate) of each bird were recorded.

2.6.2. External

In order to estimate the body surface area prior to external dosing, DCCO carcasses (n=7) that ranged in weight from 1.6 to 2.4 kg were skinned by making a ventral cut from head to vent. A cut was also made on the ventral side of each leg to allow for complete flattening of the feathered surface of the legs. Wings remained attached to the skins and severed from the body at the midpoint of the humerus. Skins were spread out on a flat surface and photographed with a Nikon D200 digital camera. Images were imported into Image J (National Institutes



Fig. 1. Determination of the mean surface area of DCCO carcasses used as the basis for estimating the area of bird requiring oil application.

of Health). Total surface area was calculated based on the entire feathered surface of the body. All non-feathered areas of the carcass were excluded (face, beak, bare leg, and feet). Wings remained folded in a relaxed position to duplicate the posture of oiled birds observed in the field. The surface area of the body excluded the tail due to high variation in tail feathers among individuals, and no clear pattern in molting of retrices in DCCOs (Dorr et al., 2014). The mean surface area of DCCO carcasses was $1403.1 \pm 86.5 \text{ cm}^2$ (n=7), and was used as the basis for estimating the area of bird that required oil application (Fig. 1).

Prior to initiation of the external dosing study, oil was applied to DCCO carcasses to determine the most efficient method for application of oil to their feathers. Coverage of 20% was targeted, which was equivalent to 280 cm^2 of the surface area of a DCCO while in the resting or loafing position. This oiling rate (20%) is the high limit of the light-oiling category (6–20%) used by the U.S. Fish and Wildlife Service for the DWH NRDA (Deepwater Horizon Natural Resource Damage Assessment Trustees, 2016). To achieve this coverage, oil was applied to the breast (140 cm^2) and back (140 cm^2) feathers. Plastic stencils, which measured $8 \times 17.5 \text{ cm}$ and $7 \times 20 \text{ cm}$ for the breast and back, respectively, were used as a guide for application of oil. The total weight of oil applied to each DCCO during each application totaled 13 g, approximately 6.5 g to the breast and 6.5 g to the back (approximately 13 mL).

A total of 25 DCCOs allocated to two groups were used in this trial. DCCOs were assigned to either control or 20% coverage oiling groups based on blood samples collected at the initiation of the three-week quarantine period. Complete blood count values were used to ensure equal division of birds with potential health concerns between groups. Monocyte counts greater than 2.0×10^9 cells/L were considered abnormal (severe monocytosis). Additionally, a small oil spill took place on November 8, 2013 not far from where some of the DCCOs were collected, which could potentially have affected some plasma values associated with oxidative stress, or liver and kidney function. These birds were also evenly distributed between groups.

The initial sample size of the control (no oil applied) and treated groups were 12 and 13 individuals, respectively. 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 both the control and treated groups was 11. The amount of oil applied to each bird on the days of application was determined by subtracting the weight of the beaker, oil and brush after application of oil from the initial weight. The control group had 6.5 g of water applied to the breast and another 6.5 g applied to the back to ensure similar treatment and handling as oiled birds. Oil or water was applied every three days through day 15 of the trial and cumulative oil ingested was calculated based on a study by Hartung (1963).

2.7. Blood sample collection

In the oral dosing study a blood sample was taken from each bird on day 0 (i.e., the day before oil dosing began) as a baseline comparison, and then once weekly until the study ended or the bird died or was

euthanized. In the external dosing study, all birds had a blood sample taken during quarantine to provide baseline data. Once dosing began, blood was collected every six days (on days 0, 6, 12 and 18), prior to external application of oil. At the end of the 21-day trial, birds were sampled for blood before euthanasia and necropsy.

To collect the sample, the brachial vein was punctured with a 25-gauge, 19-mm butterfly needle attached to a 300-mm tube (600- μ L volume). On day 0 only, the needle and tube were flushed with a 100-international unit (IU) sodium heparin solution before use; thereafter, the needle and tube were flushed with a 100-IU lithium heparin solution. Approximately 3–4 mL of blood were collected and transferred to labeled lithium heparin Vacutainer™ tubes and kept on ice for subsequent processing. In the external dosing study, serum samples (1 mL) were collected from the brachial vein first using syringes and tubes containing no anticoagulant. Following the 1 mL collection, heparinized syringes were used to collect approximately 10 mL of blood from the jugular vein that was divided for hematology and biochemical analyses.

