Diagnostic techniques for detecting exposure and anemia in birds exposed to crude oil

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ABSTRACT

Oil spills have long been recognized as a significant threat to wildlife. Historically, mortality estimates have served as the basis for assessing impact to natural resources. However, these mortality estimates alone neglect the more wide-spread impact of oil spills on wildlife including birds, many of which may not immediately succumb to exposure, but instead suffer sublethal injury that may negatively affect physiological homeostasis, reproduction, and long-term survival. Therefore, there is a need to improve our understanding of the risk of exposure and effect of sublethal oiling during damage assessments. In this dissertation I evaluated the extent of sublethal oil exposure in the immediate aftermath of the Deepwater Horizon spill on American oystercatchers (Haematopus palliatus), black skimmers (Rynchops niger), brown pelicans (Pelecanus occidentalis), clapper rails (Rallus crepitans), and seaside sparrows (Ammodramus maritimus) through both visual evaluation of and under the application of ultraviolet light to individual birds potentially exposed to oil. I found that there were many individual birds with modest oil exposure, demonstrating that more birds are exposed to oil than are accounted for by mortality estimates. Additionally, I developed a field-adapted technique using an in vitro method in brown pelicans that was effective in determining oxidative hematologic injury as measured by a suite of parameters including a reduction in circulating erythrocytes and hemoglobin, formation of Heinz bodies, and an increase in reticulocytes, in birds exposed to oil. I then applied this suite of parameters to individual birds affected in the aftermath of the Deepwater Horizon spill, and found that birds with modest visible or UV-detectible oil exposure suffer hematologic injury, a quantifiable adverse sublethal effect of modest oil exposure. Finally, I used an experimental approach to evaluate the pathologic effects of crude oil exposure in zebra finches (Taeniopygia guttata), evaluating the same suite of hematologic parameters as well as gross pathology, histopathology, and electron microscopy. This controlled study provided evidence that there may be significant
variability in the response of birds to oil exposure that may be attributable to species-specific sensitivity and/or other factors such as the use of dispersants after oil spills. Collectively, this body of work demonstrated that many more birds are exposed to oil during spill events than are accounted for by mortality estimates alone, and that these birds can suffer quantifiable sublethal hematologic injury. The ability to accurately assess the extent of exposure and hematologic damage caused by oil spills is critical to determine the appropriate approach to management needed to offset impacts to fisheries, wildlife, habitats, and economic resources impacted by oil spills.
Diagnostic techniques for detecting exposure and anemia in birds exposed to crude oil

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GENERAL AUDIENCE ABSTRACT

Fossil fuels are the world’s primary energy source and are an important part of everyday life. Our reliance on petroleum requires extraction, transportation, storage, and refinement of millions of gallons of crude oil each day. As an unintended consequence, some of this oil is inadvertently spilled into the environment, and these oil spills have long been recognized as a threat to wildlife. Assessing the impact of oil spills on wildlife is a major concern to industries, government, and the general public. Historically, mortality estimates have served as the basis for assessing impact to natural resources. However, these mortality estimates alone neglect the more wide-spread impact of oil spills on wildlife including birds, many of which may not immediately succumb to exposure, but instead suffer sublethal physiologic injury that negatively affects physiology, reproduction, and long-term survival. Therefore, there is a need to improve our understanding of the risk of exposure and effects of sublethal oiling during damage assessments. In this dissertation, I evaluated the extent of sublethal exposure to oil from The Deepwater Horizon spill for several species of birds through both visual evaluation of and under the application of ultraviolet light. This demonstrated that many more birds are affected by oil exposure than are accounted for by mortality estimates. Additionally, I developed a field-adapted technique in a controlled setting that is effective in determining oxidative injury to red blood cells in birds exposed to oil, and applied this approach to several species in the field during the aftermath of the Deepwater Horizon spill. Finally, I used an experimental approach to evaluate the extent of pathologic effects of Deepwater Horizon crude oil exposure in individuals under controlled dosages. The ability to accurately assess the extent of damage caused by oil spills is critical to determine the appropriate approach to management needed to offset impacts to fisheries, wildlife, habitats, and economic resources impacted by oil spills.
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My committee members, Todd Katzner, Jeffery Walters, and Eric Hallerman, were vital to the completion of this dissertation. Their mentorship and critical analysis of this body of work were instrumental during the planning, revision, and publication process.

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Thanks to my young children, Laurel, Cora, and Stella, who have grown and developed into kind and inquisitive individuals and provided motivation for me to finish this project, even if unknowingly.

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ATTRIBUTION

Chapter 1 was coauthored by Lee Fox and William Hopkins. WH conceived and designed the study. LF facilitated field work and sample collection. All authors provided comments and approved the manuscript.

Chapter 2 was coauthored by Eric P. Smith, Nina Shoch, James D. Paruk, Evan A. Adams, David C. Evers, Patrick G.R. Jodice, Christopher Perkins, Shiloh Schulte, and William A. Hopkins. DE and WH conceived and designed the study. NS, JP, EA, PJ, and SS coordinated and conducted field work. CP provided laboratory support. ES helped with statistical design and analysis. All authors provided comments and approved the manuscript.

Chapter 3 was coauthored by Eric P. Smith, Nina Shoch, James D. Paruk, Evan A. Adams, David C. Evers, Patrick G.R. Jodice, William A. Hopkins. DE and WH conceived and designed the study. NS, JP, EA, and PJ coordinated and conducted field work. ES helped with statistical design and analysis. All authors provided comments and approved the manuscript.

Chapter 4 was coauthored by Christopher Goodchild, Sarah E. DuRant, Thomas Cecere, D. Phillip Sponenberg, and William A. Hopkins. CG, SD, and WH conceived and designed the study. TC and PS provided cytology and pathology support. All authors provided comments and approved the manuscript.
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INTRODUCTION

I. Petroleum spills and associated risks

Fossil fuels are the world’s primary energy source and are an important part of everyday life. They are used to heat our homes, operate our vehicles, power industry and manufacturing, and they provide the vast majority of the world’s electricity. Our reliance on petroleum in particular necessitates extraction, transportation, storage, and refinement of millions of gallons of crude oil each day (Riazí 2021). As an unintended consequence, some of this oil is inadvertently spilled into the environment, and these oil spills have long been recognized as a threat to wildlife (Bourne 1968; Albers 2003, Ahmed and Fakhruddin 2018, Zhang et al. 2019). Although terrestrial habitats can be affected, aquatic systems are often more severely impacted by liquid petroleum, as even small spills can disperse across a large area on the surface of water (Fingas 2016).

Petroleum spills occur from a variety of sources, including release of crude oil from tankers, offshore platforms, drilling rigs, and wells (Chang et al. 2014). The exact composition of these crude oils can vary substantially depending upon the source, as content and concentrations of polycyclic aromatic hydrocarbons (PAH) in the oil are unique to the site of extraction (Grimmer et al. 1983, Chang et al. 2014, Faksness et al. 2014). In addition to crude oil, there are many other sources of liquid petroleum spills that occur in aquatic ecosystems each year. These include refined products such as gasoline or diesel fuel, six different variations of bunker fuel oil, and refuse or waste oil (Chang et al. 2014). As with crude oil, these products have different constituents from each other, and therefore pose varying threats to aquatic systems (Albers 1995).
Table 1. Oil spills that occurred in navigable U.S. waters between 1995-2018 excluding the Deepwater Horizon spill in 2010. This particular incident, which resulted in an unprecedented 207,000,000 gallons spilled (more than twice the sum of all volumes below), was omitted from this table to provide a more representative average spill volume. (Data collected from U.S. Department of Transportation [https://www.bts.gov/content/petroleum-oil-spills-impacting-navigable-us-waters](https://www.bts.gov/content/petroleum-oil-spills-impacting-navigable-us-waters)).

<table>
<thead>
<tr>
<th>Source</th>
<th>Gallons spilled</th>
<th>Incidents</th>
<th>Average gallons/spill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>31,678,714</td>
<td>117,407</td>
<td>270</td>
</tr>
<tr>
<td>Vessel sources, total</td>
<td>14,398,838</td>
<td>66,836</td>
<td>215</td>
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<tr>
<td>Tankship</td>
<td>2,490,379</td>
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<td>1789</td>
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<tr>
<td>Tank barge</td>
<td>6,605,920</td>
<td>3,412</td>
<td>1936</td>
</tr>
<tr>
<td>Other vessels(^a)</td>
<td>5,302,539</td>
<td>62,030</td>
<td>85</td>
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<tr>
<td>Nonvessel sources, total</td>
<td>16,405,490</td>
<td>27,828</td>
<td>590</td>
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<tr>
<td>Offshore pipelines</td>
<td>439,776</td>
<td>441</td>
<td>997</td>
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<tr>
<td>Onshore pipelines</td>
<td>1,414,372</td>
<td>154</td>
<td>9184</td>
</tr>
<tr>
<td>Other(^b)</td>
<td>14,550,055</td>
<td>27,061</td>
<td>538</td>
</tr>
<tr>
<td>Mystery(^c)</td>
<td>874,387</td>
<td>22,741</td>
<td>38</td>
</tr>
</tbody>
</table>

\(^a\) Other vessels include commercial and industrial vessels, fishing boats, freight vessels, oil recovery vessels, public and research vessels, recreational boats, tug boats, offshore drilling units and supply vessels, publicly owned ships, as well as vessels not fitting any particular class.

\(^b\) Other nonvessel sources include ports, waterfront facilities, nonmarine land facilities, fixed platforms, mobile facility, municipal facility, aircraft, land vehicles, railroad equipment, factories, fleeting areas, industrial facilities, intakes, locks, marinas, nonvessel common carrier facilities, sewers, drains, and shipyards.

\(^c\) Mystery spills are spills from unknown or unidentified sources.

The frequency and volume of petroleum spilling into the environment is concerning. According to the United States Department of Transportation, there were 117,408 incidents of oil spills totaling 238,678,714 gallons in navigable U.S. waters between 1995 and 2018, with an average of more than 5,100 spills occurring each year (Data collected from U.S. Department of Transportation [https://www.bts.gov/content/petroleum-oil-spills-impacting-navigable-us-waters](https://www.bts.gov/content/petroleum-oil-spills-impacting-navigable-us-waters)). These include in numerous high profile, large-scale spills such as the Exxon Valdez and Deepwater Horizon spills, which led to widespread and long-lasting impacts on ecosystems, including bird populations (Piatt et al. 1990, Wiens et al. 1996, Iverson and Esler 2010, Munilla et al. 2011, Haney et al. 2014a). The number and volume of oil spills that occur suggest that the risk to aquatic birds and other wildlife is substantial (Table 1).
The multitude of large and small petroleum spills pose a significant risk for food webs, local economies that rely on the environment in which the spills occur, and for many species of wildlife. Being able to accurately assess the damage caused by these spills is critical to determine the appropriate approach to mitigation restoration needed to offset impacts to fisheries, wildlife, habitats, and economic resources impacted by oil spills. However, damage estimation following oil spills is complex and requires a holistic, yet structured approach to at-risk organisms and ecosystems.

II. Natural Resource Damage Assessments

The Natural Resource Damage Assessment (NRDA) process is a highly structured legal process that federal agencies, together with affected states, use to evaluate the impacts of oil spills and ship groundings on natural resources, both along the nation's coast and throughout its interior. Natural Resource Damage Assessment partners, referred to collectively as "natural resource trustees", work through a formal process authorized under the Comprehensive Environmental Response, Compensation, and Liability Act (1980) and the Oil Pollution Act (1990) to identify the extent of natural resource injuries, and the quantity and quality of restoration necessary to compensate the public for those damages. During oil spill damage assessments, the United States Fish and Wildlife Services (USFWS) and the Nation Oceanic and Atmospheric Administration (NOAA) collaborate to represent the United States. These agencies are supported by the US Department of Justice (DOJ), which facilitates settlements and litigation against parties responsible for the spill event.

The most visible application of the NRDA process occurs during large-scale spill events, which can have destructive consequences on local ecosystems, capture public attention, and cause dramatic, long-lasting negative effects on birds and wildlife (Norse and Amos 2010). The intense media and public scrutiny surrounding these spills has solidified their negative effects in the minds of the public, and accurate damage assessments of high-profile spills are critically important to facilitate fair and adequate determination of natural resource harm. Prior to the Deepwater Horizon spill of 2010 -- which was particularly devastating because of the historic volume of crude oil spilled, the importance and extent of the aquatic and terrestrial habitat damaged, the number of birds and wildlife affected, and the
loss of human life in the immediate aftermath of the explosion -- damage assessments on wildlife populations in the aftermath of spills were based in large part on the economic value of dead birds and other wildlife as a means to quantify injury (Rodgers et al. 2005).

While mass mortality is an important aspect to help assess natural resource damage, it underestimates the true damage to wildlife, as there are many individual animals that are exposed to oil contamination but do not immediately succumb and die. Many individuals also likely suffer injury from exposure, which may result in reduced reproductive output, movement perturbations, and latent mortality that can affect populations (Wiens et al. 1996, Iverson and Esler 2010). Therefore, the ability to assess sublethal injury can contribute to a more accurate estimation of damage that occurs to populations following oil spill events. Assessing sublethal physiologic damage to birds exposed to oil is the focus of this dissertation, which evaluated the physiological effects of oil in several species of birds exposed to oil in the aftermath of the Deepwater Horizon spill in the Gulf of Mexico.

III. Deepwater Horizon Spill and the immediate aftereffects

On April 20th, 2010, an explosion on the offshore oil platform Deepwater Horizon operating 64 Km off the coast of Louisiana, USA, killed 11 crewmen and ignited an inextinguishable fire. Two days later, the rig sank, and the well began gushing crude oil into the Gulf of Mexico. The Deepwater Horizon oil spill resulted in the largest accidental offshore oil spill in history, with estimates indicating that 207,000,000 gallons of crude oil poured into the sea, spreading over 180,000 square kilometers along the surface (Graham et al. 2011, Barron et al. 2020). The spill caused unprecedented damage to the flora and fauna, resulting in dramatic effects on the fishing and tourism industries, and substantial damage to the natural resources, including birds and other wildlife, of the region.

The ecosystem affected by the Deepwater Horizon spill is home to more than 8,000 species, including 214 species of birds (Shirley et al. 2010). Along with injury to marine mammals, fishes, aquatic reptiles, benthic organisms, seagrasses, and corals, the spill resulted in massive avian mortality, with estimates suggesting that bird deaths may have
reached as high 600,000 to 800,000 individuals in the aftermath of the spill (Haney et al 2014a,b). The scale of this die-off has obvious implications for bird populations throughout the area affected by the spill.

The damage caused by the Deepwater Horizon spill to the region’s ecology directly impacted the region’s economy. Although the cost of tourism dollars is difficult to quantify, estimated projections for the overall impact of lost or degraded commercial, recreational, and mariculture fisheries in the Gulf were $2.3 billion with a loss of 22,000 jobs over the same time-frame (Sandifer et al. 2021, Sumaila et al. 2012). This dramatic economic impact resulted in considerable loss of income to households and businesses in the Gulf states, even without the losses associated with clean-up cost, value of lost oil, effect of environmental damage beyond fisheries, and other negative impacts on direct uses such as bird and wildlife watching and other non-fishing tourism (Sumaila et al. 2012). Aside from the economic impacts, 11 people died in the explosion and 17 were injured. These losses affected families not only from the region directly impacted by the spill, but in the United States at large.

The environmental damage and NRDA efforts on the Deepwater Horizon spill provided an opportunity to examine the mechanisms underlying potential sublethal effects on individual birds to improve damage assessments for this spill and inevitable future spill events. This dissertation focuses on assessment of sublethal physiological injury of birds exposed to the Deepwater Horizon oil as a case study, both to improve the accuracy of estimates of natural resource damage caused by this unprecedented spill, and to develop methodology that can be employed during future oil spill events. Additionally, the work presented here also offers an opportunity to improve damage assessments that reach beyond acute bird mortality events.

IV. Hematologic injury to birds exposed to oil

The most visible effect of crude oil exposure on birds is the contamination of plumage, which results in disturbance in the physical characteristics of feathers, thereby reducing the ability to fly, dive, swim, and thermoregulate (Helm et al. 2015). Although the mortality associated with heavy oiling of feathers is well-known, a
more wide-spread impact of oil spills on birds is likely through ingestion of contaminated food or water, or from preening oiled feathers (Hartung 1964, Holmes and Cronshaw 1977, Alexander et al. 2017).

A cascade of physiologic damage from the ingestion of crude oil in aquatic birds has been described, including inflammation, immunosuppression, endocrine disruption, and oxidative damage to cells (Fry et al. 1986, Leighton 1993, Briggs et al. 1996, Golet et al. 2002, Troisi et al. 2007, 2016, Fallon et al. 2018, 2020, 2021). This undoubtedly contributes to the acute mortality that occurs in the aftermath of spill events. However, these negative effects can also occur without being immediately lethal. Sublethal effects of petroleum exposure can negatively impact growth, alter organ function, reduce reproductive success, and increase risk of disease (Briggs et al. 1996, Esler et al. 2000, Giese et al. 2000, Eppley and Rubega 1990, Alonso-Alvarez et al. 2007). These factors can coalesce to reduce fitness in individuals and negatively impact populations on a much larger scale than do acute mortality events alone (Esler et al. 2002, Golet et al. 2002). Of the sublethal physiological impacts resulting from avian exposure to oil spills, oxidative damage to erythrocytes and subsequent anemia are of particular interest to oil spill investigators, as parameters associated with anemia can be quantified and are linked to a variety of performance and physiologic, and therefore possible fitness, ramifications.

Exposure to the polycyclic aromatic hydrocarbons (PAHs) found in crude oil results in oxidative injury to erythrocyte cell membranes and cytoplasmic hemoglobin (Hb), the metalloprotein responsible for oxygen transport from the respiratory organs to other tissues (Leighton et al. 1983, Latimer et al. 2003). This damage occurs as the result of insult caused by metabolites of PAHs, generated from actions of cytochrome P450 enzymes (Troisi et al. 2007, 2016, Desnoyers 2010). When exposed to these metabolites, the Hb molecule undergoes changes in sulfhydryl bonds resulting in denaturation of the oxygen-carrying protein (Jandl et al. 1960). Aggregates of this damaged Hb can be highlighted by certain vital staining techniques (e.g., new methylene blue), resulting in inclusion bodies within red blood cells (RBCs) that are readily identifiable using light microscopy (Latimer et al. 2003). These aggregations of denatured Hb, called Heinz body inclusions (also, Heinz-Ehrlich bodies) are pathognomonic for oxidative damage to
RBCs. These damaged, abnormal erythrocytes undergo lysis or are destroyed in vivo, a process known as hemolysis (Latimer et al. 2003). Hemolysis can subsequently result in anemia, defined as a reduction in circulating erythrocytes and oxygen-carrying hemoglobin.

In vertebrates, the appropriate physiological response to compensate for anemia is the release of immature blood cells, termed reticulocytes because of their meshwork of retained RNA, from hematopoietic organs (Goodnough et al. 2000, Campbell and Ellis 2007). A decrease in circulating oxygen, due to anemia, triggers the release of reticulocytes into the bloodstream, a process designated as a regenerative response (Rosse and Waldmann 1962). These cells can be identified using the same staining techniques that highlight Heinz bodies, allowing for quantification of another endpoint associated with hemolytic anemia.

Hemolytic anemia is an important insult to birds following oil exposure for two important reasons. First, individuals with anemia experience functional difficulties. Anemia causes reduced availability of oxygen to tissues, which consequently leads to anaerobic metabolism, altered cell membrane permeability, cellular and tissue dysfunction, and if it progresses, ultimately organ failure (Greenburg 1996, Fried 2009). Heinz body hemolytic anemia in particular induces a reduction in energy availability for metabolic processes (Butler et al. 1986). Consequently, anemic animals suffer from muscle fatigue, lethargy, and reduced cognitive function (Jackson 2013). These physiological manifestations of anemia have obvious implications for food acquisition, reproduction, and survival (Figure 1). Second, oxidative hemolytic anemia could be another useful symptom of sublethal injury during natural resource damage assessments, but a reliable, field-deployable technique to assess and quantify oxidative anemia in individual birds needs to be developed. Such a technique would be invaluable to ensure accurate damage assessments in birds potentially exposed to oil.
Figure 1. Conceptual diagram of the mechanism of polycyclic aromatic hydrocarbons (PAH) induced oxidative damage on red blood cells (RBCs) and hemoglobin (Hb), its effects, subsequent physiologic response, and potential outcome in birds.