Each blood sample collected during the oral and external dosing studies was aliquoted for the following assessments: Heinz bodies, CBCs, electron microscopy, PCV, hemoglobin concentration (Harr et al., 2017a, this issue), total antioxidant capacity (Pritsos et al., 2017, this issue), and plasma clinical chemistries (Dean et al., 2017b, this issue). In the external dosing study, a whole blood sample was collected using no anticoagulant to determine activated clotting time. A glass tube containing 3 mg of diatomaceous earth was incubated at 37 °C prior to the addition of blood. A timer was started when 0.5 mL of whole blood was transferred to the tube, which was then inverted to several times to mix the diatomaceous earth with the whole blood. The tube was incubated for 30 s at 37 °C and visually examined for microclot formation by inverting the tube. This last step was repeated until clots formed. The time that elapsed from introduction of blood into the tube until evidence of the first clot was activated clotting time.

2.8. Necropsy

Depressed birds were euthanized, following multiple consultations with the onsite veterinarian. A depressed bird was one that tucked its head under its wings, was lethargic and unresponsive, experienced weight loss, and had a cloacal temperature of 39.4 °C or less.

After birds were euthanized, they were necropsied as soon as possible to ensure fresh tissues adequate for analysis. The remaining birds were necropsied on the last day of the respective studies. Birds were weighed and euthanized by cervical dislocation according to IACUC-approved protocols. Necropsy blood samples were obtained by direct cardiac puncture with a non-heparinized needle (20-gauge, 25.4-mm) and 10-mL syringe for serum samples, followed by subsequent heparinized (100-IU lithium heparin) needles (20-gauge, 25.4-mm) and 10-mL syringes for whole blood and plasma samples. An attempt was made to collect 30 mL of blood from each bird for a larger suite of endpoints than those evaluated at day 0.

All organs were assessed grossly for abnormalities and documented by digital images. The brain, heart, lungs, kidneys, thyroid gland, liver, gastrointestinal (GI) tract, spleen, and adrenal glands were collected and weighed to the nearest mg. In addition, if gonads, thymus, and bursa were present, they were collected and weighed.

Organs were placed in an appropriately labeled specimen jar containing 10% neutral buffered formalin; one adrenal and one thyroid gland were each placed in individual micro-centrifuge tubes, also containing 10% neutral buffered formalin, for subsequent histopathological assessment (Harr et al., 2017b, this issue). Two samples each of the liver, kidney and GI tract, and the remaining thyroid and adrenal glands were flash-frozen in liquid nitrogen for subsequent analysis of cytochrome P450 activity (Alexander, submitted for publication et al., this issue) and oxidative damage assessment (Pritsos et al., 2017, this issue).

2.9. Additional external dosing study methods

All birds in the external dosing study had an Advanced Telemetry Systems (ATS, Isanti, MN USA) F1815T implantable very high frequency (VHF) temperature transmitter surgically implanted in the coelom prior to study initiation for daily determination of internal body temperature. External body temperature was taken just prior to initial oiling and every six days thereafter (on days 3, 9, 15 and 20) with a handheld scanning thermograph camera (FLIR ThermoCAM T640; FLIR Systems, Boston, MA, USA) (Mathewson, submitted for publication et al.). Echocardiograms were used to evaluate DCCO cardiac structure and function (Harr et al., 2017c, this issue).

2.10. Statistical methods

The following statistical methods were used in the present studies in addition to other studies in this issue. Oxidative stress endpoints, 3-methyl histidine, and organ weights were compared by one-way analyses of variance (ANOVAs). Statistical significance was assessed based on *p*-values determined for each distinct ANOVA model (i.e., there was no attempt to control for experiment-wise Type I error). Hematologic and plasma clinical chemistry values, FLIR data, body weight data, and body temperature data collected across multiple time points were compared using linear mixed effects regression models with a repeated measures structure, where treatment was modeled as a fixed effect and the individual bird within elapsed days was modeled as a random effect. Regression models included effects for elapsed days, treatment, and a treatment*days interaction term. For the oral dosing study, elapsed days and treatment (oil dose level as mL/kg bw/day) 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. Graphical inspection of data distributions using boxplots and scatterplots overlaid with fitted regression models indicated that data for endpoints analyzed using ANOVA or regression were generally symmetric about their means and did not span more than an order of magnitude; thus, transformations were deemed to be unnecessary. Differences among treatment groups on day 0 were evaluated using Kruskal-Wallis test.

Box plots were used to provide a quick visual summary of distributions for endpoints not analyzed over time and for endpoints for which the regression model was not appropriate, illustrating the range, shape, and extremes of the distributions. Side-by-side box plots allow for a visual comparison of these characteristics across treatment groups. Calculations were performed using TIBCO Spotfire S-PLUS 8.2 for Windows.

3. Results

Results associated with endpoints assessed in the oral and external dosing studies with DCCOs that are not covered in the present report include oil-induced increases in CYP1A protein expression and catalytic activity (Alexander, submitted for publication et al., this issue), changes in CBC estimates and plasma chemistry and electrophoresis endpoints (Dean et al., 2017b, this issue), hemolytic anemia as indicated by decreased PCV, relative reticulocytosis with an inadequate regenerative response, and presence of Heinz bodies (Harr et al., 2017a, this issue), and increases in liver and kidney weights and the presence of lesions in kidney, liver, heart and thyroid gland (Harr et al., 2017b, this issue). Results related to a decrease in cardiac systolic function and internal body temperature and heat loss in externally dosed DCCOs are presented in Harr et al. (2017c) and Mathewson et al. (2017), respectively. Results related to changes in oxidative stress endpoints in orally dosed DCCOs are presented in (Pritsos et al., 2017, this issue).

Table 1
Effect of daily oral dosing with artificially weathered MC252 oil on food intake and oil ingestion by double-crested cormorants (*Phalacrocorax auritus*).

	Control		5 mL/kg bw		10 mL/kg bw	
	Mean	SE	Mean	SE	Mean	SE
Days 1–7						
n	6		8		6	
Oil ingestion	0	0	5.3	0.1	8.8	0.4
Food intake	361.8	38.9	290.9	26.7	267.2	17.8
Days 8–14						
n	6		7		5	
Oil ingestion	0	0	5.1	0.1	8.5	0.3
Food intake	574.1	13.0	398.4	21.1	211.6	18.4
Days 15–20						
n	6		7		.	
Oil ingestion	0	0	5.2	0.2	.	.
Food intake	545.1	30.8	431.0	37.1	.	.
All days^a						
n	6		7		5	
Oil ingestion	0	0	5.2	0.3	8.4	0.9
Food intake	484.9	58.5	367.7	58.5	247.5	33.0

^a Days 1 – 11 for birds in 10 mg/kg bw group.

3.1. Oral

3.1.1. Oil intake, food consumption and body weight

Mean oil consumption by treatment is presented in Table 1. The 5 mL/kg bw treatment group averaged 5.2 mL during the course of the study and the 10 mL/kg bw group never reached the target dose and averaged 8.4 mL/kg bw due to reduced intake of oil-injected catfish.

The control birds increased food intake until day 8 when consumption peaked at an average of 621 g of catfish consumed per day and then ranged from 518 to 606 g of catfish consumed per day for the rest of the study. The 5 mL/kg bw group increased intake until day 9 when consumption peaked at 558 g of catfish consumed per day and then decreased reaching the lowest average consumption, 233 g of catfish, by day 12. Consumption rebounded to 601 g of catfish by day 21 to approach control levels of intake. The 10 mL/kg bw group had a reduced intake from day 1 and peaked on day 9 at 330 g of catfish per day. This treatment group was terminated on day 14 due to mortality and health of the remaining DCCOs in the group.

All dosed and control groups experienced a similar decrease in body weight over the 21-day trial. Body weight at necropsy was not significantly different among treatment groups (1801.2 ± 63.5 g, 1680.8 ± 33.5 g, 1565.5 ± 75.7 g for the control, 5 mL/kg bw and 10 mL/kg bw treatment groups, respectively).

3.1.2. Clinical signs and mortality

Of the 26 adult, mixed-sex DCCO used in this 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. Prior to day 21, double crested cormorants were euthanized based on veterinary assessment of severe distress, or when the animals were moribund, to ensure that necropsies could be performed on fresh carcasses and that a complete suite of endpoints could be sampled. All birds in the 10 mL oil/kg bw group died or required euthanasia before the end of the study. One DCCO in the 5 mL oil/kg bw group and 0 control birds died before the end of the study.

Clinical signs of toxicity in birds orally dosed with oil included reduced cloacal temperature, lower body weight, lethargy, feather damage, morbidity, and death. These signs were first observed on day 5 in birds dosed with 10 mL oil/kg bw and on day 7 in birds dosed with 5 mL oil/kg bw (Table 2). There was a reduced ability of oiled birds to control their cloacal temperatures. The cloacal temperatures of the control group were consistent during the study, ranging from 41.2 to

43.0 C. Cloacal temperatures of the 5 mL oil/kg bw group ranged from 39.5 to 41.7. The 10 mL oil/kg bw group's cloacal temperatures ranged from 40.0 to 41.2 on day 7 of the study to 38.3–39.7 C just prior to their death or euthanasia. Abnormal excreta containing blood (hematochezia) was first observed in the 10 mL oil/kg bw birds on day 8 and in the 5 mL oil/kg bw birds on day 13. During blood collections, it was noted that in oil-dosed DCCOs the time required for blood to clot was longer compared to controls. Upon necropsy of birds in the 5 mL oil/kg bw group, the remaining blood was pooling and not forming clots, whereas clot formation was observed in all control birds.