Anemia has been identified in many species of birds naturally exposed to crude oil during spill events. For example, rehabilitation facilities treating oiled birds have reported that common murres (*Uria aalge*) have lower packed cell volume (PCV) than unoiled rescued birds of the same body mass (Duerr et al., 2016; Parsons et al., 2018). Common terns (*Sterna hirundo*) exposed to crude oil from a spill in Buzzards Bay, Massachusetts had lower packed cell volumes than birds sampled in the same region during years without a large spill event (Nisbet et al. 2013). White-
winged scoters (*Melanitta fusca*) contaminated with fuel oil (Bunker C oil) from a capsized cargo ship near Hokkaido, Japan had reduced PCV, erythrocyte counts, and hemoglobin compared to unaffected birds. Circulating PAHs in overwintering common loons (*Gavia immer*) were associated with lower packed cell volumes in areas affected by the Deepwater Horizon spill (Paruk et al. 2018). Similarly, PAH concentrations in oiled common guillemots (*Uria aalge*) exposed to oil and recovered near the coast of the United Kingdom were positively correlated with anemia (Troisi et al. 2007). These field studies highlight the importance of hematologic damage resulting from exposure to oil spills. However, the majority of studies to date have focused on severely oiled birds, making it unclear whether more modest oiling would induce anemia. This factor, coupled with the variability in hematologic parameters reported and limited species evaluated in these studies prompted the need for further field evaluation of oxidative hemolytic anemia of additional species that experienced more subtle oiling in the field.

Controlled experimental oil-exposure studies in birds also have demonstrated anemia. Leighton et al. (1983) first described the direct, toxic hematologic effect of petroleum-induced hemolytic anemia by exposing nestling herring gulls (*Larus argentatus*) and Atlantic puffins (*Fratercula arctica*) to Prudhoe Bay crude oil, demonstrating large reductions in hematocrit and Heinz body formation after oral ingestion of oil (Leighton 1986). Similarly, ring-billed gulls (*Larus delawarensis*) dermally exposed to Deepwater Horizon crude oil had reduced PCV compared to control birds (Dannemiller et al. 2019). Signs of hemolytic anemia in adult birds exposed experimentally to fuel oil has also been demonstrated in mallard ducks (*Anas platyrhynchos*) (Hartung and Hunt, 1966; Lee et al., 2012) and weathered crude oil in ring-billed gulls (*Larus delawarensis*) (Dannemiller et al., 2019) and double-crested cormorants (*Phalacrocorax auritus*) (Harr et al., 2017a). While there is experimental evidence of anemia in birds exposed to oil, there are also a few studies that failed to find anemia in some species (e.g., Fleming et al. 1982, Newman et al. 1999, Bursian et al. 2017, King et al. 2021). There are a variety of potential reasons for these discrepancies including differences in dosing protocols, age and species studied, and diagnostic techniques employed. For these reasons, further
evaluation of the hematologic effects of oil exposure using a robust and consistent suite of parameters and diagnostic tools both in the field and the laboratory are needed.

The ability to identify sublethal injury in live birds is important in assessing damage resulting from oil spill events. The methods needed to identify oxidative injury to erythrocytes and subsequent anemia are relatively simple, and blood volume collected from captured individuals need not be large to confirm the presence of Heinz bodies and anemia. If anemia is widespread among birds with modest visible oiling, a much greater number of birds can be assumed to potentially have suffered physiological injury. Thus, demonstrable sublethal hematologic injury can play an important role in NRDA efforts, as it demonstrates injury in a much larger number of birds then are accounted for from acute mortality estimates. Including these individuals with sublethal injury improves the accuracy of assessments, and can be used to establish more appropriate mitigation and restoration efforts as well as appropriate accountability of offending parties.

**III. Ultraviolet-assisted oiling assessment**

While visual evaluation of oil present on plumage is obviously important to determine which birds are exposed to oil during spill events, it is likely that small quantities of oil could be missed, particularly on birds with dark plumage. Thus, there is a need to develop simple tools that can be used to more accurately evaluate birds that are captured for oil exposure. For example, ultraviolet light shows promise as a means of enhancing detection of oiled birds because it has long been used as a tool to evaluate for oil slicks on the surface of the water following spills (De Kerf 2020). If this technology could be applied to individual animals, it is likely that small amounts of oil present on the plumage of birds could be identified, increasing the accuracy of determining which individuals are exposed.

Crude oil fluoresces under ultraviolet (UV) light (Burlamacchi 1983; Colligan and LaManna 1993; Fingas and Brown, 2014). This attribute has led to its use to improve detection of oil in several abiotic matrices. For example, ultraviolet light can be used to enhance detection of oil on or below the surface of a body of water as well as on snow and ice (Fingas and Brown 2000; Fingas and Brown 2013). Remote UV sensors are commonly used to monitor oil
films associated with spills at very thin layers down to 0.1 µm (Fingas and Brown 1997, Brekke and Solberg 2005; Jha et al. 2008). Despite the historic and ongoing use of UV light to determine the extent of oil slicks, this tool has not been used to evaluate individual animals for oil exposure. Application of UV light to the plumage of birds in the area of oil spills has the potential to identify oil in small quantities. These lightly-oiled birds may not be identified on visual exam alone, yet may still suffer physiologic injury. Therefore, the application of UV light on the plumage of birds may better define the extent of damage to individuals during spill events.

Focusing on acute mortality as a sole means for assessing the impact of oil spills greatly underestimates the negative effects of oil on aquatic birds. Sublethal effects from oil exposure are far more widespread, and are known to cause negative impacts on numerous species (e.g., Butler et al. 1998, Balserio et al. 2005, Alonso-Alvarez et al. 2007, Parez et al. 2010). Oxidative injury to erythrocytes and anemia is one of the few sublethal effects that can be quantified in live birds with a single blood sample, making it a valuable tool in natural resource damage assessments. Due to the paucity of studies incorporating the importance of sublethal injury into damage assessments, more research is needed to enhance detection of mild oiling in birds as well as to determine whether mild oiling can cause anemia.

My dissertation sought to develop and evaluate a suite of physiologic parameters in both experimental and field setting to determine the extent of hemolytic anemia in birds exposed to Deepwater Horizon crude oil. Further, I tested the utility of ultraviolet light to identify modestly-oiled birds that would otherwise be treated as unaffected during oil spill events. My approach included a combination of methods development in a controlled setting, application of the techniques in the field in the aftermath of the Deepwater Horizon spill, and evaluation in an experimental dosing study. My research used this integrative approach to accomplish two overarching goals. First, I sought to advance our understanding of the extent of sublethal oil exposure in birds. Second, I aimed to identify physiologic parameters that are important effects of oil exposure and to quantify the hematologic effects of this sublethal exposure. Specifically, I:

1. Utilized laboratory techniques in a controlled in vitro setting to define a suite of physiological parameters that provide a thorough evaluation of the hematologic status of birds exposed to crude oil.
2. Developed field-applicable techniques using an *in vitro* approach that can be applied to birds exposed to oil in both experimental settings and during spill events.

3. Improved our understanding of sublethal exposure through an oral dosing of zebra finches and by applying a novel field technique using UV light as a means to identify birds with modest oiling that would otherwise be missed on visual exam.

4. Advanced our understanding of the effects of sublethal oil exposure in a diversity of bird species with different natural histories oiled during the Deepwater Horizon spill by quantifying hematologic parameters including Heinz body formation, erythrocyte counts, reticulocyte numbers, and other useful hematologic parameters using the methodology developed in the laboratory to a rapid-response field scenario.

5. Further defined the effects of oil exposure using both light and electron microscopy in a laboratory dosing study of zebra finches (*Taeniopygia guttata*) to better understand the severity of effects and the potential for species differences in hematologic response to oil exposure.
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CHAPTER 1: A practical quantification method for Heinz bodies in birds applicable to rapid response field scenarios

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ABSTRACT

Oil-induced oxidative injury to red blood cells results in Heinz body hemolytic anemia. Here, we evaluated three Heinz body staining techniques in brown pelican (Pelecanus occidentalis) blood. Using a range of in vitro acetylphenylhydrazine incubations, we validated a field-adapted technique against laboratory wet-mounts and verified the stability of this technique for one month following preparation. Employing this technique during petrochemical spill responses allows for delays between sample collection and analysis.

Keywords: Heinz body, oxidative hemolysis, hemolytic anemia, Pelecanus occidentalis

INTRODUCTION

Industry, government, and the general public are concerned about the impact of oil spills on wildlife. Historically, wildlife mortality has served as a foundation for assessing the impact of oil spills on ecosystems, as well as for directing resources toward restoration and mitigation efforts. For example, acute avian mortality has been well described for many large-scale oil spills, such as the Exxon Valdez, Prestige, and, more recently, Deepwater Horizon (Piatt et al. 1990, Iverson et al. 1993, Camphuysen et al. 2002, Henkel et al. 2012). Although quantifying acute mortality is clearly important, this information alone may underestimate the more widespread impact of sublethal oil exposure on individuals, populations, and ecosystems (Iverson et al 2010, Henkel et al. 2012, Velando et al. 2005). Sublethal injury from oil exposure spans an array of physiological effects, including inflammation,
immunosuppression, and hemolytic anemia (Fry et al. 1986, Leighton 1993, Briggs et al. 1996, Golet et al. 2002). Anemia is of particular importance, as it causes reduced availability of oxygen to tissues, which consequently leads to anaerobic cellular metabolism, altered cell membrane permeability, cellular and tissue dysfunction, and, if it progresses, ultimately organ failure (Latimer et al. 2003, Houston and Myers, 1993).

Hemolytic anemia can result from a variety of etiologies, including oxidative injury to cytoplasmic hemoglobin (Hb), the metalloprotein responsible for oxygen transport from the respiratory organs to other tissues (Latimer et al. 2003). When exposed to reactive oxygen compounds, the Hb molecule undergoes changes in sulfhydryl bonds resulting in denaturation of the oxygen-carrying protein (Jandl et al. 1960). Aggregates of this damaged Hb can be highlighted by certain vital staining techniques (e.g., new methylene blue), resulting in inclusion bodies within red blood cells (RBCs) that are readily identifiable using light microscopy (Latimer et al. 2003). These aggregations of denatured Hb, called Heinz body inclusions (also, Heinz–Ehrlich bodies) are pathognomonic for oxidative damage to RBCs. Affected cells may lyse spontaneously or be removed from circulation by phagocytic cells, potentially resulting in anemia, depending on the extent and duration of RBC damage (Jandl et al. 1960). Heinz body–induced hemolytic anemia promotes fatigue and a reduction in energy availability for metabolic processes, and ultimately can decrease fitness (Butler et al. 1986).

Although many compounds can induce oxidative RBC injury, the formation of Heinz bodies and subsequent hemolytic anemia in birds has been most thoroughly demonstrated following exposure to oil (Leighton et al. 1985, Fry and Lowenstein 1985, Leighton 1986, Yamato et al. 1996, Troisi et al. 2007). Oil-induced oxidative damage is believed to be mediated by metabolites of polycyclic aromatic hydrocarbons generated from the metabolic actions of cytochrome P450 enzymes (Leighton et al. 1985). Consequently, it has been suggested that Heinz bodies can be used as a marker for exposure to crude oil (Troisi et al. 2007). However, previous studies often have relied on a wet-mount staining technique to quantify Heinz bodies, a technique that is impractical in the field, as these slides must be evaluated immediately after they are prepared and cannot be stored for later analysis (Troisi et al. 2007). Thus, the use
of Heinz body formation as a practical monitoring tool for oxidative injury in wild birds would benefit from a reliable technique that can be better applied to field situations.

The objectives of the present study were to evaluate the efficacy and longevity of two field-adapted Heinz body-staining techniques compared with the traditional wet-mount technique. In our first experiment, we applied three staining techniques to blood of brown pelicans (*Pelecanus occidentalis*, hereafter pelicans) blood following *in vitro* incubation with multiple concentrations of acetylphenylhydrazine, an oxidizing compound known to induce Heinz body formation (Rifkind 1964, Rifkind and Danon 1965, Peisacha et al. 1975, Ogawa et al. 1992, Gutzwiller 1998). In the second experiment, we sequentially evaluated slides prepared using the field-adapted staining technique to determine longevity of cell morphology over four weeks. In many field studies, there is a delay between time of blood collection and analysis; thus a field-adapted technique must be reliable for several days or weeks after samples are collected. Finally, we discuss the utility and significance of enumeration of Heinz bodies as it applies to assessing the impact of oil exposure in birds.

**MATERIALS AND METHODS**

*Blood sample collection*

We collected 3 ml of blood directly into vacutainers containing ethylenediaminetetraacetic acid (EDTA; BD Diagnostics) via 23-G butterfly needles from the medial metatarsal vein of 10 pelicans (three males and seven females) maintained in captivity at Save our Seabirds in Sarasota, Florida, USA, in July 2011. We filled two heparinized hematocrit tubes with untreated whole blood collected from the butterfly tubing. We then placed the blood on ice and transported it to the laboratory the same day for analysis, and we followed all guidelines of Virginia Tech and the American Veterinary Medical Association for animal care and use.

*Hematologic parameters*
We measured the packed cell volume (PCV), total plasma solids, and Hb for each bird. We quantified PCV (%) following centrifugation in a microhematocrit centrifuge (Zipocrit, LW Scientific) for 5 min at 4,400 g. We determined total plasma solids (g/dl) via refractometer, and total Hb (g/dl) using a Hemocue Hb Analyzer Hb201 (HemoCue) (Velguth et al. 2010).

Sample treatments

We incubated blood from each bird with acetylphenylhydrazine (Sigma-Aldrich) within 4 h of collection to induce Heinz body formation in vitro. We prepared five concentrations of acetylphenylhydrazine (10, 1, 0.5, 0.1, and 0.01), as well as one control using 1× phosphate-buffered saline (Fisher Bioreagents) as a diluent (Palasuwan et al. 2006). For each bird, we prepared six 100-µl aliquots of EDTA-treated whole blood for incubation. We incubated these aliquots with 100 µl of the different concentrations of acetylphenylhydrazine or with 100 µl of phosphate-buffered saline (control) at room temperature (\(\sim 25^\circ C\)), such that blood from each bird was exposed to each concentration of acetylphenylhydrazine in a 1:1 volume. Final acetylphenylhydrazine incubation concentrations ranged from 0.033 through 33 mM.

Slide preparation and analysis

We conducted two different experiments with these samples. In the first experiment, we compared the effects of three different slide preparation techniques and varying concentrations of acetylphenylhydrazine. Immediately following incubation with acetylphenylhydrazine, we prepared slides from each sample via three techniques. Techniques A and B are field-adapted but differ in how blood cells are stained with new methylene blue (0.5% [w/v], RICCA Chemical), whereas technique C uses clinically based wet-mount staining. In the first field-adapted technique (technique A), we used treated blood to make smears that were allowed to air-dry following a standard two-slide wedge method (Clark et al. 2009). After drying, we flooded the slides with new methylene blue for 20 min, followed by rinsing and air-drying. For the second (field-adapted) and the third (wet-mount) technique (techniques B and C,
respectively), we prepared slides after incubation of treated whole blood with new methylene blue stain. Staining preparations similar to technique B have been used to quantify reticulocytes, which are young red blood cells with a reticular (mesh-like) network of ribosomal RNA (Campbell and Ellis 2007, Tvedten and Moritz 2010, Harvey 2012). Newman et al. (1999) reported an air-dried technique for identifying Heinz bodies in rhinoceros auklets (*Cerrorhinca monocerata*) but their study failed to detect any Heinz body formation. Therefore, this type of staining technique requires validation in a controlled, experimental setting before it can be reliably applied to field scenarios. For these two techniques, we added 25 µl of new methylene blue stain to 25 µl of erythrocyte suspension and incubated the mixture at 21 ± 2°C for 20 min before slide preparation. For field-adapted technique B, we then prepared routine blood smears using a two-slide wedge technique and allowed the slides to air-dry (Clark et al. 2009, Tvedten and Moritz 2010). Technique B slides were retained for time-dependent analysis. For technique C, we made a traditional wet-mount preparation by placing a drop of each stained blood sample onto a microscope slide with a coverslip (Troisi et al. 2007).

For all three techniques, immediately after preparation we counted the number of cells affected with Heinz bodies per 1,000 erythrocytes under 1,000 × light microscopy (Tvedten and Moritz 2010). The same individual performed all cell counts and evaluated each slide once for each technique. We retained slides made using technique B for the time-dependent analysis described below. We did not retain slides prepared by technique A for time-dependent analysis, as these slides did not result in Heinz body counts consistent with the wet-mount technique C, nor did we retain slides prepared by wet-mount technique C, as these preparations rapidly dry, leading to distorted cell morphology and inaccurate Heinz body counts.

In the second experiment, we determined the effects of time on Heinz body counts. We evaluated air-dried slides prepared following incubation with new methylene blue via field-adapted technique B, described above, on days 1, 2, 3, 7, 14, 21, and 28 following initial preparation with the above concentrations of acetylphenylhydrazine. For this repeated-measures evaluation, we counted the number of cells containing Heinz bodies per 500 erythrocytes under
1,000 × light microscopy. The same individual (JF) performed all cell counts and evaluated each slide once on each day. We stored these slides in a standard slide box at 21 ± 3°C for the duration of the study.

Statistical analyses

We calculated mean and standard deviation for PCV, total plasma solids, and total Hb. Incubation of pelican blood with 0 and 0.033 mM acetylphenylhydrazine resulted in no Heinz body formation in any of the blood samples, and thus we did not include these concentrations in the analyses. Data from the first experiment comparing staining techniques did not meet the assumption of normality, and data transformation did not improve the distribution. Therefore, we used Friedman's test to compare the effects of the different techniques and different acetylphenylhydrazine concentrations on Heinz body formation (Pereira et al. 2015). We used Wilcoxon tests with Bonferroni adjustment to assess differences between individual techniques and concentrations (Rice 1989).

For the second experiment evaluating stability of slides over time, we used one-way repeated-measures analysis of variance following log(x + 1) transformation to better meet assumptions of normality, with concentration of acetylphenylhydrazine as the between-subject factor and time as the within-subjects factor. We applied a Greenhouse–Geisser (1958) correction to account for violation of sphericity. For each analysis, we used SAS Version 9.2 and set the level of significance at \( \alpha = 0.05 \) throughout except for when we used the Bonferroni adjustment.

RESULTS

Packed cell volume (mean = 43.3, standard deviation [SD] = 2.50), total plasma solids (mean = 4.54, SD = 0.83), and total Hb concentration (mean = 14.36, SD = 1.32) of pelicans in the present study were consistent with those reported in other studies (Wolf et al. 1985, Zaias et al. 2000). Sex had no effect on PCV, total plasma solids, or Hb concentration. We found a significant dose-dependent increase in Heinz body counts with increasing concentrations of acetylphenylhydrazine (Friedman S = 99.26, df = 3, \( p < 0.001 \)). Staining technique overall had a marginal effect on
Heinz body counts (Friedman $S = 5.62$, $df = 2$, $p = 0.06$), and technique A consistently generated lower Heinz body counts than techniques B ($Z = -1.8973$, $p = 0.03$) and C ($Z = 1.7245$, $p = 0.04$). We found no difference in Heinz body counts between the field-adapted technique B and the wet-mount technique C ($Z = -0.2407$, $p = 0.41$; Fig. 1).

*In vitro*

For the second experiment examining the stability of slides prepared with technique B over time, repeated-measures analysis of variance with Greenhouse–Geisser correction revealed that storage time had no effect on the number of cells affected by Heinz bodies ($F_{7,21} = 0.98$, $p = 0.43$), whereas concentration of acetylphenylhydrazine had a dose-dependent effect on Heinz body formation ($F_{3,36} = 274$, $p < 0.001$; Fig. 2).

**DISCUSSION**

Oil spills can have dramatic consequences to wildlife, particularly seabirds (e.g., Piatt et al. 1990). Monitoring the physiological effects of petroleum exposure is an important component of oil spill response. Although there are a myriad of negative effects from oil exposure, oxidative injury to RBCs and resulting Heinz body hemolytic anemia are likely important to individual survival and serve as a valuable monitoring tool during oil spill responses.

The present study demonstrated that acetylphenylhydrazine induces dose-dependent Heinz body formation *in vitro* in pelican blood at concentrations of 0.33 mM and above. To our knowledge, with the exception of domestic chickens (*Gallus domesticus*), this is the first demonstration of Heinz body formation *in vitro* for blood from members of the class Aves (Datta et al. 1990). Heinz bodies are pathognomonic for oxidative injury to RBCs, and their formation has been described in vivo in birds following exposure to dimethyl disulfide 35, n-butyl mercaptan and n-butyl disulfide (Abdo et al. 1983), white phosphorus (Sparling et al. 1998), phenylhydrazine (Sorrell and Weiss 1982), and phenylhydrazine-hydrochloride (Datta et al. 1990). However, the most frequently reported chemical cause of Heinz

It has been suggested that Heinz bodies should be used as a biomarker for oil-induced oxidative injury, yet techniques for quantifying Heinz bodies have not been consistent (Leighton et al. 1985, Fry and Lowenstine 1985, Leighton 19986, Yamato et al. 1996, Troisi et al. 2007). Staining techniques for enumeration of Heinz bodies have often relied on clinical wet-mount preparations with fresh blood (Troisi et al. 2007, Hawkey and Dennett 1989). These wet-mount slide preparations (technique C), however, can be impractical in field situations, as they must be evaluated microscopically immediately after preparation (Troisi et al. 2007, Hawkey and Dennett 1989). In the present study, we found that field-adapted technique B provided Heinz body counts (percentage of RBCs affected) consistent with clinically based wet-mount slides. Although air-dried techniques have been described for evaluation of reticulocyte numbers, a standard technique for Heinz body quantification in birds that can be applied to field scenarios has not been described and validated against wet-mount preparations (Campbell and Ellis, 2007, Harvey 2012, Newman et al. 1999). Conversely, when compared with wet-mounts, slides that were air-dried prior to being flooded with new methylene blue (technique A above) resulted in an underestimation of the number of cells affected by Heinz bodies, and therefore should not be used.

In the present study, the field-adapted slide preparation technique B provided consistent Heinz body counts for up to 28 d after preparation in pelicans. This is important, as there is often an unavoidable time delay between sample collection in the field and analysis in the laboratory during petrochemical spill response scenarios. Even in an experimental laboratory setting, this stability can provide advantages over wet-mount techniques. We expect that the duration of morphological stability after preparation could likely be extended, particularly following application of a permanent coverslip.

Based on our findings, we recommend that the field-adapted technique B described and validated here is appropriate for field-response situations in which Heinz body counts are a desired endpoint in birds. Specifically, we
recommend that whole blood be collected directly into and gently inverted in EDTA-treated blood tubes, and incubated for 20 min with new methylene blue in a 1:1 ratio (e.g., 25 µl of each); then, this mixture can be used to prepare a minimum of two air-dried slides. After screening 1,000 RBCs per slide, Heinz body counts should be reported as a percentage of RBCs affected. This staining technique has the added advantage of allowing for quantification of reticulocytes (i.e., immature red blood cells), which is an important parameter in cases of anemia. In small avian species in which only limited blood can be safely collected, EDTA-treated microtainers or hematocrit tubes can be used in place of larger blood tubes. Because the RBCs of some species including ostriches (*Struthio camelus*) (Campbell and Ellis 2007), black-crowned cranes (*Balearica pavonina*) (Clark et al. 2009), and laughing kookaburra (*Dacelo novaeguineae*) (Clark et al. 2009), and members of the Corvidae (Hawkey and Dennett 1989) and Megapodiidae (Clark et al. 2009) families, can be damaged when incubated with EDTA, another anticoagulant, such as lithium heparin, should be considered. However, whenever possible, EDTA is preferable to heparin when staining for cellular morphology, as heparin results in extensive clumping of hematological cells (Campbell and Ellis 2007).

The physiological implications of Heinz body formation and their relationship to petroleum exposure make them an important parameter for health assessments following oil spill events. Heinz body quantification in a rehabilitation setting may have prognostic value, and if repeated in an individual, could provide a temporal evaluation of response to treatment. The field-adapted technique B evaluated here provides a means for assessment of hematological injury that can be applied to free-ranging birds in nearly any field situation, including oil spill responses, as it affords the opportunity for samples to be conveniently prepared and transported for evaluation. Although the present study focused on birds, our findings may be applicable to other wildlife including reptiles and mammals that are also known to form Heinz bodies (Basile et al. 2011, LeiNarurkar et al. 2002). During oil spill response scenarios, we recommend that field researchers and rehabilitation facilities routinely collect blood and stain for Heinz bodies using this technique.
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Figure 1. *In vitro* Heinz body generation in brown pelican (*Pelecanus occidentalis*, mean $\pm$ 1 standard error, $n = 10$ per time/dose combination) blood treated with different concentrations of acetylphenylhydrazine (mg/ml) quantified with three different slide preparation techniques. Open bars represents field-adapted technique A (slide preparation followed by flooding with new methylene blue), shaded bars represent field-adapted technique B (blood incubated with new methylene blue followed by slide preparation), and hashed bars represent traditional wet mount technique C.
Figure 2. The effect of time after preparation of blood on mean (mean ± 1 standard error, n = 10 per time/dose combination) Heinz body counts in brown pelican (Pelecanus occidentalis) red blood cells exposed *in vitro* to different concentrations of acetylphenylhydrazine (33 mM ▲, 3.3 mM ●, 1.7 mM ■, 0.33 mM ○) using field-adapted staining technique B.
Chapter 2: Hematological indices of injury to lightly oiled birds from the Deepwater Horizon oil spill


*Formatted for Ecotoxicology and Environmental Chemistry

ABSTRACT

Avian mortality events are common following large-scale oil spills. However, the sublethal effects of oil on birds exposed to light external oiling are not clearly understood. We found that American oystercatchers (area of potential impact (API) \(n = 42\), reference \(n = 21\)), black skimmers API \(n = 121\), reference \(n = 88\)), brown pelicans (API \(n = 91\), reference \(n = 48\)), and great egrets (API \(n = 57\), reference \(n = 47\)) captured between 20 June, 2010 and 23 February, 2011 following the Deepwater Horizon oil spill experienced oxidative injury to erythrocytes, had decreased volume of circulating erythrocytes, and showed evidence of a regenerative hematological response in the form of increased reticulocytes compared to reference populations. Erythrocytic inclusions consistent with Heinz bodies were present almost exclusively in birds from sites impacted with oil— a finding pathognomonic for oxidative injury to erythrocytes. Average packed cell volumes were 4% to 19% lower and average reticulocyte counts were 27% to 40% higher in birds with visible external oil than birds from reference sites. These findings provide evidence that small amounts of external oil exposure is correlated with hemolytic anemia. Furthermore, we found that some birds captured from the area impacted by the spill but with no visible oiling also had erythrocytic inclusion bodies, increased reticulocytes, and reduced packed cell volumes when compared to birds from reference sites. Thus, birds suffered hematologic injury despite no visible oil at the time of capture. Together, these findings suggest that adverse effects of oil spills on birds may be more widespread than estimates based on avian mortality or severe visible oiling.
Key words: Deepwater Horizon, oil spills, Heinz bodies, oxidative hemolytic anemia, polycyclic aromatic hydrocarbons

INTRODUCTION

Oil spills can have substantial and long-term effects on ecosystems. Seabirds, waterfowl, and colonial waterbirds are at particular risk, and extensive avian mortality has been described for many large-scale oil spills, such as the Exxon Valdez (Iverson et al. 2010), Prestige (Munilla et al. 2011), and more recently, Deepwater Horizon (DWH) (United States Fish and Wildlife Service 2011). Acute mortality data are used to assess impact to natural resources and ultimately to direct mitigation and restoration efforts. Acute mortality data alone, however, likely underestimate the more widespread impact of oil exposure on individuals and long-term consequences on populations and ecosystems (Iverson et al. 2010, Velando et al. 2005, Votier et al. 2005). Prolonged adverse effects of oil spills include ongoing population declines after the initial mortality event and slow population recovery (Peterson et al. 2003), but the effects of modest oil exposure on individuals remain poorly understood (Seiser et al. 2000, Golet et al. 2002, Alonso-Alverez et al. 2007). Because sublethal exposure (i.e., small amount of external oiling that does not result in immediate death) puts more birds at risk than are represented by acute mortality data, a thorough understanding of the adverse effects of light oiling is important.

Exposure to polycyclic aromatic hydrocarbons (PAH) found in crude oil and metabolites formed through metabolic enzymes (e.g., cyp450) triggers an array of pathologic effects in birds, including inflammation, immunosuppression, and oxidative damage to cells (Golet et al. 2002, Fry et al. 1986, Leighton 1986, Brigs et al. 1996). These sublethal effects can negatively impact growth, alter organ function, reduce reproductive success, and increase risk of disease, thereby reducing fitness in individuals and ultimately impacting populations on a larger scale than mortality events alone (Golet et al. 2002, Alonso-Alverez et al. 2007, Briggs et al. 1996, Eppley and Rubegga 1990, Esler et al. 2002, Patuk et al. 2016). Of the sublethal impacts resulting from avian exposure to oil spills, oxidative...
damage to erythrocytes and subsequent anemia are of particular interest because these changes can be evaluated from single blood samples taken from live birds.

Exposure to PAHs and their metabolites can result in oxidative injury to cytoplasmic hemoglobin (Hb), and aggregates of this damaged Hb can be highlighted by cell staining techniques (e.g., new methylene blue vital staining), resulting in inclusion bodies within erythrocytes (Brockus and Andreasen 2003, Fallon et al. 2013). These aggregations of denatured Hb, called Heinz body inclusions (also, Heinz-Ehrlich bodies) are pathognomonic for oxidative damage to erythrocytes. Subsequent to injury, these damaged, abnormal erythrocytes undergo lysis (i.e. hemolysis). Heinz body hemolytic anemia induces fatigue and a reduction in energy availability for metabolic processes (Butler et al. 1986). Hemolytic anemia has been demonstrated in several species of birds exposed to crude oil under experimental and natural conditions, and in cases where vital staining techniques were used, Heinz body inclusions were found within erythrocytes (Leighton et al. 1983, Leighton et al. 1985, Fry and Lowenstine 1985, Yamato et al. 1996, Troisi et al. 2007). While some of these studies provide evidence of a correlation between oil exposure, PAH levels in the blood, and Heinz bodies, they focus on a limited number of species and rely on relatively heavy oiling or large experimental oral doses of oil. Thus, further work is needed to understand variation in species-specific hematological responses, especially to the light and moderate oil exposures that are likely more common in most oil spill events.

In this investigation, we evaluated the hematologic response of birds exposed to oil from the DWH oil spill. The DWH was the largest accidental offshore oil spill in history, releasing an estimated 4.9 million barrels (210,000,000 US gal, + 10%) into the Gulf of Mexico (McNutt et al. 2012). Surface oil from this spill ultimately affected more than 175,000 km2 of surface water and coastal marshes in the Gulf of Mexico (Norse and Amos 2010). We evaluated the relationships between visible oiling, a suite of hematologic endpoints, and plasma proteins associated with oxidative hemolysis. Based on the existing literature, we made a priori predictions of the biological response of birds exposed to the DWH spill (Figure 1). Unlike other studies that have predominately focused on heavily oiled birds, the approach...
used here provides a comprehensive assessment of the hematological effects of trace and light amounts of crude oil on these species (Figure 1, Leighton et al. 1985, Yamato et al. 1996, Troisi et al. 2007).

METHODS

Study area and focal species

We focused our research on American oystercatchers (*Haematopus palliatus*, AMOY), black skimmers (*Rynchops niger*, BLSK), brown pelicans (*Pelecanus occidentalis*, BPRE), and great egrets (*Ardea alba*, GREG). These species were selected because they are common in the area affected by the spill and they represent a diversity of ecological niches which could influence their relative susceptibility to oil exposure (Fallon et al. 2014). We captured all birds from reference areas and areas of potential impact (hereafter API) from 20 June, 2010 until 23 February, 2011. Age classes and sex were similar between API and reference sites. We captured AMOY (API \(n = 43\), reference \(n = 21\)) and BLSK (API \(n = 121\), reference \(n = 88\)) with decoy-noose traps, box traps, and cannon-nets, BPRE (API \(n = 91\), reference \(n = 48\)) with noose and padded leg-hold traps, and GREG (API \(n = 57\), reference \(n = 47\)) with net guns (Table 1).

Impacted sites included locations along coastal Louisiana, with five BPRE collected from coastal Mississippi, where exposure to oil from the DWH spill was likely. Reference sites included various locations along coastal South Carolina and Georgia, USA where no recent oiling events had been recorded.

Visible oiling

We evaluated the majority of birds captured in both API and reference sites for evidence of visible oiling, and assigned an oiling score of none (0% of plumage affected with visible oil), trace (<5% plumage affected), light (6-20% plumage affected), moderate (21-40% plumage affected), or heavy (> 40% plumage affected) (Fallon et al. 2014).

Blood collection and sample handling
We collected blood from the medial metatarsal vein or superficial ulnar vein using 21 or 23G butterfly needles and deposited it directly into lithium heparin and ethylenediaminetetraacetic acid (EDTA) vacutainers as well as heparinized hematocrit tubes. The volume of blood collected was less than 10% of circulating blood volume which we assumed to be 8% of body mass. Immediately following blood collection, we prepared new methylene blue-stained blood smears in the field to quantify Heinz bodies and reticulocytes as described below. Blood samples in vacutainers and hematocrit tubes were maintained on ice and transferred to a field laboratory within 12 hours of collection. Heparinized plasma was promptly separated from cells via centrifugation and transferred to cryovials. Heparinized plasma for ferritin and HAP analyses was frozen and stored at -80 °C until processing.

We quantified erythrocytic inclusions consistent with Heinz bodies, reticulocytes, packed cell volume (PCV), hemoglobin (Hb), mean corpuscular hemoglobin concentration (MCHC), red blood cell count (RBC), mean cell volume (MCV), haptoglobin-like protein (HAP), and ferritin, as described below. In all cases, individuals performing these analyses were blinded to oiling status, capture location, and results of other analyses.

**Heinz bodies and reticulocytes**

To prepare the new methylene blue stained slides in the field, we transferred 25ul of blood from the EDTA vacutainer to a 0.5ml centrifuge tube containing 25ul of new methylene blue stain (Fallon et al. 2013). We mixed this combination and allowed it to incubate for 20 minutes, and then prepared two blood smears via the standard two-slide technique. We evaluated slides within 72 hours of collection. We identified Heinz bodies as blue-staining inclusion bodies within erythrocytes or, less commonly, found along the cell margin. Retained reticular material has an affinity for new methylene blue stain and is common in avian erythrocytes. However, the less intense stain affinity and relatively uniform size of the Heinz body inclusions distinguished them from the cytoplasmic organelle material and retained reticular material (Leighton 1986). Cells that have sustained oxidative damage undergo lysis or are removed from circulation, resulting in a reduced PCV and RBC, which subsequently triggers the release of young erythrocytes termed reticulocytes from hematopoietic centers (Campbell and Ellis 2007). We identified reticulocytes as erythrocytes
with reticular remnants encircling > 50% of the circumference of the nucleus (Johns et al. 2008). We counted 1000 erythrocytes under 1000X light microscopy, noting the number of cells affected by Heinz bodies as well as the number of reticulocytes.

### PCV, Hb, MCHC, RBC, and MCV

Anemia is defined as reduced PCV, Hb, or RBC (Campbell and Ellis 2007). We determined PCV (%) and Hb (g/dl) heparinized samples within 12 hours of collection. Packed cell volume was calculated using a standard hematocrit reader following centrifugation at 11,000 rpm for 5 minutes. Total Hb (g/dl) was quantified using a Hemocue Hb Analyzer Hb201. Red blood cell count (RBC, cells/mm³) was estimated via standard manual methodology using a hemocytometer at a commercial laboratory (Avian and Exotics Clin Path Lab, Wilmington, OH, USA). With hemolytic anemia, the MCHC, or the average hemoglobin per cell, typically does not change from baseline concentration or may be artifactually increased due to free Hb in the bloodstream. We calculated MCHC (g/dl) by dividing the total Hb (g/dl) X 100 by PCV (expressed as a proportion, Brockus et al. 2003). Vertebrates respond to oxidative hemolytic anemia with a regenerative response, resulting in increased number of reticulocytes in circulation. For most species, these cells tend to be larger than mature erythrocytes, leading to an increase in MCV. We calculated mean cell volume (MCV, in femtoliters or fL per cell) as 10 X PCV (as a proportion) divided by RBC count (cells/mm³).

### Plasma HAP and ferritin

In addition to hematologic changes following exposure to crude oil, there is evidence that concentrations of the plasma proteins, haptoglobin-like protein (HAP) and ferritin, may be correlated with oxidative hemolysis (Troisi et al. 2007). Haptoglobin-like protein, an acute-phase glycoprotein, functions primarily to bind free Hb, and during hemolytic anemia may be depleted resulting in low plasma concentration (Troisi et al. 2007, Prichard et al. 1997, Chamanza et al. 1999, Trevisan et al. 2001). Ferritin (FT) is also important in this cascade, as it functions to store free iron and has been demonstrated to increase in cases of PAH-induced hemolytic anemia in oiled Guillemots (Figure 1,
We determined plasma HAP (mg/ml) levels using a standard microplate colorimetric assay following the manufacturer’s specifications (Tri-Delta Phase Range haptoglobin colorimetric bioassay). Levey-Jennings charts were maintained throughout the study to evaluate for systematic and random error. Using high and low control samples, the average inter-assay coefficient of variation (CV) was 4.8%. Using results from replicate samples, the average intra-assay CV was 5.5%. These results are similar to those reported by the manufacturer (Tri-Delta Phase RangeTM haptoglobin colorimetric bioassay, Biognosis Ltd., UK).

We determined plasma ferritin using a standard colorimetric assay (Troisi et al. 2007, Aoki et al. 1992). Ferritin standards derived from purified equine spleen ranged from 18.75 to 300 ng/ml. We prepared a protein precipitation solution (10% w/v trichloroacetic acid, 3% w/v HCl plus 40% v/v thioglycolic acid) and color reagent (1.5M sodium acetate containing bathophenanthroline disulfonic acid). We added twenty-five µl of protein precipitation solution to 25 µl of each prepared standard, samples, controls, and blanks. Then we gently vortexed mixtures and incubated them at 56° C for 15 minutes, before cooling on ice. We then added 150 µl of color developing solution and allowed mixtures to incubate at 25° C for 5 minutes. We centrifuged these at 5000 rpm for 5 minutes, then transferred 150 µl of supernatant to microplate wells in duplicate and read the plate at 535 nm absorbance immediately. We employed standard quality assurance procedures including analysis of standard reference materials, duplicate samples, method blanks, and laboratory control samples with each analytical batch. The average inter-assay CV was 5.4% and the average intra-assay CV was 6.1%.