3.2. External

3.2.1. Oil intake, food consumption and body weight

Hartung and Hunt (1966) estimated that a duck with 7 g of oil on its feathers would ingest approximately 1.5 g of oil on the first day of oil exposure. Based on previous studies (Hartung, 1963), it was estimated that DCCOs would ingest 21% of the oil applied to their feathers within the first day and 50% by day 8. In the present study, based on external application of 13 g oil to DCCO every three days over 15 days, a cumulative ingestion of approximately 38 g of oil was estimated (Table 3).

Food consumption had a significant elapsed day ($p=0.006$) and treatment*day interaction ($p < 0.001$) (Fig. 2). Birds treated with oil consumed more fish than control birds during the trial period beginning on day 7 of the trial. Average food consumption over the 21-day trial for the control group was 403 g compared to 421 g in the oiled group. From day 1 through day 6 of the trial, mean daily food consumption was 410 g for the control group and 337 for the oiled group. From day 7 through day 20 of the trial, daily food consumption averaged 399 g for the control group and 464 g for the oiled birds.

There was a significant treatment group effect ($p=0.02$), elapsed day effect ($p < 0.001$), and treatment*day ($p=0.04$) interaction with respect to changes in DCCO body weights during the trial period. Body weights increased over the trial period in both oil treated birds and control birds; however, birds treated with oil gained weight at a faster rate than control birds (Fig. 3).

3.2.2. Clinical signs and mortality

Three DCCOs died after the study was initiated. A control bird died on day 0 immediately after being sampled for blood. A dosed bird was found dead on day 14 and a second dosed bird was found dead on day 18, both from unknown causes that may or may not have been related to oil exposure. Upon necropsy of the treated bird that died on day 14, there were fish in the esophagus and stomach, indicating that the bird had eaten recently. The heart appeared slightly flaccid after removal. Unclotted blood was present in the body cavity. Bile collected from the gall bladder was hemolytic. Packed cell volume determined from a blood sample collected from the heart ranged from 26% to 28%. Upon necropsy of the bird that died on day 18, there were petechial hemorrhages on the heart and pancreas and there were hemorrhages in the omentum in the region of the small intestine. There were multiple beige focal lesions located on the surface of the liver and the bile was very dark in color. The kidney appeared mottled. There was frothy fluid on the surface of the right lung and there were petechial hemorrhages on the cerebral hemispheres, cerebellum and brain stem.

A number of clinical signs were observed in birds externally dosed with oil including deterioration of feather integrity, abnormal feces, excessive preening, feather plucking, and lethargy (Table 3). Feathers of all oiled birds appeared matted and rough by day 3. As the study progressed, two DCCOs first began to pluck feathers on day 14 of the study and by day 16 all oiled birds were engaged in this activity. The skin on the breast and back of some oiled birds was noticeably discolored by day 6 and by day 9, oil covered much of the surface area of all birds and the integrity of the feathers was deteriorating. Plucked feathers were largely downy material, but also included some contour

Table 2

Average and cumulative oil consumed and the progression of clinical signs and mortality in double-crested cormorants orally exposed to oil.

Day	5 mL oil/kg bw		10 mL oil/kg bw		Dose	Event	Total oil at death		
	n	Daily oil intake	Cumulative oil intake	n				Daily oil intake	Cumulative oil intake
1	9	8.8	8.8	9	13.4	13.4	Study initiation: first day of oil exposure		
2	9	8.9	17.7	9	15.3	28.7			
3	9	8.7	26.4	9	13.3	42.0			
4	9	10.0	36.4	9	13.3	55.3			
5	9	9.8	46.2	9	14.5	69.8	10	1 died	40 mL
6	9	10.0	56.2	8	16.3	86.1	5, 10	Lethargic, reduced body temp; 1 5 mL/kg bw died	120 mL
7	8	10.0	66.2	8	14.0	100.1			
8	8	8.0	74.2	8	13.8	113.9	10	Lethargic, abnormal excreta, reduced body temp	120 mL
9	8	8.3	82.5	8	15.3	129.2			
10	8	8.3	90.8	7	15.7	144.9	10	Lethargic, abnormal excreta	120 mL
11	8	8.3	99.1	7	14.0	158.9	10	Lethargic; 1 died	
12	8	8.3	107.4	5	14.4	173.3	5, 10	Lethargic, abnormal excreta, reduced body temp; 2–10 mL oil/kg bw euthanized	139 mL
13	8	8.3	115.7	2	10.0	183.3	5, 10	Lethargic, reduced body temp	207 mL
14	8	8.3	124.0	2	10.0	193.3	5, 10	Lethargic, abnormal excreta, reduced body temp; 1–10 mL oil/kg bw euthanized	182 mL
15	8	8.3	132.3				10	Lethargic, reduced body temp; 2–10 mL oil/kg bw euthanized	223 mL
16	8	8.0	140.3				5, 10	Abnormal excreta	
17	8	8.3	148.6				5, 10	Abnormal excreta	
18	8	8.3	156.9				5, 10	Abnormal excreta	
19	8	8.3	165.2					Control DCCO abnormal excreta	
20	8	8.3	173.5						
21	8	8.3	181.8					Necropsy remaining birds	