**Statistical Analyses**

We used SAS software (version 9.3 SAS Institute Inc.), and R 3.0.0 (Foundation for Statistical Computing) for all analyses. Where appropriate, we evaluated normality and homogeneity of variance using graphing methods and Shapiro-Wilk and Levene’s tests, respectively. Because of the large number of individual birds for which we had missing values for one or more variables (primarily due to limited blood volume from some individuals), we used a series of univariate statistical tests rather than taking a multivariate approach. Because data were not normally
distributed, we used non-parametric tests in these analyses. To account for the lack of independence of hematologic responses compared in our univariate models, we applied a conservative $\alpha < 0.01$ to assess significance, while also noting cases where $\alpha > 0.01$ and $\alpha < 0.05$.

Oil exposure risk and PAH absorption and metabolism are likely species-dependent because of differences in behavior and physiological differences in absorption and metabolism of petroleum. Likewise, the biological responses to PAHs and PAH metabolites, such as changes in erythrocytes or biochemical variables, are also likely species-dependent. Therefore, we first evaluated whether species differed in severity of visible oiling and the suite of physiological variables for each capture site type (reference and API locations) using Kruskal-Wallis tests with species as the independent variable. Because these analyses revealed significant differences in most values among species regardless of oil exposure (Table 2, 3), we analyzed blood endpoints separately for each species.

To determine the effects of visible oiling on Heinz body formation, reticulocytes, PCV, Hb, MCHC, RBC, MCV, ferritin, and HAP, we used Kruskal-Wallis with subsequent post-hoc analysis to compare capture site type and oiling classifications (using the SAS Multtest procedure). Because of a limited range of severity of visible oiling (Table 1), we classified birds for statistical models as either birds from reference sites, birds from API with visible oil, or birds from API with no visible oil. These three categories were used in all blood analyses.

RESULTS

Visible oiling

In reference areas, the only birds with any oiling were 7 GREGs with trace quantities of visible oiling. We retained these birds in their a priori designated reference group for our statistical analyses and thus were conservative in our analysis. In APIs, we found numerous birds with trace and light oiling, but we captured no birds that were moderately or heavily visibly-oiled (Table 1). Visible oiling of birds captured from API sites ranged in frequency from 25% in BRPE to 75% in AMOY.
Birds from API sites, both those with visible oil and those without, had higher Heinz body counts than birds from reference sites (Figure 2). Kruskal-Wallis analysis among birds from reference sites, API without visible oil, and API with visible oil revealed significant differences in Heinz body occurrence in BLSK \((p<0.001)\), BRPE \((p=0.024)\) and GREG \((p=0.003)\), but not in AMOY. Post hoc analyses suggested that API oiled BRPE \((p=0.012)\) and GREG \((p=0.003)\) had significantly higher Heinz body counts than birds from reference sites, but neither differed from API non-visibly oiled birds. Oiled BLSK from API sites also had significantly higher Heinz body counts than birds from reference sites \((p<0.001)\), and API BLSK without visible oil had significantly higher Heinz body counts than reference birds \((p=0.002)\).

Across species, birds from API sites had, on average 35% higher reticulocytes counts than birds from reference sites (Figure 3). Kruskal-Wallis analysis revealed significantly different reticulocyte counts between sites in AMOY \((p<0.001)\), BLSK \((p=0.004)\), BRPE \((p<0.001)\) and GREG \((p=0.01)\). Post hoc analyses revealed that API oiled and API non-visibly oiled BLSK \((p=0.019\) and \(p=0.013\) respectively) and BPRE \((p<0.001\) and \(p<0.001\) respective) had significantly higher reticulocyte counts than birds from reference sites. Oiled AMOY \((p<0.001)\) and GREG \((p=0.004)\) had significantly higher reticulocyte counts than birds from reference sites while neither differed from API birds without external oil.

Birds from API sites had lower PCV values than birds from reference sites (Figure 4). We found a significant difference in PCV in AMOY \((p<0.001)\), BRPE \((p<0.001)\), and GREG \((p<0.001)\) but not BLSK \((p=0.164)\). Post hoc analyses revealed that API oiled and API not visibly oiled AMOY \((p<0.001\) and \(p=0.005\) respectively), BRPE \((p<0.001\) and \(p<0.001\) respectively), and GREG \((p<0.001\) and \(p<0.001\) respectively) had significantly lower PCV values than reference birds. Across species, birds from API sites averaged 12% lower PCV than reference birds.

Birds from API sites also had lower Hb concentration than birds from reference sites (Figure 5). We found significant differences in Hb concentrations in BRPE \((p=0.002)\) and GREG \((p<0.001)\) but not BLSK \((p=0.876)\). Hemoglobin was not determined in AMOY from API sites and thus this species was excluded from this analysis. Post
Post hoc analyses revealed that API oiled and API not visibly oiled BRPE ($p=0.01$ and $p=0.003$ respectively) had significantly lower Hb concentrations than reference birds. Similarly, API oiled and API not visibly oiled GREG ($p=0.003$ and $p=0.001$ respectively) had significantly lower Hb concentrations than birds from reference sites. Across species, birds from API sites averaged 8% lower Hb than birds from reference sites. However, we found no statistically significant difference in MCHC between birds from API and reference sites.

Although RBC at treatment and reference sites did not differ statistically in BLSK and BRPE, GREG from API had lower RBC counts ($p=0.003$) than birds from reference sites (Figure 6). Post hoc analyses revealed that GREG from reference areas had significantly higher (16%) RBC counts than birds from API areas with no visible oiling ($p=0.006$), and marginally higher (9%) RBC counts than birds from API sites with visible oil ($p=0.05$). Red blood cell count was not determined in AMOY and thus this species was excluded from this analysis.

Although MCV did not differ among sites for BLSK, we found a significant difference between sites in BRPE ($p<0.001$) and GREG ($p<0.001$). Post hoc analyses revealed that BRPE from API sites with and without visible oiling had lower MCV than reference birds ($p<0.001$), while GREG from API sites with visible oil had lower MCV ($p<0.001$) and without visible oil had marginally lower MCV than birds from reference sites ($p=0.035$). Mean cell volume was not determined in AMOY and thus this species was excluded from this analysis.

We found no significant relationship between visible oiling and HAP concentration in AMOY, BRPE, or GREG. However, in BLSK we found a marginally significant difference in HAP concentration ($p=0.014$). Black skimmers from API sites with no visible oil had lower HAP concentrations than those from reference sites ($p=0.008$).

We found no significant relationship between visible oiling and ferritin concentration in AMOY, BLSK or BRPE. However, we found a significant difference among sites in ferritin concentration in response to visible oiling in GREG ($p<0.001$). Post hoc analyses revealed that GREG from reference areas had significantly higher ferritin than birds from API areas with visible oiling ($p<0.001$) and without visible oiling ($p<0.001$).
DISCUSSION

The DWH spill released an unprecedented volume of crude oil into the Gulf of Mexico (McNutt et al. 2012). While there were thousands of dead birds found in the weeks following the disaster, there were many more that were likely exposed to oil that suffered sublethal injury (United States Fish and Wildlife Service 2011). We demonstrated in this study that even birds with small amounts of oil present on their feathers can experience oxidative injury to erythrocytes and decreased numbers of circulating erythrocytes (PCV), and show evidence of a regenerative hematological response (i.e., increased reticulocytes) (Table 4). We also found hematological injury in birds from API sites without visible evidence of oiling when compared to reference birds. This finding is important, as it suggests that birds present in the API were experiencing oxidative injury even without visible evidence of oil exposure. While oil that was previously present on the feathers may have been preened and ingested, oil exposure can also occur from drinking and foraging on contaminated prey. A variety of negative effects of oil ingestion without external oiling has been reported in numerous experimental studies (Alonso-Alverez et al. 2007, Leighton 1986, Butler et al. 1986, Leighton et al. 1985). Together these findings provide strong evidence that relatively modest oil exposure may cause hematologic injury in coastal and marine birds.

The presence of intracellular inclusions consistent with Heinz bodies in birds of all four species with visible oil from impacted sites indicates that oxidative injury to erythrocytes had occurred in each of these species. Further, this oxidative injury was either the result of recent onset or was ongoing, as cells with Heinz bodies undergo lysis and are rapidly removed from circulation (Campbell and Ellis 2007). Conversely, in all of the individual birds from the reference sites \( n = 157 \) we found only a single bird with Heinz bodies present, indicating that oxidative injury that causes Heinz body formation is quite uncommon in unexposed populations of these four species. Such low background occurrence of Heinz bodies in unexposed populations makes this an attractive cellular response variable for field studies in these species.
Avian erythrocytes often contain retained reticular material that has affinity for new methylene blue stain. Some authors have found it difficult to distinguish Heinz bodies from retained reticular material in nestling birds, because very young birds have an abundance of immature cells containing large amounts of this reticulum (Leighton et al. 1985). Although the birds that we sampled represented a variety of age classes, none were nestlings. It is likely that light microscopy may underestimate the number of cells affected by oxidative injury, as not all cells that have undergone oxidative insult will develop Heinz body inclusions (Fallon et al. 2014). When possible, electron microscopy may provide a more sensitive and complementary screening tool for oxidative injury to erythrocytes, as it can identify additional ultrastructural changes to cells that may be overlooked by light microscopy such as damaged mitochondria and disruption of nuclear membranes (Leighton 1985).

Oxidative injury to erythrocytes is expected to result in a reduction in PCV or RBC and a subsequent regenerative response that includes an increase in reticulocytes. We found the predicted presence of Heinz bodies, increased reticulocytes, and decreased PCV in AMOY, BRPE and GREG from impacted sites. This cascade can cause a decrease in oxygen availability to tissues which can subsequently induce muscle fatigue, lethargy, decreased energy availability for metabolic processes, adverse reproductive impacts, and ultimately may have implications for survival and fitness (Brockus and Andreasen 2003, Butler et al. 1986, Piersma et al. 1996, Ots et al. 1998).

Our results indicate that there are species-specific differences in our target variables within both reference and API sites, highlighting the importance of species considerations when evaluating effects of oiling on avian physiology (Table 2, Harr 2002, Ferguson et al. 2014). In addition to inherent physiological variation among species, differences in foraging strategies, habitat preferences, and behaviors may play a role in differences in both exposure and effects among species. For example, we found that GREG was the only species with reference individuals that had evidence of visible oiling (n = 7 out of 48, Table 1). Among species in this study, GREG are most apt to frequently forage in drainage ponds and in pooled, standing water from residential or industrial run-off which may contain petroleum waste.
Therefore, individuals of this species may be at higher risk for exposure to petroleum from other sources than other species examined in this investigation.

Haptoglobin-like protein and ferritin have been suggested as markers for oxidative injury from crude oil exposure (Troisi et al. 2007). However, the documentation for the relationship between HAP and oil exposure is inconsistent and based on exposure to large amounts of oil (Troisi et al. 2007, Prichard et al. 1997). We found only marginally lower HAP in BLSK from API than reference birds and a trend towards decreased HAP in oiled individuals of all species when compared to birds from reference sites. Haptoglobin-like protein levels have been shown to vary widely in both reference and exposed individuals, and differences have been described among age classes (Prichard et al. 1997). Additionally, circulating HAP is known to increase in response to inflammation (Delers et al. 1998). Therefore, it is possible that oil-induced inflammation increases circulating HAP while hemolysis is consuming HAP, leading to a limited net effect.

We found that ferritin was related to oil exposure only in GREG (lower in birds from API than reference birds) and not in the other species evaluated in this study. This result contrasts with previous work that suggested that ferritin may be elevated in birds exposed to oil in order to protect against oxidation of bio-molecules (Troisi et al. 2007). Ferritin concentration in the serum can be affected by a number of environmental factors. It functions primarily to store iron, but it also operates to quench free radicals, such as hydrogen peroxide, and serves as an acute phase inflammatory protein. Moreover, ferritin has been found to vary by age and species (Theil 1987). In humans, serum ferritin has been found to vary by diet, activity level, and reticulocyte percentage, as erythropoietin-stimulated RBC production can reduce serum ferritin concentration (Alexander et al. 1994, Dufaux et al. 1981, Cavill 1999. Finally, a large proportion of ferritin within the body is found within cells, not in plasma where it has been historically measured in wild birds (Fallon et al. 2013, Troisi et al. 2007, Prichard et al. 1997, Ong et al. 2005). Taken together, our results suggest that ferritin and HAP may not be useful as specific biomarkers of trace and light oil exposure in contrast to their apparent utility in heavily oiled birds (Troisi et al. 2007).
Mean corpuscular volume and MCHC are values that can be used to help determine if anemia is regenerative (e.g., from hemolysis) or non-regenerative (e.g., chronic disease). Birds with hemolytic anemia have increased reticulocyte counts, which can often manifest with increased MCV and MCHC, because young red blood cells tend to be larger (i.e., increased MCV), and during hemolysis, there can be artifactually elevated MCHC due to increased free hemoglobin in the bloodstream. Here, we found that MCV did not differ between API and reference sites. These results may have occurred because while PCV was determined in a field laboratory shortly after collection, RBC was determined approximately 48 hours post collection. The time delay from collection to RBC determination may have resulted in a small amount of evaporation of plasma leading to an artifactually higher RBC count compared to PCV values [16]. Consequently, because MCV is the result of PCV/RBC, an artifactually elevated RBC would cause a relative decrease in MCV. Further, although MCHC was higher in all species of birds from API sites than birds from reference sites, the results were not statistically significant. It is also possible that chronic inflammation from ongoing crude oil exposure may have confounded these results, as chronic inflammation can contribute to reduced MCHC (Brockus and Andreasen 2003).

While circulating PAH concentrations may be important for identifying recent exposure and understanding dose-response relationships, the methodology for quantifying blood PAH levels is time consuming, expensive, and not always practical in field studies, particularly if large sample sizes are desirable or funding is limited. During oil spill events external visible oiling can be determined quickly and inexpensively following only minimal personnel training. We demonstrated here that trace and light visible oiling are correlated with adverse effects that include oxidative hemolysis and decreased PCV. Therefore, visible oiling represents a relatively cost- and time-effective strategy for assessing sub-lethal exposure under rapid response scenarios in the field and should be emphasized as a key component in future oil spill events.

In summary, our results demonstrated that birds exposed to oil from the DWH spill had evidence of oxidative injury to erythrocytes, decreased numbers of erythrocytes in circulation, and evidence of an erythrocytic regenerative
response. These changes are consistent with oxidative hemolytic anemia caused by exposure to oil. The pathologic implications of Heinz body formation and their relationship to petroleum exposure make them an important endpoint for health assessments following oil spill events, as Heinz body formation in birds is a pathological abnormality. In wild bird populations, anemia has been associated with decreased survival and has been linked to increased stress and immunosuppression as well as decreased reproductive success (Yorinks and Atkinson 2000, Applegate and Beaudion 1970, Hatch et al. 2010). These repercussions of anemia have obvious implications for individual fitness, and suggest that sublethal physiological injury associated with small amounts of oil exposure may have important negative long-term repercussions for individuals. Additionally, our work demonstrates that proper new methylene blue staining to enumerate reticulocytes and erythrocytic inclusions consistent with Heinz bodies is an important technique that can be applied under field conditions during petroleum-associated wildlife incidents (Fallon et al. 2013).

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### TABLE 1

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Table 1. Number of individuals and the degree of visible oiling for American oystercatcher (AMOY), black skimmer (BLSK), brown pelican (BRPE), and great egret (GREG) from reference sites and area of potential impact from the Deepwater Horizon oil spill (API).

### TABLE 2

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<tr>
<td>Hemoglobin</td>
<td>33.008</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MCHC</td>
<td>4.594</td>
<td>0.094</td>
</tr>
<tr>
<td>MCV</td>
<td>24.327</td>
<td>0.012**</td>
</tr>
</tbody>
</table>

* $p \leq 0.01$

** 0.05 $> p > 0.01$
Table 3. Species mean and standard error for Heinz bodies (cells with Heinz bodies/1000 erythrocytes), reticulocytes (%), haptoglobin (mg/ml), ferritin (ng/ml), PCV (%), hemoglobin (g/dl), RBC (cells/mm³), MCHC (%), and MCV (fl), found in AMOY, BLSK, BRPE, and GREG in reference areas and API (see Tables 1 and 2 for acronyms)

<table>
<thead>
<tr>
<th>Variable</th>
<th>AMOY</th>
<th>BLSK</th>
<th>BRPE</th>
<th>GREG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference</td>
<td>API</td>
<td>Reference</td>
<td>API</td>
</tr>
<tr>
<td></td>
<td>Range of</td>
<td>Sample size</td>
<td>Range of</td>
<td>Sample size</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10-21</td>
<td></td>
<td>18-29</td>
</tr>
<tr>
<td>Heinz Bodies</td>
<td>0(0)</td>
<td>6.71(2.69)</td>
<td>0(0)</td>
<td>10.22(3.27)</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>44(2.28)</td>
<td>59.86(2.15)</td>
<td>53.58(1.70)</td>
<td>66.75(2.81)</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>0.25(0.02)</td>
<td>0.26(0.02)</td>
<td>0.62(0.07)</td>
<td>0.39(0.04)</td>
</tr>
<tr>
<td>Ferritin</td>
<td>40.70(4.42)</td>
<td>30.1(3.88)</td>
<td>64.47(6.00)</td>
<td>56.22(4.30)</td>
</tr>
<tr>
<td>CV</td>
<td>47.38(0.76)</td>
<td>42(0.96)</td>
<td>44.66(0.86)</td>
<td>42.66(0.81)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>15.22(0.83)</td>
<td>NA</td>
<td>17.00(0.38)</td>
<td>16.67(0.61)</td>
</tr>
<tr>
<td>MCHC</td>
<td>NA</td>
<td>NA</td>
<td>2.71(0.03)</td>
<td>2.59(0.07)</td>
</tr>
<tr>
<td>MCV</td>
<td>36.27(1.77)</td>
<td>NA</td>
<td>38.16(1.25)</td>
<td>39.26(1.80)</td>
</tr>
<tr>
<td>PCV</td>
<td>NA</td>
<td>NA</td>
<td>162.77(2.91)</td>
<td>162.74(7.31)</td>
</tr>
</tbody>
</table>

*Endpoint not quantified due to limited blood quantity*
Table 4. Summary of the response of birds exposed to oil from the Deepwater Horizon Spill including Kruskal-Wallis results for Heinz bodies (cells with Heinz bodies/1000 erythrocytes), reticulocytes (%), PCV (%), RBC (cells/mm$^3$), hemoglobin (g/dl), MCHC (%), RBC (cells/mm$^3$), MCV (fl), haptoglobin (mg/ml), and ferritin (ng/ml) found in AMOY, BLSK, BRPE, and GREG (see Tables 1 and 2 for acronyms).

<table>
<thead>
<tr>
<th>Variable</th>
<th>AMOY</th>
<th>BLSK</th>
<th>BRPE</th>
<th>GREG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A priori prediction</td>
<td>$p$ value</td>
<td>Percent* difference</td>
<td>Consistent with prediction?</td>
</tr>
<tr>
<td>Heinz bodies</td>
<td>Increase 0.267 NA$^b$</td>
<td>&lt;0.001* NA$^b$</td>
<td>Yes</td>
<td>0.024** NA$^b$</td>
</tr>
<tr>
<td>reticulocytes</td>
<td>Increase &lt;0.001* 40.16%</td>
<td>0.004* 25.63%</td>
<td>Yes</td>
<td>&lt;0.001* 47.38%</td>
</tr>
<tr>
<td>PCV</td>
<td>Decrease &lt;0.001* -11.54%</td>
<td>0.164 -3.67%</td>
<td>Yes</td>
<td>&lt;0.001* -13.83%</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>Decrease NA$^c$ NA$^c$</td>
<td>0.876 -1.08%</td>
<td>Yes</td>
<td>0.002* -10.29%</td>
</tr>
<tr>
<td>MCHC</td>
<td>Increase NA$^c$ NA$^c$</td>
<td>0.878 3.31%</td>
<td>Yes</td>
<td>0.134 3.42%</td>
</tr>
<tr>
<td>RBC</td>
<td>Decrease NA$^c$ NA$^c$</td>
<td>0.613 -2.59%</td>
<td>Yes</td>
<td>0.561 -1.55%</td>
</tr>
<tr>
<td>MCV</td>
<td>Increase NA$^c$ NA$^c$</td>
<td>0.360 -5.97%</td>
<td>No</td>
<td>0.002* -15.39%</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Decrease 0.319 -7.03%</td>
<td>0.014** -24.97%</td>
<td>Yes</td>
<td>0.762 -14.73%</td>
</tr>
<tr>
<td>Ferritin</td>
<td>Increase 0.073 -32.58%</td>
<td>0.568 -6.87%</td>
<td>No</td>
<td>0.184 -41.85%</td>
</tr>
</tbody>
</table>

$^a$ Calculated from the mean values of reference area birds and birds from API sites with visible oil relative to the reference mean

$^b$ Percent difference in Heinz bodies could not be calculated because none were found for any reference birds except one GREG

$^c$ Endpoint not quantified due to limited blood quantity

* $p \leq 0.01$

** $0.05 > p > 0.01$
Proposed mechanism of oil-induced oxidative damage, its effects, and subsequent response

- Oil exposure → PAHs in circulation
- PAHs in circulation → Oxidative metabolites of PAHs
- Oxidative damage to Hb → Heinz body formation
- Heinz body formation → Hemolysis and liberation of Hb, iron
- RBCs in circulation → Decreased PCV and RBC
- Anemia: Decreased PCV and RBC
- Regenerative response: Increased reticulocytes, Stable or increased MCHC, Increased MCV
- Increased FT Decreased HAP

Figure 1. Conceptual diagram of a proposed mechanism of polycyclic aromatic hydrocarbons (PAH) induced oxidative damage, its effects, and subsequent response in birds. Included are a priori predictions of the response of variables Heinz bodies, reticulocytes, hemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBC), ferritin (FT), haptoglobin (HAP), mean corpuscular hemoglobin concentration (MCHC), and mean cell volume (MCV) to oil exposure. Inset shows photomicrograph of avian reticulocyte (R) and Heinz body inclusions (HB) in a black skimmer.
Figure 2: Number of erythrocytes containing Heinz bodies/1000 erythrocytes (Mean +/- SE) found in American oystercatcher (AMOY, $n = 38$), black skimmer (BLSK, $n = 108$), brown pelican (BRPE, $n = 83$), and great egret (GREG, $n = 87$) from reference areas (non-shaded bars), areas of potential impact with no visible oil (shaded bars), and areas of potential impact (API) from the Deepwater Horizon oil spill with visible oil (hashed bars). Asterisk indicates significant difference ($p<0.01$).
Figure 3: Number of reticulocytes/1000 erythrocytes (Mean +/- SE) found in American oystercatcher (AMOY, n = 38), black skimmer (BLSK, n = 108), brown pelican (BRPE, n = 83), and great egret (GREG, n = 87) from reference areas (non-shaded bars), areas of potential impact (API) from the Deepwater Horizon oil spill with no visible oil (shaded bars), and areas of potential impact with visible oil (hashed bars). Asterisks indicate significant difference (p≤0.01) from reference sites.
Figure 4: Percent packed cell volume (PCV) (Mean +/- SE) found in American oystercatcher (AMOY, n = 50), black skimmer (BLSK, n = 103), brown pelican (BRPE, n = 81), and great egret (GREG, n = 89) from reference areas (non-shaded bars), areas of potential impact (API) from the Deepwater Horizon oil spill with no visible oil (shaded bars), and areas of potential impact with visible oil (hashed bars). Asterisks indicate significant difference ($p \leq 0.01$) from reference sites.
Figure 5: Hemoglobin (Hb) (Mean +/- SE) found in black skimmer (BLSK, \(n=78\)), brown pelican (BRPE, \(n=71\)), and great egret (GREG, \(n=89\)) from reference areas (non-shaded bars), areas of potential impact with no visible oil (shaded bars), and areas of potential impact (API) from the Deepwater Horizon oil spill with visible oil (hashed bars). Asterisks indicate significant difference (\(p<0.01\)) from reference sites. American oystercatchers were excluded from this figure because no Hb data were collected for birds from reference areas.
Figure 6: Red blood cell count (RBC) (Mean +/- SE) found in black skimmer (BLSK, n = 93), brown pelican (BRPE, n = 47), and great egret (GREG, n = 74) from reference areas (non-shaded bars), areas of potential impact with no visible oil (shaded bars), and areas of potential impact (API) from the Deepwater Horizon oil spill with visible oil (hashed bars). Asterisks indicate significant difference (p<0.01) and ** indicates marginal (0.01 > p ≤ 0.05) from reference sites. American oystercatchers were excluded from this figure because no RBC data were quantified for this species.
Chapter 3. Ultraviolet-assisted oiling assessment improves detection of oiled birds experiencing clinical signs of hemolytic anemia after exposure to the Deepwater Horizon oil spill


*Formatted for Ecotoxicology

ABSTRACT

While large-scale oil spills can cause acute mortality events in birds, there is increasing evidence that sublethal oil exposure can trigger physiological changes that have implications for individual performance and survival. Therefore, improved methods for identifying small amounts of oil on birds are needed. Because ultraviolet (UV) light can be used to identify thin crude oil films in water and on substrate that are not visually apparent under normal lighting conditions, we hypothesized that UV light could be useful for detecting small amounts of oil present on the plumage of birds. We evaluated black skimmers (Rynchops niger), brown pelicans (Pelecanus occidentalis), clapper rails (Rallus crepitans), great egrets (Ardea alba), and seaside sparrows (Ammodramus maritimus) exposed to areas affected by the Deepwater Horizon oil spill in the Gulf of Mexico as well as from reference areas from 20 June, 2010 to 23 February, 2011. When visually assessed without UV light, 19.6% of birds evaluated from areas affected by the spill were determined to be oiled (previously published data), whereas when examined under UV light, 56.3% of the same birds were determined to have oil exposure. Of 705 individuals examined in areas potentially impacted by the spill, fluorescence under UV light assessment identified 259 oiled birds that appeared to be oil-free on visual exam, supporting its utility as a simple tool for improving detection of modestly oiled birds in the field. Further, UV assessment revealed an increase in qualitative severity of oiling (approximate % of body surface oiled) for 40% of birds
compared to what was determined on visual exam. Additionally, black skimmers, brown pelicans, and great egrets exposed to oil as determined using UV light experienced oxidative injury to erythrocytes, had decreased numbers of circulating erythrocytes, and showed evidence of a regenerative hematological response in the form of increased reticulocytes. This evidence of adverse effects was similar to changes identified in birds with oil exposure as determined by visual examination without UV light, and is consistent with hemolytic anemia likely caused by oil exposure. Thus, UV assessment proved useful for enhancing detection of birds exposed to oil, and identified birds that were experiencing clinical signs of anemia. We conclude that UV light evaluation can help identify oil exposure in many birds that would otherwise be identified visually as unexposed during oil spill events.

Key words: Deepwater Horizon, oil spill, Heinz bodies, ultraviolet fluorescence, hemolytic anemia

INTRODUCTION

Acute avian mortality associated with large-scale oil spills is well documented, with seabirds, waterfowl, and colonial waterbirds at particular risk (e.g., Piatt et al. 1990; Iverson and Esler 2010; Munilla et al. 2011; USFWS 2011). However, there is increasing evidence that sublethal exposure (modest external oiling that does not result in rapid mortality) can have important impacts upon individual health and may affect population dynamics, which could influence damage assessments and subsequent restoration and mitigation efforts (Seiser et al. 2000; Trust et al. 2000; Golet et al. 2002; Alonso-Alvarez et al. 2007; Harr et al. 2017; Fallon et
al. 2018). Consequently, it is important not only to identify birds that have died from oil exposure, but also to identify birds with sublethal exposure to better estimate the number of birds at risk following both small- and large-scale oil spill events. However, identifying small amounts of oil on feathers can be difficult, especially in birds with dark plumage, under natural lighting conditions. Failure to detect modest oiling may result in inaccurate estimates of the number of birds exposed during oil spills events. Therefore, there is a need for simple, reliable techniques to identify small amounts of oil on birds.

Crude oil fluoresces under ultraviolet (UV) light (Burlamacchi 1983; Colligan and LaManna 1993; Fingas and Brown, 2014). This attribute has led to its use to improve detection of oil in several abiotic matrices. For example, UV light can be used to enhance detection of oil on or below the surface of a body of water as well as on snow and ice (Fingas and Brown 2000; Fingas and Brown 2013). Remote UV sensors are commonly used to monitor oil films associated with spills in very thin layers down to 0.1 µm (Fingas and Brown 1997, Brekke and Solberg 2005; Jha et al. 2008). Thus, we hypothesized that UV light may also be useful to detect small amounts of oil on the feathers of captured birds that might not be apparent under normal light conditions.

Birds experiencing modest exposure to crude oil can experience myriad physiological effects, including inflammation, immunosuppression, and oxidative damage to cells (Fry et al. 1986; Leighton 1995; Briggs et al. 1996; Golet et al. 2002). These sublethal effects can negatively impact growth, alter organ function, reduce reproductive success, and likely increase risk of disease (Briggs et al. 1996; Esler et al. 2000; Giese et al. 2000; Eppley and Rubega 1990; Alonso-Alvarez et al. 2007). Of the sublethal, physiological impacts resulting from avian exposure to oil spills, oxidative damage to erythrocytes and subsequent anemia are of particular interest during oil spill investigations, as such injury can be evaluated in blood samples taken from live birds.
Hemolytic anemia has been demonstrated in several species of birds exposed to crude oil under both experimental (e.g., Leighton et al. 1983; 1985; Fry and Lowenstein 1985; Harr et al. 2017) and natural conditions (Yamato et al. 1996; Troisi et al. 2007; Fallon et al. 2018). Although most work has focused on severe oiling, recent evidence indicates that small amounts of visible oiling correlate with oxidative injury to erythrocytes in several species of birds (Fallon et al. 2018).

In this investigation, we evaluated the utility of UV fluorescence as a tool to identify birds with small amounts of oil on their plumage that might otherwise be missed by traditional visual oiling assessment during and in the immediate aftermath of the 2010 Deepwater Horizon oil spill in the Gulf of Mexico, USA. To achieve this objective, we determined the presence and severity of oiling (% of body surface oiled) by visual inspection under natural light (visual oiling assessment) and then again under UV light (UV oiling assessment) in black skimmers (Rynchops niger, BLSK), brown pelicans (Pelecanus occidentalis, BRPE), great egrets (Ardea alba, GREG), clapper rails (Rallus crepitans, CLRA), and seaside sparrows (Ammodramus maritimus, SESP). Second, we evaluated relationships between severity of visible oiling, UV oiling, and a suite of hematologic parameters characteristic of adverse effects from oil exposure in BLSK, BRPE, and GREG. We evaluated these relationships to determine whether UV oiling assessment improved detection of the number of birds experiencing adverse clinical signs compared to those detected through visual assessment alone.

METHODS

Study area and focal species

Our five focal species represent a diversity of ecological niches, which could influence their relative susceptibility to oil exposure. Clapper rails and seaside sparrows are year-round
residents along the Gulf Coast, inhabit salt marshes surrounded by open water, and are omnivorous, eating seeds and marine invertebrates (Post and Greenlaw 2009; Rush et al. 2012). Black skimmers, recognized as a species with declining populations (Vieira et al. 2018), were selected because of their unique surface water foraging strategy and because their nesting habits (sand and shell beaches and islands) put them at high risk for exposure to oil (Gochfeld and Burger 1994). Brown pelicans eat mostly fish and are at high risk of dermal exposure to oil because they capture their food most often by diving after prey (Shields 2002). Great egrets forage for food in a wide range of habitats and are unique among the other species evaluated in this study because they are a wading bird that has diverse prey items, including fishes, insects, marine invertebrates, small mammals, reptiles, and amphibians (McCrimmon et al. 2011).

The procedures involving animals were conducted by Biodiversity Research Institute personnel with approval from the US Fish and Wildlife Service. We captured BLSK ($n = 120$), BRPE ($n = 66$), CLRA ($n = 100$), GREG ($n = 54$), and SESP ($n = 365$) from reference areas and areas impacted by the Deepwater Horizon spill from 20 June, 2010 until 23 February, 2011. We captured BLSK with noose mats, box traps, and cannon-nets, BRPE with noose traps, padded leg-hold traps, and net guns, CLRA by hand with night lighting from airboats as well as with drift fences leading to box traps, GREG with net guns, and SESP with targeted mist netting (Mills and Ryder 1979; Crozier and Gawlik 2003; Herring et al. 2008; Perkins et al. 2010).

Sites affected by the Deepwater Horizon spill included locations along coastal Louisiana, with five BRPE collected from coastal Mississippi, where exposure to oil from the Deepwater Horizon spill was likely (Figure 1). Reference sites for BLSK, BRPE, and GREG included various locations along coastal South Carolina and Georgia, USA where no recent oiling events had been recorded (Figure 2). Because CLRA and SESP maintain small home ranges, reference sites for
these two species included saline *Juncus* marshes, saline *Spartina* marshes, and brackish *Phragmites* marshes with no visible oil along coastal Louisiana, Mississippi, and Alabama (Figure 1). We banded all birds with leg bands appropriate for each species and released them at the capture location after we completed oiling assessments and sample collection.

*Visible and UV oiling assessment*

We evaluated the majority of birds captured in both oiled and reference sites for evidence of visible oiling under natural lighting conditions, and assigned a visible oiling score of none (0% of plumage affected with visible oil), trace (<5% plumage affected), light (6-20% plumage affected) moderate (21-40% plumage affected) or heavy (> 40% plumage affected; see Supplemental Figures 1 and 2). We completed this examination under physical restraint appropriate for each species with wings in both extension and normal standing posture. We then placed each bird under an opaque canvas cover to block out natural light and exposed the plumage to UV light (Labino compact PH135 UV spotlight, Labino AB Solna, Sweden, 365 nm peak UV-A). We assigned a separate UV oiling score using the same five oil score categories. Thus, the same birds were categorized using two independent techniques (visible and UV oiling) to determine whether the use of a UV light source improved detection and severity of oiling (see example in Supplemental Figure 3).

*Blood collection and sample handling*

We collected blood in a subset of individual BLSK, BRPE, and GREG from the medial metatarsal vein or superficial ulnar vein using a 21G or 23G butterfly catheter and lithium heparin and ethylenediaminetetraacetic acid (EDTA) vacutainers. These three species were selected for hematological analyses due to ease of blood collection and their relatively large body size. Immediately following collection, we filled two heparinized hematocrit tubes for packed cell
volume (PCV) analysis. At this time, we also prepared new methylene blue-stained blood smears to quantify Heinz bodies and reticulocytes as described below. We placed remaining blood samples on ice and transferred them to the field laboratory. Once in the field laboratory, we prepared two additional EDTA-treated blood smears for complete blood cell analysis using a standard two-slide technique (Aird 2010).

**Hematologic parameters**

The hematological assessment methods and results, including our new methylene blue staining technique as well as reticulocyte and Heinz body identification, have been described in detail previously (Fallon et al. 2018). For BLSK, BRPE, and GREG, we prepared new methylene blue-stained blood smears in the field after incubating for 20 minutes (Fallon et al. 2013). We evaluated 1000 erythrocytes under 1000X light microscopy, counting the number of cells affected by Heinz bodies as well as the number of reticulocytes. The individual performing these analyses (JAF) was blinded to oiling status, capture location, and results of other analyses.

We determined PCV and hemoglobin (Hb) from heparinized samples within 12 hours of collection. Packed cell volume (%) was calculated using a standard hematocrit reader following centrifugation at 11,865 x g for 5 minutes. Total Hb (g/dl) was quantified using a Hemocue Hb Analyzer Hb201 (Velguth et al. 2010). Red blood cell count (RBC, cells/mm3) was estimated via standard manual methodology using a hemocytometer at a commercial laboratory (Avian and Exotics Clinical Pathology Laboratory, Wilmington, OH, USA) (Campbell 1995). Individuals performing these analyses were blinded to oiling status, capture location, and results of other analyses. Hematological results of these parameters in birds from reference sites and birds with visible oiling were first reported in Fallon et al. (2018), but are reanalyzed here in relation to the current UV oiling assessment.
Statistical analyses

We used SAS software (version 9.3 SAS Institute Inc., Cary, NC, USA) for all analyses. Where appropriate, we evaluated normality and homogeneity of variance using Shapiro-Wilk and Levene’s tests, respectively. We used univariate statistical tests for physiological variable analyses, as this dataset contained missing values for one or more values from several birds (for more details, see Fallon et al. 2018). To account for the lack of independence of physiological responses compared in our univariate models, we applied a conservative \( \alpha < 0.01 \) to assess statistical significance in these models, while also noting cases where \( \alpha > 0.01 \) and \( \alpha < 0.05 \).

To determine the utility of UV light assessment as a tool to identify birds with small amounts of oil on the plumage that would otherwise be missed by visual oiling assessment, we used McNemar’s exact test to compare the number of birds with visible oil to the number of birds with UV oiling from areas affected by the Deepwater Horizon spill within each species. Additionally, we calculated the number of birds that increased one or more category in oiling severity under the application of UV light (e.g., a bird that was categorized as light oiling under visual assessment appeared as moderate oiling under UV assessment) and compared the effect size of this change in severity using Cliff’s delta (Cliff 1993, Romano et al. 2006, Macbeth et al. 2011). Cliff’s delta is a measure of the degree of overlap between two populations, and ranges from \(-1\) to \(+1\), with +/-0.147 representing a small effect (percent of non-overlap is 14.7%), +/-0.33 representing a moderate effect (percent of non-overlap is 33%), and > +/-0.474 representing a large effect (percent of non-overlap is 47.4% (Cohen 1988).

To determine whether UV oiling assessment improved detection of birds experiencing adverse clinical signs compared to those detected through visual assessment alone, we first determined the effects of UV-detectable oiling on Heinz body formation, reticulocytes, PCV, Hb,
and RBC using Kruskal-Wallis tests for each species with subsequent post-hoc analysis (SAS Multtest procedure). Additionally, we used Mann-Whitney tests to compare physiologic parameters (Heinz bodies, reticulocytes, PCV, and Hb) from birds with visible oiling to the subset of birds that had no evidence of visible oiling but tested positive for oiling under UV light.

Because we had physiological data on a limited number of birds with UV oil but no visible oil, we pooled species for this analysis (Heinz bodies: BLSK \( n = 18 \), BRPE \( n = 8 \), GREG \( n = 5 \) \([n = 31\) total]; reticulocytes: BLSK \( n = 18 \), BRPE \( n = 8 \), GREG \( n = 5 \), \([n = 31\) total]; PCV: BLSK \( n = 19 \), BRPE \( n = 8 \), GREG \( n = 7 \), \([n = 34\) total]; Hb: BLSK \( n = 11 \), BRPE \( n = 6 \), GREG \( n = 7 \), \([n = 24\) total]).

**RESULTS**

**UV oiling assessment**

From our sample of 705 birds from areas impacted by the Deepwater Horizon spill, we identified 138 birds with evidence of visible oiling. However, the number of oiled birds from the same sample population increased by 259 individuals to 397 birds once we viewed them under UV light. Therefore, use of UV light increased the overall percentage of oiled birds in our sample population from 19.6% to 56.3% (Table 1, Figure 3). Importantly, improved detection with the aid of a UV light occurred in all five species (Table 1, Figure 3). Additionally, we found evidence that the qualitative categorization of oiling severity increased when we evaluated birds under UV light; 40% of birds increased by at least one category (e.g., an increase from lightly to moderately oiled) after using UV compared to a standard visual exam (Figure 4). Cliff’s delta analysis revealed a moderate effect size on oiling severity with UV evaluation (delta = 0.288). No BLSK \( n = 87 \), BRPE \( n = 39 \), or SESP \( n = 55 \) from reference locations were found to have oil on 71
their plumage either by visual or UV assessment. We found no CPRL \((n = 30)\) from reference sites with oil on standard visual assessment, but one individual was found to have trace amounts under UV assessment. In contrast, of 47 GREG from reference sites, 7 individuals had evidence of trace visible oil and 27 had trace or light UV oil.