feathers from the breast and back. Skin in those areas was observed to have become thickened, discolored and irritated. Abnormal excreta was 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. Microscopic examination of opportunistic fecal samples revealed large numbers of RBCs, documenting GI hemorrhage. Only one control bird had abnormal excreta that consisted of green diarrhea with no evidence of gelatinous protein or blood as noted in the oiled birds. Six of the 11 birds that survived to necropsy were described as being lethargic within four days of necropsy.

3.2.2.1. Blood coagulation. It was observed during the oral dosing study that blood in oil-dosed birds clotted very slowly or did not clot during necropsy. The decision was made to examine this apparent clotting dysfunction at necropsy of externally oiled birds by determination of activated clotting time and time required for untreated blood to clot. Clotting dysfunction was evidenced by a significant increase ($p < 0.001$) in activated clotting time in oiled birds (359 ± 90.5 s) compared to controls (172 ± 21.5 s) at necropsy. Blood of control birds clotted within three to four minutes compared to no clotting after eight minutes for several treated birds.

4. Discussion

Modifications in the oral dosing technique for DCCOs described here resulted in development of clinical signs and changes in a number of hematological, biochemical, and tissue endpoints consistent with petroleum intoxication that are presented and discussed in other reports comprising this special issue (Harr et al., 2017a, 2017b, Pritsos et al., 2017, Alexander, submitted for publication et al., Dean et al., 2017b). The hemolytic anemia (Harr et al., 2017a, this issue) and changes indicative of liver and kidney damage (Harr et al., 2017b, this issue) observed in the oral study have been reported in other studies assessing the effects of oil exposure on avian species. However, these results are specific for the source of oil, the species, and age class of the birds, the dosing methodology, and the husbandry employed, and should be used with caution. New findings from the oral dosing study included

cardiovascular abnormalities documented upon gross necropsy that prompted further diagnostic evaluation in the external dosing study (Harr et al., 2017c). Evidence of coagulopathy found on gross necropsy has not been reported before in oil-dosed birds and has been minimally investigated using MC252 oil in any species.

The methods developed in the external dosing study of DCCO with artificially weathered MC252 oil, also resulted in clinical signs and changes in a number of endpoints consistent with petroleum intoxication. Hemolytic anemia and clotting dysfunction (Harr et al., 2017a, this issue), cardiac abnormalities (Harr et al., 2017c, this issue), and changes indicative of liver and kidney damage (Harr et al., 2017b, this issue) were similar to those observed in the DCCO oral dosing study, indicating that the method of oil application to DCCO feathers is an appropriate means of assessing oil toxicity in this species.

4.1. Oil intake, food consumption and body weight

The mean daily intake of oil by orally dosed DCCOs over the course of the present 21-day study was a reflection of food consumption in each group. Those birds in the 5 mL oil/kg bw group consumed an average of 5.2 mL oil/kg bw per day, which was slightly more than the targeted dose. Birds in the 10 mL oil/kg bw group consumed an average of 8.5 mL oil/kg bw per day, which was 15% below the targeted dose and is a reflection of the 19% decrease in food consumption compared to the quarantine period. The total quantity of oil ingested by the nine DCCOs in the 10 mL/kg bw group that died during the course of the trial ranged from 40 (day 5) to 223 mL (day 15).

In the external dosing study, a cumulative ingestion of 38 g of oil through preening was estimated based on Hartung (1963). This estimate is close to the minimum quantity of oil associated with mortality in the orally dosed DCCOs.

Birds orally dosed with oil consumed less food than control birds, whereas food consumption of externally oiled birds was greater compared to controls. Mean food consumption during the oral dosing study for the 5 mL oil/kg BW group was 76% of control consumption; food consumption for the 10 mL oil/kg BW group was 51% of control consumption. Mean daily food consumption for all three groups for the

Table 3
Cumulative oil applied, estimated oil consumed and the progression of clinical signs and mortality in double-crested cormorants exposed to external oiling.

Study Day	Cumulative oil applied (mL)	Estimated cumulative oil ingested (mL) ^A	Treatment		Event
			Control n birds affected	Oiled n birds affected	
0	13	0	1	0	Study initiation: first day of exposure to oil; one control bird died prior to water application
1		3	0	13	Visible oil on feathers
2		3	0	13	Visible oil on feathers
3	26	4	0	13	Feathers matted and rough
4		7	0	13	Feathers matted and rough
5		8	0	13	Feathers matted and rough
6	39	9	0	13	Feathers matted and rough, feather damage observed, skin noticeably discolored
7		13	0	13	Feathers matted and rough, skin noticeably discolored
8		15	0	13	Feathers matted and rough, skin noticeably discolored
9	52	16	0	13	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating
10		20	0	13	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating
11		21	0	13	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating
12	65	22	0	13	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating (4 birds with abnormal excreta containing RBCs)
13		26	0	13	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating (6 birds with abnormal excreta containing RBCs)
14		28	0	13	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating (2 birds - feather plucking observed in pens, 7 birds with abnormal excreta containing RBCs)
15	78	29	0	12	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating (10 birds - feather plucking, 1 oil treated bird mortality)
16		33	0	12	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating, feather plucking
17		34	0	12	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating, feather plucking
18		35	0	12	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating, feather plucking (4 birds lethargic)
19		36	0	11	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating, feather plucking (1 oil treated bird mortality)
20		37	0	11	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating, feather plucking
21		38	0	11	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating, feather plucking (7 birds with abnormal excreta containing RBCs); necropsy ^B
22		38	0	11	Feathers matted and rough, skin noticeably discolored, oil covering much of surface area of birds, feather integrity deteriorating, feather plucking; necropsy ^C

^A Calculation based on Hartung (1963), % ingestion of oil = 1.0409x + 0.1731.

^B Eleven DCCO necropsied on day 21.

^C Eleven DCCO necropsied on day 22.

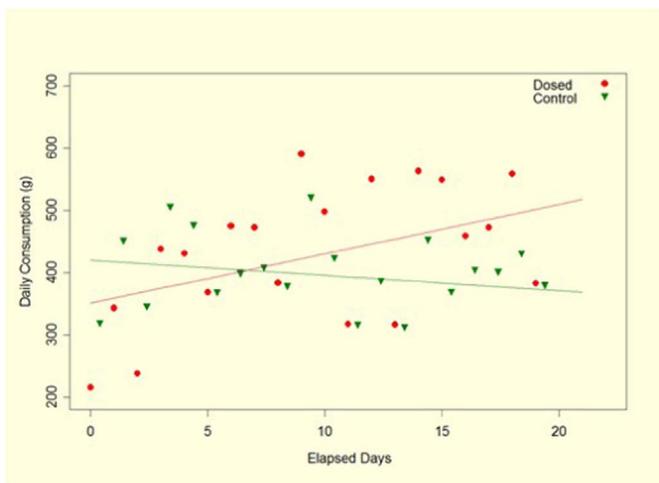


Fig. 2. Treated (dosed) and control group DCCOs daily feeding (g).

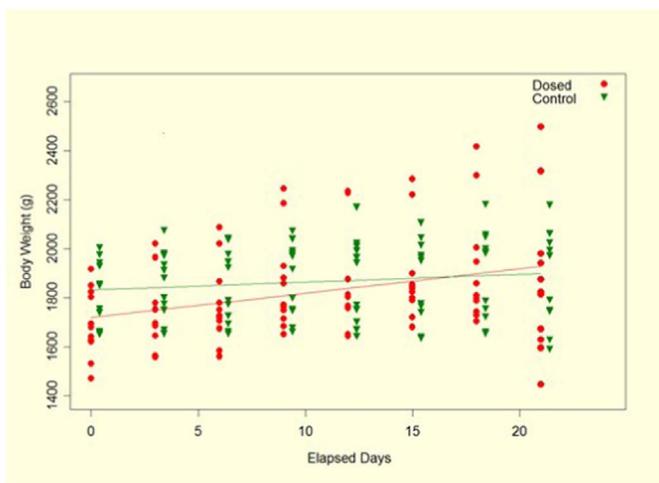


Fig. 3. Treated (dosed) and control group DCCOs changes in body weights over time during the 21-day treatment period.