**Physiological Correlates of UV Oiling**

The physiological responses associated with visible oiling were originally reported elsewhere (Fallon et al. 2018). Here, we focus on comparing the physiological responses detectable based on the two oiling assessment protocols. We found significantly more Heinz bodies and reticulocytes in BLSK, BRPE, and GREG with UV oiling compared to reference populations (Table 2). Additionally, we found significantly lower PCV and Hb in BRPE and GREG, as well as decreased RBC in GREG with UV oiling compared to reference populations (Table 2). Based on visual comparison of statistical outcomes of our previous study and the current study, we found that the effect of oiling on Heinz body formation, reticulocytes, PCV, Hb, and RBC counts was similar for both visual (data from Fallon et al. 2018) and UV assessment techniques (Table 3). Additionally, we found no significant difference between the mean number of Heinz bodies, reticulocytes, PCV, or Hb between birds that had only oiling detectable with UV light versus birds with oil apparent on both visual and UV oiling assessment (Table 4).

**DISCUSSION**

It is important to understand the extent of exposure and injury to birds and other wildlife during oil spill events to develop an accurate damage assessment. While there were thousands of dead birds found in the weeks following the Deepwater Horizon spill, there were many more that
were likely exposed to oil but did not immediately succumb (Peterson et al. 2003; USFWS 2011). We hypothesized that the use of hand-held UV lights could enhance the sensitivity of visible oiling assessments, because even trace amounts of oil have been shown to fluoresce (Chase et al. 2005). To test this, we evaluated 705 individuals from sites affected by the Deepwater Horizon oil spill with both visual and UV assessment. Ultraviolet assessment identified oiling on 259 individual birds (a 97% increase in detection) that appeared to be oil-free on initial visual examination. Ultraviolet assessment resulted in a significant increase in the number of individuals determined to be oiled for all species, although this effect was least pronounced in GREG (Table 1, Figure 3). Adult GREG have exclusively white plumage, which likely makes even trace amounts of oil more apparent on visual exam. Additionally, we found that UV assessment revealed that birds had more extensive exposure than was apparent on visual assessment, with 40% of birds increasing by at least one category of oiling severity after application of the UV light (Figure 4). Cliff’s delta analysis confirmed that this effect size was statistically significant. Together, these findings suggest that UV assessment can more accurately determine the number and severity of birds exposed to oil during spill events compared to visual assessment without the aid of UV light.

We found that birds with oil identified with UV light were experiencing adverse effects similar to those we had observed in our prior visual assessment (Fallon et al., 2018). Birds with small amounts of oil on their plumage as determined by UV evaluation had hematological changes consistent with oxidative injury to red blood cells (Table 2, 3). The presence of Heinz bodies combined with increased reticulocytes found in oiled BLSK, BRPE, and GREG with UV oiling suggests that this method can be used to detect birds with modest oiling that may be experiencing sublethal physiological injury. Additionally, BRPE and GREG from impacted sites
had decreased PCV and Hb. These three features—presence of Heinz bodies, decreased PCV or Hb, and increased reticulocytes—are indicative of oxidative injury, anemia, and a physiological regenerative response. This physiologic cascade decreases oxygen availability to tissues (Latimer et al. 2003) which can induce muscle fatigue, lethargy, and decreased energy availability for metabolic processes, and adversely affect reproduction (Butler et al. 1986; Piersma et al. 1996; Walton et al. 1997; Ots et al. 1998; Hylton et al. 2006). These physiological changes have implications for survival and fitness, suggesting that sublethal physiological injury associated with modest oil exposure may have important negative long-term repercussions for individuals.

Although there is no clearly established threshold for what degree of reduced erythrocyte volume leads to decreased survival, anemia in oiled birds at admission to rehabilitation facilities is correlated with higher mortality rates (Duerr et al. 2016). Our results suggest that UV assessments can be useful in identifying birds with very small amounts of oil that also have experienced adverse effects, and that these hematological changes mirror those found with visible oiling (Fallon et al. 2018).

Although all BLSK, BRPE, and SESP and all but one CPRL from reference sites had no evidence of visual or UV oil on their plumage, GREG appeared to be at increased risk of oil exposure in our reference sites. Of 47 GREG from reference sites evaluated by both visual and UV assessment, 7 birds (15%) had trace visual oiling and 27 (57%) had trace (n = 26) or light (n = 1) UV oiling. Of our study species, GREG are the only species with exclusively white plumage, which may make small amounts of oil more easily discernible. Additionally, this species is unique among those in this study, as it is a wading bird that spends a great deal of foraging time standing or slowly wading through water, and frequents man-made drainage ponds and pooled, standing water from residential, agricultural, or industrial run-off which may contain petroleum waste.
(Trail 2006; McCrimmon et al. 2011). Consistent with their exposure, this was the only species in which Heinz bodies were identified in the reference population. Our results suggest that further investigation into the frequency of exposure to petroleum products in this species is warranted.

There are several limitations to consider when incorporating UV light assessment during oil spill events. First, the individual bird must be evaluated under minimal natural light, which is cumbersome with large birds. Second, although application of UV light increased the detection of oiled birds, the majority of birds that appeared to be oil-free on visual examination but were determined to be oiled under UV light application had only trace or light amounts of oil on their plumage (5-20% of plumage affected). Because of this, the severity of hematologic changes in the UV-oiled population of birds was similar to that of visibly oiled birds (Table 3, Figure 4). Thus, UV assessment not only proved useful for enhancing detection of birds exposed to oil, but these birds also suffered hematologic injury similar to visibly-oiled birds. Finally, there is the possibility of false positive fluorescence with naturally occurring oils. Further work in an experimental setting may help determine the frequency of false positive results.

In summary, our results demonstrate that UV assessment can identify small amounts of oil present on birds that appear oil-free on visual exam. Additionally, UV light allowed detection of oiled feathers over a larger proportion of surface area on individuals than can be seen on visual exam. Therefore, UV assessment of individual birds could be considered as an additional tool following both large and small oil spill events to help formulate a more complete damage assessment. This technique may be most useful to categorize birds with trace oiling that would otherwise be missed on visual exam, particularly in birds with dark plumage. Further, UV-oiled birds exposed to the Deepwater Horizon spill had evidence of oxidative injury to erythrocytes, decreased numbers of erythrocytes in circulation, and evidence of an erythrocytic regenerative
response, similar to birds with visible oiling. These changes are consistent with formation of Heinz bodies and oxidative hemolytic anemia, a pathological abnormality caused by exposure to oil.

Compliance with Ethical Standards:

Conflict of Interest: Jesse A. Fallon declares that he has no conflict of interest. Eric P. Smith declares that he has no conflict of interest. Nina Schoch declares that she has no conflict of interest. James D. Paruk declares that he has no conflict of interest. Evan A. Adams declares that he has no conflict of interest. David C. Evers declares that he has no conflict of interest. Patrick G.R. Jodice declares that he has no conflict of interest. Marie Perkins declares that she has no conflict of interest. Dustin E. Meattey declares that he has no conflict of interest. William A. Hopkins declares that he has no conflict of interest.

Ethical Approval: The procedures involving animals were conducted by Biodiversity Research Institute personnel with approval from the U.S. Fish and Wildlife Service. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

ACKNOWLEDGEMENTS

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Jason Fidorra who led the collection of the GREG samples; Mike Yates and Jim Dayton who coordinated sample collection from BRPE. Additionally, BLSK, BRPE, GREG samples were processed in BRI’s field laboratory by a dedicated team who provided excellent assistance, including Dr. Michelle Walsh, Dr. Lee Friedman, Ruth Valentine, Judi Ellal, Michelle Brown, Alishia Zyer, Tim Watson, and Ken Weber. Jeff Walters, Eric Hallerman, and Todd Katzner provided helpful comments that improved the manuscript. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

LITERATURE CITED


Harr KE, Cunningham FL, Pritsos CA, Pritsos KL, Muthumalage T, Dorr BS, Horak KE, Hanson-Dorr KC, Dean KM, Cacela D, McFadden AK (2017) Weathered MC252 crude oil-
induced anemia and abnormal erythroid morphology in double-crested cormorants


evaluating group differences on the NSSE and other surveys: Are the t-test and Cohen’s d indices the most appropriate choices? In Proceedings of the Annual Meeting of the Southern Association for Institutional Research, Arlington, VA, USA, pp14–17.


### Table 1

Number of individual black skimmer (BLSK), brown pelican (BRPE), clapper rail (CLRA), great egret (GREG), and seaside sparrow (SESP) from areas affected by the Deepwater Horizon oil spill with McNemar’s test comparing visible oiling and UV fluorescence (*statistical significance).

<table>
<thead>
<tr>
<th></th>
<th>Total (N)</th>
<th>Visible Oil</th>
<th>UV Oil</th>
<th>Chi²</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLSK</td>
<td>120</td>
<td>47</td>
<td>92</td>
<td>45.00</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BRPE</td>
<td>66</td>
<td>21</td>
<td>35</td>
<td>14.00</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CLRA</td>
<td>100</td>
<td>14</td>
<td>82</td>
<td>65.06</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>GREG</td>
<td>54</td>
<td>38</td>
<td>46</td>
<td>8.00</td>
<td>0.005*</td>
</tr>
<tr>
<td>SESP</td>
<td>365</td>
<td>18</td>
<td>142</td>
<td>142.00</td>
<td>0.001*</td>
</tr>
<tr>
<td>Total</td>
<td>705</td>
<td>138</td>
<td>397</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Summary of the response of birds exposed to oil based on UV oiling assessment from the Deepwater Horizon oil spill.

Kruskal-Wallis results are reported for Heinz bodies (cells with Heinz bodies/1000 erythrocytes), reticulocytes (%), PCV (%), hemoglobin (g/dl), and RBC (cells/mm$^3$) found in black skimmers (BLSK), brown pelicans (BRPE), and great egrets (GREG). Percent differences calculated from the mean values from birds from reference areas (BLSK $n = 57$, BRPE $n = 32$, GREG $n = 46$) and birds from Deepwater Horizon affected (impacted) sites with UV detected oil (BLSK $n = 51$, BRPE $n = 44$, GREG $n = 51$) relative to the reference mean. Asterisk represents significance ($p \leq 0.01$) and ** indicates marginal significance (0.05 $\geq p > 0.01$). N/A indicates that results were not determined for this variable in a particular bird except one GREG. Mean reference results previously published in Fallon et al. 2018).

<table>
<thead>
<tr>
<th>Variable</th>
<th>BLSK</th>
<th>BRPE</th>
<th>GREG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Reference</td>
<td>Mean</td>
</tr>
<tr>
<td>Heinz bodies</td>
<td>0.000</td>
<td>9.34</td>
<td>N/A</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>53.6</td>
<td>67.3</td>
<td>0.004</td>
</tr>
<tr>
<td>PCV</td>
<td>44.7</td>
<td>43.1</td>
<td>0.137</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>17.0</td>
<td>16.8</td>
<td>0.841</td>
</tr>
<tr>
<td>RBC</td>
<td>2.71</td>
<td>2.64</td>
<td>0.905</td>
</tr>
</tbody>
</table>
Table 3. Summary of results ($p$ values) from Kruskal-Wallis analysis of oiled birds as determined by visual assessment under natural lighting conditions (data from Fallon et al. 2018) compared to reference populations and UV- assisted assessment compared to reference populations. Response variables were Heinz bodies (cells with Heinz bodies/1000 erythrocytes), reticulocytes (%), packed cell volume (PCV, %), hemoglobin (Hb, g/dl), and red blood cell count (RBC, cells/mm$^3$) found in black skimmers (BLSK), brown pelicans (BRPE), and great egrets (GREG).

<table>
<thead>
<tr>
<th></th>
<th>Heinz Bodies</th>
<th>Reticulocytes</th>
<th>PCV</th>
<th>Hb</th>
<th>RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLSK</td>
<td>UV oiled</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>0.137</td>
<td>0.841</td>
</tr>
<tr>
<td></td>
<td>Visibly oiled</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>0.164</td>
<td>0.876</td>
</tr>
<tr>
<td>BRPE</td>
<td>UV oiled</td>
<td>0.028</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Visibly oiled</td>
<td>0.024</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>GREG</td>
<td>UV oiled</td>
<td>&lt;0.001</td>
<td>0.007</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Visibly oiled</td>
<td>0.003</td>
<td>0.010</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 4. Mann-Whitney comparison of mean number of Heinz bodies (number of cells/1000 erythrocytes), reticulocytes (%), packed cell volume (PCV, %), and hemoglobin (g/dl) between birds (pooled species including black skimmers, brown pelicans, and great egrets) that tested positive for oiling under both UV and natural (visible) light versus those that tested positive under UV light only from sites impacted by the Deepwater Horizon oil spill.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Visible and UV oil</th>
<th>UV oil only</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heinz bodies</td>
<td>79 8.22 (1.99)</td>
<td>31 2.87 (1.58)</td>
<td>0.355</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>79 69.08 (2.10)</td>
<td>31 64.84 (3.09)</td>
<td>0.597</td>
</tr>
<tr>
<td>PCV</td>
<td>78 40.40 (0.57)</td>
<td>34 40.24 (0.94)</td>
<td>0.730</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>73 14.88 (0.38)</td>
<td>24 15.40 (0.53)</td>
<td>0.320</td>
</tr>
</tbody>
</table>

*
**Fig. 1** Area of potential impact capture locations in Louisiana, Mississippi and Alabama for American oystercatcher, black skimmer, brown pelican, and great egret. Also shown are reference capture locations and area of potential impact capture locations for clapper rail and seaside sparrow. Because these two species maintain small home ranges, we classified *Juncus* marshes, saline *Spartina* marshes, and brackish *Phragmites* marshes with no known direct connectivity to the oil spill area as reference sites.
Fig. 2 Reference capture locations in South Carolina for American oystercatcher, black skimmer, brown pelican, and great egret.
Fig. 3 Percent of black skimmer (BLSK), brown pelican (BRPE), clapper rail (CLRA), great egret (GREG), and seaside sparrow (SESP) in areas of potential impact from the Deepwater Horizon oil spill with visible oiling and oil detected under UV fluorescence. Asterisks indicate statistically significant difference ($p < 0.05$) between visible and UV detection techniques.
Fig. 4 Severity of visible oiling (none is 0% of plumage affected with UV oiling, trace <5% plumage affected, light 6-20% plumage affected, moderate 21-40% plumage affected or heavy > 40% plumage affected) and oil detected under ultraviolet fluorescence in birds from areas affected by the Deepwater Horizon spill.
Supplemental Fig. 1 Evaluation of severity of oiling requires extension of the wing and spreading of the tail. In the image above, there is oil present on the tail feathers of a great egret (Ardea alba) with light oiling (left) and on the primary flight feathers of another great egret with trace oiling (right).
Supplemental Fig. 2 Restraint and visual examination of a clapper rail (*Rallus crepitans*) during evaluation for oil exposure in normal standing posture (left) and during wing extension (right) in the aftermath of the Deepwater Horizon oil spill. Extension of the wings reveals more severe oiling than is apparent when the bird is restrained in a standing position. This individual is an example of heavy oiling (>40% of plumage affected).
Supplemental Fig. 3 Restraint of a seaside sparrow (*Ammodramus maritimus*) during evaluation of plumage for oil exposure under natural light (left) and ultraviolet light (right). During visual exam, trace amounts of oil are difficult to identify on the dark wing plumage of this seaside sparrow (left). When viewed under ultraviolet light, trace amounts of oil are identified as more obvious fluorescent yellow-green material (arrows, right). Fluorescent areas were not present on seaside sparrows in areas unaffected by the oil spill.
Chapter 4. Hematological and histological changes from ingestion of Deepwater Horizon crude oil in zebra finches (Taeniopygia guttata)

Coauthors: Christopher Goodchild, Sarah E. DuRant, Thomas Cecere, D. Phillip Sponenberg, William A. Hopkins

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ABSTRACT

Exposure to crude oil during spill events causes a variety of pathologic effects in birds, including oxidative injury to erythrocytes, which is characterized in some species by the formation of Heinz bodies and subsequent anemia. However, not all species appear to develop Heinz bodies or anemia when exposed to oil, and there are limited controlled experiments that use both light and electron microscopy to evaluate structural changes within erythrocytes following oil exposure. In this study, we orally dosed zebra finches (Taeniopygia guttata) with 3.3 or 10 mL/kg of artificially weathered Deepwater Horizon crude oil or 10 mL/kg of peanut oil (vehicle control) daily for 15 days. We found that birds receiving the highest dosage experienced a significant increase in reticulocyte percentage, mean corpuscular hemoglobin concentration, and liver mass, as well as inflammation of the gastrointestinal tract and lymphocyte proliferation in the spleen. However, we found no evidence of Heinz body formation based on both light and transmission electron microscopy. Although there was a tendency for packed cell volume and hemoglobin to decrease in birds from the high dose group compared to control and low dose groups, the changes were not statistically significant. Our results indicate that additional experimental dosing studies are needed to understand factors (e.g., dose- and species-specific sensitivity) and confounding variables (e.g., dispersants) that contribute to the presence and severity of anemia resulting from oil exposure in birds.
**Key words:** Deepwater Horizon oil spill, Heinz bodies, hemolytic anemia, oil toxicity

**INTRODUCTION**

Oil spills cause acute mortality events in wildlife, including birds, but sublethal effects are likely even more common (Perez et al. 2009, Alonzo-Alvarez 2007, Golet et al. 2002, Eppley and Rubega 1990, Fry et al. 1986). Sublethal crude oil exposure can have a variety of negative effects on vertebrates including kidney and liver damage, gastrointestinal disturbance, altered immune function, and anemia (King et al. 2021, Fallon et al. 2018, Alonso-Alvarez et al. 2007; Leighton et al. 1985, Leighton 1986, Peakall et al., 1981, 1989, Szaro et al., 1978). Of these, the only change that can be readily quantified in live birds from a single blood sample is anemia. Anemia is a reduction in circulating erythrocytes or of the oxygen-carrying protein hemoglobin (Hb) that causes reduced availability of oxygen to tissues, leading to anaerobic metabolism, altered cell membrane permeability, and cellular and tissue dysfunction (Greenburg 1996, Fried 2009). The polycyclic aromatic hydrocarbons found in crude oil cause oxidative injury to erythrocyte membranes and cytoplasmic hemoglobin, resulting in membrane deformation and the formation of intracellular inclusions of aggregates of damaged hemoglobin called Heinz bodies in some species of birds (Latimer et al. 2003, Harr et al. 2017a). These damaged, abnormal erythrocytes undergo lysis or are destroyed *in vivo*, which can result in hemolytic anemia (Butler et al. 1986).

Hemolytic anemia has been demonstrated in several species of birds exposed to crude oil during spill events (Fry and Lowenstine 1985, Yamato et al. 1996, Troisi et al. 2007, Fallon et al. 2018, 2020). Experimental exposure of birds to a variety of crude oils has also resulted in anemia
(Leighton et al. 1983, Fry and Lowenstine 1985, Harr et al. 2017a). Conversely, some studies have failed to find significant oxidative changes in erythrocytes or hemolytic anemia when birds are exposed to crude oil (Fleming et al. 1982, Newman et al. 1999, Bursian et al. 2017). However, very few studies have quantified the time course of changes to circulating erythrocytes and tissue histopathology under rigid controlled dosing regimens in a species that is well-adapted to captivity (Harr et al. 2017a, b). This approach would allow for a thorough evaluation of the physiological effects of crude oil ingestion by birds without the confounding uncertainties associated with the unknown exposure history of wild birds stressed by time in captivity.

We evaluated hematological, histopathological, and erythrocyte morphological changes associated with ingestion of crude oil from the Deepwater Horizon spill by zebra finches \textit{(Taeniopygia guttata)}. Oil from the Deepwater Horizon spill, the largest accidental oil spill to date, triggers hemolytic anemia in several species of wild birds (Fallon et al. 2018, 2020, Harr et al. 2017a), but exposure history (e.g., dose of exposure, duration of exposure) of these wild birds and other confounding factors such as possible exposure to oil dispersants remain unknown. Our approach allowed us to assess whether systemic anemia, organ and tissue damage, and cellular and subcellular changes to erythrocytes progress over time with oil exposure. The species we selected is well-adapted to captivity, which reduces confounding experimental factors commonly reported in other dosing studies (Newman et al. 1999, Leighton 1986, Fry and Lowenstein 1985). Additionally, the zebra finch is an ecologically relevant avian model for understanding toxic effects in songbirds exposed to the Deepwater Horizon oil spill (Perez-Umphrey et al. 2018).
METHODS

Oil Weathering

We obtained oil from the Mississippi Canyon 252 (Gulf Coast Restoration Organization, Batch #: SO-2011116-MPDF-003) and then artificially weathered it to more closely replicate the substance that would be encountered by wild birds. We weathered the oil by heating at 80° C with a stir bar under a fume hood until the volume was reduced to 80% original volume, a standard method for artificial weathering (Forth et al. 2017, Goodchild et al. 2020). The weathering process removes water-soluble and more volatile aromatic compounds (Hartung 1967; McEwan and Koelink 1973; Zurcher and Thuer 1978; Lambert et al. 1982).

Birds and dosing protocol

We maintained forty-eight adult zebra finches (male = 24, female = 24) in a controlled laboratory animal facility at Oklahoma State University (temperature, 24 ± 2°C; fluorescent lighting, 14 hours light and 10 hours dark). We housed birds in pairs in 40 X 61 X 91 cm wire cages and fed them ad libitum a diet of a millet seed. Oklahoma State University Animal Care and Use Committee approved the study protocol. We assessed all birds as clinically healthy based on physical examination, behavior, posture, and body condition. We randomly assigned sixteen birds (8 males and 8 females) to each of three dosing categories: control (10 mL/kg peanut oil), low dose (3.3 mL/kg Deepwater Horizon crude), or high dose (10 mL/kg Deepwater Horizon crude). Doses were similar to those used in the literature (e.g., Harr et al. 2017a, Harr et al. 2017b, Leighton 1986, Leighton et al. 1985) and to recent work using the same source of weathered oil (Goodchild et al. 2020). To normalize for volume, we diluted crude oil with peanut oil in the low dose group. Total volume of each gavage did not exceed 0.2 mL. We administered oil daily for 15 days using metal gavage tubes.
Sample collection and processing

We collected blood from the basilic vein with heparinized capillary tubes at day 0 (prior to the first dose), 7, and 15. For each of these time points we quantified body mass, reticulocytes, Heinz bodies, packed cell volume (PCV), hemoglobin (Hb), and mean corpuscular hemoglobin (MCHC). To determine the number of reticulocytes and evaluate for Heinz bodies, we prepared new methylene blue-stained blood smears after mixing 10µL of blood with 10µL of new methylene blue stain (Rica Chemical Company; Arlington, TX, USA) and incubating the mixture for 25 minutes at room temperature (Fallon et al. 2013). For each bird we evaluated 1000 erythrocytes at 1000X magnification, identified Heinz bodies as blue-staining round intracellular inclusion bodies, and identified reticulocytes as erythrocytes with reticular remnants encircling > 50% of the circumference of the nucleus (Fallon et al. 2013). A single individual (JF) who was blinded to sample treatment identity performed all microscopic evaluation of erythrocytes. We calculated PCV using a standard hematocrit reader following centrifugation at 11,000 rpm for 5 minutes. Total Hb (g/dL) was quantified using a Hemocue Hb Analyzer Hb201, which relies on an azidemethemoglobin reaction (Velguth et al. 2010). We calculated MCHC (g/dL) by dividing the total Hb by PCV (expressed as a proportion). On day 15, we determined red blood cell count (RBC) via standard manual methodology using a hemocytometer and calculated mean cell volume (MCV, in femtoliters or fL per cell) by dividing PCV (as a proportion) by RBC count (in cells/mm³) and multiplying the quotient by 10⁹ in a subset of 30 birds (10 from each treatment group) (Thrall et al. 2012). After final blood collection and weighing, birds were euthanized using isoflurane followed by cervical dislocation.

Necropsy and Histopathology
We performed a *post-mortem* evaluation on a subset of 30 birds (10 from each treatment group). From these birds, we obtained liver mass and performed gross evaluation of all coelomic viscera. For histopathologic evaluation, we fixed samples of liver, kidney, gonad, adrenal gland, spleen, proventriculus, ventriculus, small intestine, and pancreas in 10% buffered formalin, embedded them in paraffin and sectioned them at 5 µm. We stained all tissues with hematoxylin-eosin.

*Electron microscopy*

On a subset of 18 birds (6 from each group), we prepared blood for analysis of cellular morphology by transmission electron microscopy. We placed 20 µl of heparinized whole blood in 1.5 mL polypropylene microcentrifuge tubes containing 500 µl 2.5% glutaraldehyde buffered to pH 7.2 (Electron Microscopy Services, Hatfield, PA, USA). We post-fixed aliquots in 1% osmium tetroxide, dehydrated in a graded acetone series, and infiltrated and embedded in Poly/Bed 812 resin. We cut sections (70 nm) with a Reichert Jung Ultracut E ultramicrotome (Vienna, Austria) collected on 200 mesh copper grids, and stained with lead citrate and uranyl acetate. We used a JEM-1400 transmission electron microscope (JEOL, Inc., Tokyo, Japan) at an accelerating voltage of 80 kV to evaluate erythrocyte ultrastructure. We took representative images of each treatment group with a Orius SC1000 Model 832 CCD digital camera with Microscopy Suite Digital Micrograph software (Gatan, Inc., Pleasanton, CA). We assessed images at 4000X, 12,000X and 30,000X, and evaluated a minimum of 100 erythrocytes from each bird.

*Statistical Analysis*

We used SPSS Statistics 25 (IBM Corp., Armonk, NY, USA) for all analyses. We first confirmed normality and evaluated homogeneity of variance using quantile-quantile plots,
Shapiro-Wilk, and Levene’s tests. We then used repeated measures multivariate analysis of covariance (MANCOVA) and Wilks lambda to determine the effect of time, treatment, and sex and their interactions on PCV, Hb, MCHC, and reticulocyte percentage with initial body mass as a covariate. There was no effect of sex or its interaction with time or treatment, and therefore we pooled sexes in our final model. We tested for sphericity and when data did not meet this assumption we used the Greenhouse-Geisser correction. We performed subsequent post hoc univariate analysis on each variable to further differentiate underlying causes of significant results. On day 15 we tested the effect of treatment on RBC and MCV with analysis of variance (ANOVA) and Tukey’s post-hoc tests, and liver mass with analysis of covariance (ANCOVA) using body mass at day 15 as a covariate and post-hoc pairwise comparisons. We set the criterion for statistical significance at $p < 0.05$.

RESULTS

Time Sequence: Heinz bodies, body mass, PCV, Hb, MCHC, and reticulocytes

Contrary to our predictions, we found no evidence of Heinz body formation in any control, low, or high dose birds at any time point. We found no interactive effect between time and treatment on body mass ($F_{2, 90} = 1.27; p = 0.287$). However, time did have a significant effect on body mass, as all treatment groups lost mass in a similar proportion (mean mass loss of 5.5%) from day 0 (mean = 15.74g) to day 15 (mean = 14.87g, $F_{2, 90} = 18.31, p < 0.0001$).

Repeated measures MANCOVA on PCV, Hb, MCHC, and reticulocyte percentage revealed a significant interaction of time*treatment ($F_{16, 352} = 1.76, p = 0.036$) and a significant time effect ($F_{8, 172} = 3.88, p < 0.001$). Post hoc tests indicated a significant time*treatment effect on reticulocyte percentage ($F_{4, 88} = 2.85, p = 0.028$) and MCHC ($F_{4, 88} = 3.92, p = 0.006$).
Specifically, birds in all treatments started the experiment with a similar percentage of reticulocytes, but birds receiving the high dose of oil gained a significantly higher percentage of reticulocytes over the course of the study than did either control or low dose birds \((p = 0.018; \text{Figure 1})\). Additionally, MCHC was highly variable among treatments and times, but birds receiving the high dose of oil had significantly higher MCHC on day 15 than did birds receiving the low dose \((p = 0.032)\) (Figure 2). In contrast, PCV and Hb decreased slightly (overall 6.6% and 7.2% decrease, respectively) in all treatment groups over the first 7 days of the experiment and then plateaued (PCV: treatment*time \(F_{4, 88} = 1.24, p = 0.300\), treatment \(F_{2, 45} = 2.45, p=0.098\), time \(F_{2, 88} = 5.838, p = 0.005\) Figure 3; Hb: treatment X time \(F_{4, 88} = 0.436, p = 0.348\), treatment \(F_{2, 45} = 1.23, p=0.304\), time \(F_{1.44, 63.54} = 11.27, p < 0.001\) Figure 4).

**Day 15: RBC, MCV, liver mass**

On Day 15 we quantified RBC, MCV, and liver mass. We found no significant difference in RBCs (control mean: 33.13 ± 10.17 (SE) cells/mm\(^3\), 3.3mL/kg: 32.28 ± 9.66 cells/mm\(^3\), 10mL/kg: 29.19 ± 8.97 cells/mm\(^3\) \((F_{2, 26} = 1.293, p = 0.2916)\) or MCV (control: 20.67 ± 0.63fL, 3.3mL/kg: 22.64 ± 1.50 fL, 10mL/kg: 20.36 ± 1.07 fL \((F_{2, 26} = 1.211, p = 0.314)\)). However, we found a significant difference in liver mass among treatment groups (control: 400.5 ± 20.80 mg, 3.3mL/kg: 400.8 ± 13.68 mg, 10mL/kg: 464.7 ± 21.55 mg \((F_{3, 26} = 6.362, p = 0.002)\). *Post hoc* analysis revealed birds in the high dose treatment group (10 mL/kg) had significantly heavier livers than either control \((p = 0.015)\) or low dose birds \((p = 0.05)\) after accounting for body mass (Figure 5).

**Histopathology**

Gross necropsy revealed that four of the high dose birds had pale spleens, but we found no other obvious differences among treatment groups during post mortem exam.
Histopathologic analysis revealed that 40% of birds in the high dose (10 mL/kg) group had numerous, active lymphoid follicles in the spleen, which were identified in only one control and one low dose bird. Additionally, all birds dosed with 10 mL/kg of oil had moderate to extensive hepatic glycogen deposition (confirmed on PAS staining), all birds dosed with 3.3 mL/kg had mild to moderate glycogen deposition, and all control birds had minimal to mild glycogen deposition. Four control birds, 4 low dose birds, and 2 high dose birds had lymphocytic infiltration into the submucosa and mucosa of the proventriculus, ventriculus or intestines, but there was no evidence of ulceration or erosion of the gastrointestinal tract in any birds. The keratinized layer of the proventriculus of one bird in the 10 mL/kg group had extensive fungal hyphal mats, and another bird in the control group bird had bacterial colonies and heterophils in this region. No abnormalities in gonads, adrenal glands, or pancreas were found in any bird.

*Transmission electron microscopy*

Erythrocytes had uniformly gray cytoplasm indicating a consistent density (Figure 6). There was occasional nuclear membrane separation due to processing. We found no differences between control, low and high dose birds, with no evidence of inclusion bodies or differences in membrane integrity.

**DISCUSSION**

Sublethal injury in birds exposed to oil spills can adversely affect individual health and performance, and documenting injury can be useful during natural resource damage assessments (Bursian et al. 2017, Fallon et al. 2018, Esler et al. 2000, Golet et al. 2002, King et al. 2021). Results from both experimental and field studies of oil exposure have demonstrated oxidative injury to erythrocytes and anemia (e.g., Fry and Lowenstine 1985, Yamato et al. 1996, Troisi et
al. 2007, Harr et al. 2017a, Fallon et al. 2018, 2020). However, some studies have found lack of evidence for oil-induced anemia in birds (Fleming et al. 1982, Newman et al. 1999, Bursian et al. 2017). Collectively, these studies raise questions about uncertainties related to prior exposure history, confounding environmental variables, and species-specific responses, all of which indicates a need for controlled experimental dosing studies and good experimental models. In the present study, we found a significant increase in reticulocytes and MCHC in zebra finches gavaged with 10 mL/kg with oil from the Deepwater Horizon spill. This finding is consistent with a regenerative response to anemia. We also found evidence of increased liver mass in birds receiving the high dose of oil. However, we did not find evidence of Heinz body formation nor a significant reduction in PCV and Hb in oil-dosed birds compared to controls.

Similar to our findings, several previous studies have found no Heinz bodies or other morphological evidence of oxidative damage to erythrocytes after experimental exposure to crude oil. Fleming et al. (1982) found no significant hematological differences in sandhill cranes (Antigone canadensis) dosed daily for 25 days with 2 or 10 mL/kg of Prudhoe Bay crude oil compared to control cranes. Similarly, Newman et al. (1999) found neither evidence of Heinz bodies on light microscopy nor a significant difference in PCV or Hb in Rhinoceros auklets (Cerrorhinca monocerata) dosed daily for 5 consecutive days with 2.5 or 10 mL/kg of Prudhoe Bay crude oil compared to control birds. More recently, Bursian et al. (2017) found a lack of statistically significant reduction in Hb and no evidence of morphological change in erythrocytes, from western sandpipers (Calidris mauri) given 1 and 5 mL/kg of artificially weathered Deepwater Horizon crude daily for 21 days. These findings contrast with other studies, especially those conducted on wild birds in the field, that have found oxidative damage

Transmission electron microscopy has been recommended to confirm the presence of inclusion bodies or other evidence of morphological change associated with oxidative injury to erythrocytes (Harr et al 2017a; Desnoyers et al. 2010). Here, transmission electron microscopy confirmed a lack of inclusion bodies or other evidence of oxidative injury to erythrocytes in oil-dosed birds, which was consistent with our results determined from light microscopy. There are relatively few electron microscopy studies in non-poultry avian species, and this study is one of only a few that used electron microscopy in the evaluation of erythrocytes of birds exposed to crude oil (Leighton et al. 1983, Harr et al. 2017a). Electron microscopy proved valuable here by providing definitive corroborating evidence that our exposure regime did not induce hemolytic anemia in zebra finches.

There are several possible factors that could contribute to Heinz body formation in some species but not others following exposure to oil. First, there are species- and age-specific differences in gastrointestinal transit time, absorption of ingested material, and capacity for antioxidant defense, all of which can result in certain species being less susceptible to hemolytic anemia from exposure to pollutants (Levey et al. 1994, Duke 1997, Ogburn et al. 2001, Constantini 2010). For example, seed eating birds, like the experimental model species used here, have different cytochrome P450 enzymes performance than do insectivorous and piscivorous species (Verbrugge et al. 2001, Liukkonen-Anttila et al. 2003, Nelson 2018), which could contribute to inherent species-specific responses to crude oil exposure. Likewise, the age of birds may affect the occurrence of hemolytic anemia, as some studies demonstrating severe drops in PCV after oil exposure were performed on young birds (Leighton et al. 1983, Szaro et
al. 1978). A second possible contributing cause for the lack of Heinz body formation in some species may be the route of exposure to oil. Most experimental studies that evaluate hematologic parameters in birds have focused on oral exposure, but birds oiled during spills likely are exposed by both dermal and oral routes, which may illicit a different physiologic response. Dermal exposure to oil can lead to absorption of aromatic hydrocarbons and systemic effects, with the extent of absorption dependent on the anatomic location of exposure, duration of exposure, lipophilicity of the oil, and specific composition of specific hydrocarbons (Jakasa 2015). Consistent changes in hematologic parameters in rats dermally exposed to crude oil have been demonstrated previously (Feuston et al. 1997). Likewise, there is evidence of oxidative damage to hemoglobin and myocardial dysfunction in double crested cormorants dermally exposed to oil from the Deepwater Horizon spill, with histopathological differences between birds dosed orally versus dermally (Harr et al. 2017a, 2017b, 2017c). Third, in addition to the effects of route of exposure and physiological differences between species and ages, most dosing studies use experimentally weathered oil, whereas natural weathering is more complex and includes oil interactions with salt water, sunlight, and temperature changes unique to the environmental conditions at the time and location of the spill which may alter the oil’s toxicity (Jonker et al. 2006, Di Toro et al. 2007). Furthermore, birds exposed to oil in field conditions are likely exposed to oil at different stages of weathering, increasing the likelihood of exposure to volatile compounds in less weathered oil that cause oxidative damage (Overton 1994, Finch et al. 2011, Rial et al. 2013). Finally, during the Deepwater Horizon spill millions of gallons of dispersant were released to help solubilize the oil slicks (Kujawinski, 2011). Decreasing the oil droplet size as occurs with dispersant may increase the possibility of absorption and/or may alter the oil’s toxicity (Bejarano 2013). Likewise, many birds naturally exposed to Deepwater
Horizon oil were likely also exposed to dispersant as well, which itself may have some toxic effect (Wise and Wise 2011). Future experimental studies are needed to disentangle the possible interactive effects of crude oil and dispersant on hematology of birds.

Although we did not detect Heinz bodies or dose-dependent anemia in zebra finches in our study, we found two lines of evidence suggesting that a regenerative response occurred in birds exposed to the highest dose (10 mL/kg) of oil. First, we found increased reticulocytes in birds receiving the high dose of oil compared to control birds. Increased reticulocytes in circulation indicates a physiological response to reduced numbers of mature erythrocytes (Goodnough et al. 2000, Campbell and Ellis 2013). These immature erythrocytes are released from hematopoietic centers in increased numbers as a regenerative response following a decrease in circulating oxygen (Rosse and Waldmann 1962). Second, we found that birds receiving the high dose of oil had significantly higher MCHC over the course of the study than did birds in the low dose group. In cases of blood loss or hemolysis, the MCHC, or the average hemoglobin concentration per cell, is stable or may be artifactually increased due to free Hb in the bloodstream (Desnoyers 2010). This is distinct from non-regenerative anemias, which typically have reduced MCHC, resulting from decreased production in hematopoietic centers, iron deficiency, or anemia of chronic disease (Thrall et al. 2012). These two findings suggest that a regenerative response was occurring in the high-dose birds and may explain why no direct symptoms of anemia were detected, because this regenerative response may have compensated for loss of mature erythrocytes leading to no treatment effect on PCV and Hb.

There was an apparent time-dependent decrease in PCV and Hb between day 0 and 7 in all treatment groups, including controls (Figures 3 and 4). These results are similar to those reported in an experiment in adult Rhinoceros auklets (Cerrorhinca monocerata), which
described a 5-day dosing study that resulted in reductions in PCV in all groups, including control animals (Newman et al. 1999). The reductions in PCV and Hb from the present study are unlikely to be the result of experimental phlebotomy, as we collected less than 10% of circulating blood volume. Birds have been documented to rapidly replace lost blood, with return to normal erythrocyte mass occurring within 3-7 days following a 30%-35% loss via hemorrhage (Schindler and Gildersleeve 1987). We hypothesize that the reduction in erythrocyte volume and Hb in all groups is most likely the result of physiologic stress or inflammation resulting from daily capture and gavage dosing (Jain et al. 1993). For example, we found lymphocytic infiltration in the gastrointestinal tracts of several birds in each of the treatment groups on histopathology, suggesting that gavage feeding crude oil or peanut oil may trigger an inflammatory response in the gastrointestinal tract. This inflammatory response in all groups including control birds may explain the trend toward reduction in PCV and Hb as well as body mass (~5%), among all groups (Rivera and Ganz 2009, Weiss and Wardrop 2011).