first five days of the trial (control = 345 g/day, 5 mL oil/kg bw = 284 g/day, 10 mL oil/kg bw = 279 g/day) was similar to the mean consumption reported for the quarantine period (298 g/day). After day 5, mean daily consumption of the 10 mL oil/kg bw birds for the remainder of the trial (225 g/day) decreased by 19%, while average daily food consumption for the control and 5 mL oil/kg bw bird groups (559 g/day and 403 g/day, respectively) increased by 62% and 42%, respectively. Using the mean body weight of DCCOs at the beginning of the oral dosing study (1728 g for the control; 1677 g for the 5 mL oil/kg bw; and 1657 g for the 10 mL oil/kg bw groups), and assuming that food consumption is 21–24% of bw (Glahn and Brugger, 1995), the expected range of food consumed on a daily basis for the control, 5 mL oil/kg bw, and 10 mL oil/kg bw groups was 363–415 g, 352–402 g, and 348–398 g, respectively. The actual percent mean food consumption over the specified time period for the control, 5 mL oil/kg bw, and 10 mL oil/kg bw groups was 35–54% greater, 0–14% greater, and 35–43% less than the predicted food consumption, respectively. Similar to results reported here, herring gull (*Larus argentatus*) nestlings orally dosed with 10 mL of Prudhoe Bay crude oil/kg bw/day or more consumed less food beginning on the third day of dosing (Leighton, 1986). It is probable that food aversion and gastrointestinal distress induced by multi-organ system dysfunction were contributory mechanisms to the decreased food consumption in birds orally dosed with oil in the present study.

Externally oiled birds consumed more food compared to controls after the second application of oil. From day 7 through day 20 of the

trial, oiled birds consumed 38% more food compared to average intake over the first six days while control birds consumed 3% less food. For externally oiled birds, the expected range of food consumed on a daily basis was 356–407 g based on initial mean body weight of 1697 g and for controls the range was 382–437 g based on initial mean body weight of 1821 g. Over the 21-day trial, control food consumption was within the expected range and oiled bird food consumption was 3–18% greater than the predicted range of food consumed. From day 1 through day 6 of the trial, mean daily food consumption of control birds was within the expected range of food consumed and 5–17% less for the oiled group. From day 7 through day 20 of the trial, daily food consumption continued to be within the expected range of food consumed for the control group but was 14–30% greater than the predicted range for the oiled birds. The greater food consumption in externally dosed birds suggests metabolic compensation for thermal stress (Mathewson, submitted for publication et al.,). Infrared thermal images indicated significantly greater surface temperatures and heat loss in oiled birds following external oil applications, but measurements collected by coelomically implanted temperature transmitters showed that internal body temperatures were stable over the course of the study period (Mathewson, submitted for publication et al.,).

In the oral exposure study, mean body weight of control and 5 mL oil/kg bw birds at day 21 was 0.6% and 9.2% less, respectively, compared to mean body weight at day 0 and mean body weight of the 10 mL oil/kg bw birds at day 14 was 11.4% less compared to day 0 mean body weight. Although body weights were not significantly different among treatments at necropsy, the loss of body weight over time in the birds dosed with oil was a reflection of the decrease in food consumption. Herring gull (*Larus argentatus*) nestlings orally dosed with 10 mL of Prudhoe Bay crude oil/kg bw/day or more consumed less food beginning on the third day of dosing compared to controls and began to lose weight on the fifth day of dosing (Leighton, 1986).

Mean body weight of externally dosed birds increased by 10.3% over the 21-day trial (1697 g vs 1872 g) while mean body weight of control birds increased 3.7% (1821 g vs 1889 g). The increase in body weight of oiled birds is a reflection of the increase in food consumption, which, as stated above, is thought to be metabolic compensation for thermal stress experienced by these birds (Mathewson, submitted for publication et al.,).

4.2. Clinical signs and mortality

Necropsy revealed underlying disease in dosed and control birds in both studies, including bacterial infection, which was not cultured or further elucidated. Disease may have contributed to some observed clinical signs. However, because disease was present in the control population, the only variable to explain the differences in mortality and morbidity between oiled and control birds is oil intoxication.

In the oral dosing study, all of the 10 mL oil/kg bw birds died or were euthanized before the end of the 21-day study, while one bird in the 5 mL oil/kg bw group died. In the external dosing study, two oiled birds died prior to the end of the study. Orally dosed birds that died or were euthanized before the end of the study displayed clinical signs that included lethargy, reduced food consumption, hypothermia, loss of body weight, and abnormal excreta. Behaviorally, birds in the 10 mL oil/kg bw group held their heads under their wings, regardless of stimulation, while control birds were bright, alert, and responsive. In the external dosing study, oiled birds also were lethargic and had abnormal excreta, but they did not experience hypothermia, reduced food consumption or loss of body weight. Rather, the clinical signs displayed by externally oiled birds were primarily related to feather and skin integrity, as would be expected. The difference in mortality and apparent severity of clinical signs between the studies could be a reflection of the amount of oil ingested during the course of the study. It was calculated that externally oiled birds ingested less oil during the 21-day trial (38 g), when compared to orally dosed birds that consumed

between 40 and 223 g of oil prior to death or necropsy.

Mortality, weight loss, and some of the clinical signs reported here have been reported in other oral dosing studies. Pekin ducks fed diets that provided approximately 2.9 mL of South Louisiana crude oil or 1.3 mL of No. 2 fuel oil/kg bw/day experienced 22% and 36% mortality, respectively, at the end of 50 days at an ambient temperature of 27 °C; and 67% and 43% mortality, respectively, at the end of a second 50-day period at an ambient temperature of 3 °C (Holmes et al., 1978). Ducks fed a diet that provided 2.5 mL of Kuwait crude oil/kg bw per day had no mortality at 27 °C but 67% mortality at 3 °C (Holmes et al., 1978). Herring gull chicks administered daily oral doses of 10 or 20 mL Prudhoe Bay crude oil/kg bw were lethargic on and after day 4 and began to lose weight on day 5 (Miller et al., 1978). Two gull chicks in the 20 mL oil/kg bw per day group were moribund on day 5 and subsequently euthanized (Leighton, 1986). In the same study, Atlantic puffin (*Fratercula arctica*) nestlings that were administered daily oral doses of 10 mL oil/kg bw were weak by day 4 and two puffins died by day 5. Two of 16 American kestrels administered feed containing 3.0% oil from the Mexican Ixtoc I well blowout died after two weeks on treatment when there was a 24 °C drop in ambient temperature (Pattee and Franson, 1982). Both birds had lost weight (21% and 31%) at the time they died; death was attributed to weight loss followed by cold stress (Pattee and Franson, 1982). Because ambient temperatures in the present studies were cooler than those recorded in the Gulf of Mexico at the time of the oil spill, and orally dosed birds had depressed body temperatures, heat lamps were added to all cages to supply additional heat. Regardless of heat lamp placement, significant and apparent dose-dependent mortality occurred. As the temperature and heat lamp placement were identical in cages of both control and orally dosed birds, any significant changes in endpoints were because of oil exposure. Hypothermia and thermal stress were considered to be a significant component of mortality in the present oral dosing study.

In the oral dosing study, all oil-dosed birds had oil on the plumage as a result of foraging for fish in the feed tanks that contained oil excreted by the birds. External oiling can result in physical alteration of the feathers, causing matting and loss of insulation and water-repellent properties. The loss of insulation and water-repellency can lead to death of oiled birds as a result of heat loss and drowning (Leighton, 1993). In the external dosing study, disruption of feather and skin integrity was the most pronounced clinical sign. The oiled birds engaged in feather plucking, which was not unexpected because it has been reported in other birds following oil spills (Snyder et al., 1973). Feather plucking will naturally result in greater total heat loss from the birds than expected.

The exposure methods used in the oral and external dosing studies resulted in similar signs of oil toxicity and can serve as appropriate means of assessing oil toxicity in DCCOs. Birds orally dosed with oil were more severely affected based on the timeline, type and magnitude of clinical signs and mortality. In both studies, birds consumed oil either as a result of eating oil-contaminated food (oral) or preening (external). Additionally, birds in both studies experienced feather damage as a result of intentional or unintentional external oiling. Orally dosed birds had difficulty maintaining internal body temperature as compared to externally dosed birds that were losing heat but maintaining core temperature. It is possible that the greater mortality in orally dosed birds was because of hypothermia, which in turn can be attributed to a combination of ingestion of 40–233 mL oil that induced toxicity and unintentional external oiling that exacerbated heat loss. While externally dosed birds also ingested oil, the estimated amount (38 mL) was at the lower end of the range associated with mortality.

Acknowledgements

Appreciation is expressed to the technicians of the National Wildlife Service's Mississippi Field Station, Paul Fioranelli, Alex Crain, Lanna Durst and Raleigh Middleton, who participated in DCCO capture and

helped carry out each study. Appreciation is also expressed to Dr. Tacy Rupp of Veterinary Cardiopulmonary Care Center in West Palm Beach, Florida for developing a method for species-specific DCCO echocardiography. Funding for these studies was provided by the US Fish and Wildlife Service, as part of the Deepwater Horizon Natural Resource Damage Assessment.

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