An increased inflammatory response also may have contributed to the more pronounced drop in mean PCV from day 0 to 7 in birds in the high dose group compared to the other two groups (Figure 3). An upregulation of proinflammatory cytokine expression has been demonstrated in zebra finches given similar doses of crude oil (Goodchild et al. 2020). In the present study, gross necropsy identified several birds in the high dose group that had grossly pale spleens. Furthermore, histopathologic analysis revealed that 40% of birds in the high dose group had numerous, active lymphoid follicles in the spleen. These findings suggest that these birds had developed a reactive spleen (increased lymphoid proliferation or mobilization as part of an inflammatory response) rather than splenitis (Abdul-Aziz 2016). Additionally, splenic
proinflammatory cytokine mRNA expression increased in zebra finches exposed to 10 mL/kg of Deepwater Horizon crude oil, supporting these histopathologic findings (Goodchild et al. 2020). Histopathologic changes that have been reported with oral ingestion of crude oil by wild birds include gastrointestinal ulceration, adrenal necrosis and lipid depletion, increased hepatic vacuolation and enlarged Kupffer cells containing pigment, hepatic necrosis, renal hyaline droplet accumulation, squamous metaplasia of renal tubules, renal tubular necrosis, and cytoplasmic vacuolation of the exocrine pancreas (Szaro et al. 1978, Fry and Lowenstine 1985, Leighton 1986, Harr et al. 2017b). We did not find lesions in the kidneys, adrenal glands, or pancreas in birds from this study. However, in addition to the gastrointestinal infiltration of lymphocytes found in all groups and reactive spleens found in the high dose group in this study, we also found significantly higher liver mass in birds receiving the highest dose of oil. Heavier livers have been reported in other oral dosing studies possibly caused by edema, inflammatory infiltrate, or hypertrophy (Harr et al. 2017b, Duerr 2013, Pristos et al. 2017, Leighton 1986, Szaro et al. 1978). Here, we found a relative increase in glycogen stores in birds receiving the high dose of oil which may add to liver mass without substantial differences in inflammatory cells in the groups (Dwivedi et al. 1984). Additionally, it is possible that liver hypertrophy and hyperplasia occurred as an adaptive response to oil exposure (e.g., increased p450 enzyme induction).

Conclusions

We did not find evidence of Heinz bodies in erythrocytes on light or electron microscopy nor significant drops in PCV or Hb in oil-dosed birds relative to controls. However, we detected multiple lines of evidence of an apparent regenerative response in birds receiving the highest does of oil, which may account for the lack of significant symptoms of anemia. Birds receiving
the highest dose also experienced inflammation of the gastrointestinal tract and lymphocyte proliferation in the spleen. It is possible that increasing dose, route, or duration of oil exposure may result in a more pronounced effect on erythrocytes, or that other study species may suffer more pronounced hematological changes. We suggest that future experimental research using a suite of diagnostic approaches, as used here, be directed toward determining differential sensitivity among species and the role of dispersant on the physiologic effects of oil. Such studies will advance our understanding of species at most risk of sublethal injury from oil spills, explain contrasting results among species and exposure conditions, and help identify mitigation practices that could influence the toxicity of oil in the field.

Compliance with Ethical Standards
Conflict of Interest: Jesse A. Fallon declares that he has no conflict of interest. Christopher Goodchild declares that he has no conflict of interest. Sarah DuRant declares that she has no conflict of interest. Thomas Cecere declares that he has no conflict of interest. Phillip Sponenberg declares that he has no conflict of interest. William A. Hopkins declares that he has no conflict of interest.

Ethical Approval: The procedures involving animals were conducted with approval from the Oklahoma State University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.
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Figure 1. Reticulocyte percentage (Mean +/- SE) on days 0, 7, and 15 in zebra finches given 3.3 mL/kg or 10 mL/kg of oil from the Deepwater Horizon spill or 10 mL/kg of peanut oil (control) daily. Asterisk indicates statistical significance (p<0.05) between high dose and control birds based on post-hoc tests.
Figure 2. Mean corpuscular hemoglobin concentration (MCHC, Mean +/- SE) on days 0, 7, and 15 in zebra finches given 3.3 mL/kg or 10 mL/kg of oil from the Deepwater Horizon spill or 10 mL/kg of peanut oil (control) daily. Asterisk indicates significant difference (p<0.05) between 10 mL/kg and 3.3 mL/kg groups based on post hoc tests.
Figure 3. Packed cell volume (PCV) (Mean +/- SE) on days 0, 7, and 15 in zebra finches given 3.3 mL/kg or 10 mL/kg of oil from the Deepwater Horizon spill or 10 mL/kg of peanut oil (control) daily. Although there was a tendency for PCV to drop in the birds receiving the highest dose of oil, the difference was not statistically significant.
Figure 4. Hemoglobin (Mean +/- SE) on days 0, 7, and 15 in zebra finches given 3.3 mL/kg or 10 mL/kg of oil from the Deepwater Horizon spill or 10 mL/kg of peanut oil (control) daily.
Figure 5. Liver mass (LS means +/- SE, wet weight, corrected for body mass) collected at necropsy on day 15 from zebra finches given 3.3 mL/kg or 10 mL/kg of oil from the Deepwater Horizon spill or 10 mL/kg of peanut oil (control) daily. Asterisk indicates statistical significance (p<0.05) between 10 mL/kg dose and 3.3 mL/kg dose as well as control birds.
Figure 6. Erythrocyte ultrastructural images using transmission electron microscopy (TEM) for control (a), low dose (3.3 mL/kg) (b), and high dose (10 mL/kg) (c.) of oil. Higher magnification reveals gray cytoplasm indicating a uniform density with centrally placed dark grey nuclei (d). No Heinz Bodies were observed using TEM. There was occasional nuclear membrane separation due to processing.
SYNTHESIS AND CONCLUSIONS

Assessing the impact of oil spills is of concern to industries, governments, and the general public (Norse and Amos 2010). In the United States, the Natural Resource Damage Assessment and Restoration Program (NRDA) in partnership with affected states, tribes, and federal trustee agencies, conducts assessments following anthropogenic events that damage wildlife and the environment. The work presented within this dissertation was prompted by the efforts of a NRDA investigation of the Deepwater Horizon oil spill and achieved two major goals. First, I provided a better understanding of the extent of modest oil exposure in birds using novel detection techniques. Second, I improved our understanding of the effects of sublethal exposure to individuals by defining and applying a quantifiable suite of parameters to assess hematologic injury to birds exposed to oil. Together, these represent fundamental advancements that greatly expand our ability to estimate numbers of injured birds in the NRDA process.

The Deepwater Horizon spill released an unprecedented volume of crude oil into the Gulf of Mexico (McNutt et al. 2012). While there were thousands of dead birds found in the weeks following the disaster, there were many more that were exposed to oil that suffered sublethal injury, as demonstrated in this dissertation. It will take decades before the long-term implications of this spill upon bird populations are fully understood. This dissertation advances our understanding of the extent of potential oil exposure in birds, the sublethal effects of oil exposure in birds in the immediate aftermath of this massive spill, and establishes techniques that can be applied to inevitable future spills.

The application of my field-adapted techniques in the aftermath of the Deepwater Horizon spill demonstrated that there is substantial risk for exposure to modest oiling in many
birds that did not succumb to initial exposure to crude oil. Further, my findings demonstrated that handheld ultraviolet (UV) light can be used to identify exposure to small amounts of oil, identifying individuals with oil exposure that would be missed by visual evaluation alone. Together, visual and UV evaluation of individual birds allowed me to identify oil exposure that would have been missed during previous damage assessments following oil spills.

In addition to my work to improve understanding of the extent of exposure during oil spill events, I devised and implemented a straightforward approach to assess anemia and the associated regenerative hematologic response in birds. Further, I provided a means for assessment of hematological injury using a core set of parameters which can be applied to free-ranging birds in nearly any field situation, including oil spill responses, as it affords the opportunity for samples to be conveniently prepared and transported for evaluation. Finally, this body of work demonstrates that oxidative hematologic injury occurs during natural exposure to oil, even with relatively small quantities of oil present on the plumage. These hematologic effects were not only present in birds with visible oiling, but also in birds with small amounts of oil that was detected only under the application of UV light. Improving the ability to quantify the damage caused by spills has implications both in our understanding of the true impact of spill events and provides a means to more precisely determine mitigation requirements for parties responsible for spills.

Results from my field work were directly applied to the Deepwater Horizon spill damage assessment. Because I demonstrated that birds suffered adverse hematologic effects from sublethal oil exposure, estimates of birds with oil present on the plumage were assumed to have suffered injury as defined by the Natural Resource Damage Assessment, and were included in the settlement negotiations (Westerholm and Rauch 2016). Additionally, the results of my
laboratory development and field application of methodology to evaluate hematologic injury in birds exposed to the Deepwater Horizon oil spill set the stage for a series of experimental studies performed under the purview of the Department of Interior, which further validated the usefulness of these techniques. These studies included oral dosing studies, an external dosing study, metabolic and flight performance studies, and field-based flight studies (Bursian et al. 2017a). Results of these investigations demonstrated oxidative damage, changes in hematologic endpoints including formation of Heinz bodies and anemia, organ systems damage, impaired cardiac function, reduced flight performance, and adverse metabolic function (Alexander et al. 2017, Bursian et al. 2017a, Bursian et al. 2017b, Harr et al. 2017abc, Horak et al. 2017, Perez et al. 2017, Pristos et al. 2017). The results of these studies indicated that the effects of oil toxicity in several avian species, even in the case of relatively light oiling, can significantly affect the overall health of birds.

Sublethal injury suffered by birds and other wildlife following spills must be incorporated into damage assessments because these effects may be widespread and greater than previously recognized in damage assessments. The results from my work were pivotal in determining a more accurate damage assessment than had previously been performed following spill events. The end result of these efforts played a part in the ultimate financial settlement with offending parties associated with the Deepwater Horizon spill. During future oil spill response scenarios, I recommend that field researchers and rehabilitation facilities routinely collect blood and stain for Heinz bodies using the techniques described in this dissertation.

I. Unanswered Questions & Future Priorities: Exposure

My results highlight several unanswered questions and challenges to evaluating natural exposure to oil that require further research. First, the field work component of this dissertation
was both time- and labor-intensive, and challenging field circumstances during spill events may make the individual hands-on evaluation of large numbers of individual birds difficult. It is possible that visual evaluation for oil exposure may be performed from a distance with magnification, using a systematic or stratified random sampling design; however, the accuracy of distance determination vs. hands-on evaluation for severity of oil would require additional validation comparing the two techniques. Further, it is likely that there are species differences in the risk of sublethal oil exposure, and further study is needed to determine which species or families of birds are most likely to encounter oil in sufficient quantities to elicit sublethal effects.

In addition to the unanswered questions posed by visual examination of individual birds, the large-scale application of evaluation with hand-held UV light poses challenges. I used UV evaluation of individual birds that also received hands-on physical exams, which requires capture. The stress associated with capture should be more thoroughly evaluated during oil spill events to assess risks to the affected individual during handling. Although logistically challenging, it may be possible to apply UV light at night to groups of birds at a distance using remote drone technology equipped with UV light and cameras. Although it is well-known that crude oil fluoresces under UV light and the tool is used to enhance detection of oil on or below the water surface (Burlamacchi 1983, Colligan and LaManna 1993, Brown and Fingas 2005), the methods applied in Chapter 3 are the first to demonstrate the utility of this technology to evaluate individual birds for oil exposure. The use of remote UV and infrared light, as well as laser fluorosensors and microwave sensors, to evaluate and quantify the extent of oil slicks on the water is already in practice (Fingas and Brown 2014, De Kerf 2020), and the application of drone technology to evaluate individuals and groups of birds with UV light may be plausible. This tool may be particularly useful during damage assessment of more common smaller petroleum spill
events, where sublethal injury is present without large-scale mortality events. This type of evaluation has the potential to greatly increase our understanding of oil exposure risk during damage assessments.

II. Unanswered Questions and Future Priorities: Effects

The results from my field assessment of the effects of sublethal oil exposure upon individual birds point to several future research directions. First, what do these hematologic changes mean to individuals and populations of birds? That is, what is the long-term outcome for these individuals with sublethal exposure and subsequent anemia, and how does this physiologic insult affect individual survival and fecundity in proximity to the spill (Madliger et al. 2021)? There is some evidence that birds exposed to spill events have decreased survival. For example, African penguins (*Spheniscus demersus*) with fuel oil contamination of 5% or more of the surface area of feathers were less likely to be re-sighted in the next five years (Wolfaardt et al., 2008b). Additionally, Harlequin ducks (*Histrionicus histrionicus*) exposed to oiled areas were shown to have decreased survival compared to unoiled areas following the Exxon Valdez spill (Esler et al., 2000b; Iverson and Esler, 2010). However, there are relatively few studies that report survival data on living birds exposed to oil in the wild, fewer report survival where exposure is quantified in some way, and none link survival to oxidative injury. Demonstrating this connection between oxidative hematologic injury and fitness traits would be valuable during future damage assessments that incorporate the techniques described in this dissertation. While we have a good understanding of the negative effects of anemia on the physiology of individuals, how these physiological effects translate to effects on individual behavior, reproductive success, and survival, as well as ultimately population dynamics, in the wild remains an inadequately studied area in avian biology.
Second, the species-specificity of the hematologic effects of oil exposure remains poorly understood. Some species clearly show a propensity for anemia following exposure to crude oil, while others seem somewhat resistant (Newman et al., 1999, Troisi et al. 2007, Bursian et al., 2017; Horak et al., 2017). Currently, there remains very limited research on the underlying physiologic mechanisms responsible for the apparent species differences in the hematologic effects of oil exposure.

Despite the findings for the species evaluated in the field, I failed to identify Heinz body formation or substantial anemia in zebra finches (*Taeniopygia guttata castanotis*) orally dosed with weathered Deepwater Horizon crude oil using either light or electron microscopy. The doses and administration of oil that I used in this chapter are comparable to other experimental studies which demonstrated Heinz bodies and anemia (Harr et al. 2017a, King et al. 2021). Similar to my findings in experimental oil exposure of zebra finches, there are a handful of previous studies that have found no Heinz bodies or limited evidence of oxidative damage to erythrocytes after experimental exposure to crude oil including sandhill cranes (*Antigone canadensis* Fleming et al. 1982), rhinoceros auklets (*Cerrorhinca monocerata*, Newman et al. 1999), and western sandpipers (*Calidris mauri*, Bursian et al. 2017). For these reasons, there is a need to develop a different model system for experimental studies on the hematologic effects of sublethal exposure, using species that are susceptible to oxidative injury. For example, mallard ducks (*Anas platyrhynchos*), which are well-adapted to captivity, have shown signs of hemolytic anemia when exposed to fuel oil exposures (Hartung and Hunt, 1966; Lee et al., 2012). Likewise, weathered crude oil exposure of ring-billed gulls (*Larus delawarensis*) (Dannemiller et al., 2019) and double-crested cormorants (*Phalacrocorax auritus*) (Dean et al., 2017b; Harr et al., 2017a).
has been shown to induce hemolytic anemia. One of these or perhaps another yet-to-be-identified species may serve as a better model system for experimental studies than zebra finch.

The lack of Heinz body formation in zebra finches and other species in previously published studies may be the result of individual species variation in susceptibility to oxidative damage to erythrocytes. Species differences in susceptibility to oxidative insult to erythrocytes are known to occur in domestic animals, and a genetic predisposition in certain humans to Heinz body formation and subsequent anemia have been well established (Edwards and Fuller 1996, Hutchison et al. 1964). These species differences may result from several physiological mechanisms that occur from exposure through oxidative injury. For example, there is likely species-specific variation in the absorption of polycyclic aromatic hydrocarbons (PAH) from the gastrointestinal tract following oil exposure (King et al. 2021). Even in oral exposure experiments, the net amount of oil ingested and absorbed may be somewhat uncertain because petroleum can be excreted or regurgitated within minutes, making the amount available for absorption less certain (Cunningham et al., 2017).

Additionally, there is likely variability among species in the composition and quantity of breakdown products of individual PAHs. After absorption of the PAH present in oil, P450 enzymes, an assemblage of enzyme isoforms concentrated in hepatocytes (liver cells) but present within most tissues in the body, trigger biotransformation of hydrocarbons (Alexander et al., 2017, Cruz-Martinez et al., 2015, Head and Kennedy). Up-regulation of some of these enzymes, (e.g., CYP1A4 and CYP1A5) has been used as a biomarker in birds exposed to oil, both in an experimental setting and during exposure during spill events (Esler et al., 2010, 2011, Golet et al., 2002). Hence, transcriptional studies of gene expression in oil-affected and unaffected birds
would be appropriate. However, these biomarkers have appreciable interspecies differences in terms of catalytic activity (Perez-Umphrey et al., 2018, Velando et al., 2010).

Finally, there is variation in physiological protective mechanisms to reduce oxidative damage. For example, glutathione, which is present as both reduced (GSH) and oxidized forms (GSSG) in vertebrates, serves to maintain homeostasis in the face of oxidative injury (Romero-Haro and Alonso-Alvarez 2015). These forms of glutathione have been quantified in birds exposed to oil, and when exposure occurs there is a shift from GSH to GSSG, indicating a response to the free radicals produced by the oxidating effects of the PAH present in crude oil. Concentrations of glutathione forms have been demonstrated to differ across both species and age, indicating differing capacity for quenching free radicals and offsetting the effects of oxidative injury from oil exposure (Wilhelm et al. 2002, Alonso-Alvarez et al. 2010). Clearly, there is a need for further experimental and field studies to help elucidate the primary drivers of these species-level differences.

My results prompt several additional key questions separate from survival data and species differences that need to be evaluated more thoroughly. First, do dispersants play a role in oxidative insult? A variety of dispersants are commonly used during mitigation of spill events. For example, in the immediate aftermath of the Deepwater Horizon spill, 1,840,000 gallons of Corexit™ dispersant were applied throughout the area affected by the spill. These dispersants change the character of crude oil, breaking up large slicks, increasing the surface area by creating smaller oil droplets, which may increase absorption of polycyclic aromatic hydrocarbons through the gastrointestinal tract of birds (Lessard and DeMarco 2000, Zeinstra-Helfrich et al. 2015). Increased absorption would likely lead to a higher risk of physiological damage, including oxidative hematologic injury. Additionally, it is also possible that the dispersants themselves
could cause oxidative hematologic injury. For example, propylene glycol, a common constituent of dispersants including Corexit, induces Heinz body formation in some species of domestic animals (e.g., Hill et al. 2001). For these reasons, dosing studies using oil alone, oil and dispersant, and dispersant alone need to be completed to more thoroughly evaluate the effects in species known to develop Heinz body anemia.

Second, differences in natural weathering in the field as opposed to artificial weathering in the laboratory of crude oil, as was used in Chapter 4 and in many other experimental dosing studies, may influence the expression of hematological injury (Gustitus et al. 2017). There are substantial changes to crude oil that occur in the field as it undergoes weathering, which can vary depending on temperature, water chemistry and salinity, sunlight exposure, and the location of the oil in the water column (Daling and StrØm 1999, Ward and Overton 2010). Natural and artificial weathering are known to affect the concentration of different PAHs, which likely plays a role in the variability of potential for oxidative injury (Neff et al 2000, Jonker et al. 2006). Despite decades of research on the effect of weathering on crude oil, more work is needed to determine the degree of toxicity associated with weathering in regard to risk of oxidative injury to individuals and populations of animals.

Immediate Priorities and Response to Future Spills

Given our ongoing reliance on crude oil for energy production, future spill events are inevitable. These spills can have substantial negative impacts on local economies, ecosystems, and wildlife populations. Expanding the ability to identify sublethal oil exposure in individuals as well as evaluating the hematologic parameters outlined here are imperative for accurate damage assessments in the aftermath of spill events. Therefore, I recommend that the techniques
to sublethal oil exposure and injury outlined in this dissertation be applied during future oil spills. By moving beyond heavily oiled and dead birds in the damage assessment, the numbers of birds identified as injured will increase many-fold, which has important implications for the financial settlements achieved with NRDA.

My results suggest that the most immediate needs for further research include improving our understanding of the extent of sublethal exposure to wildlife through evaluation of individual animals exposed to oil, understanding the specific physiologic mechanisms of different species’ responses to oil exposure, and expanding our ability to assess and quantify hematologic and other physiological parameters in individuals with sublethal oil exposure. Evaluating the role of remote detection of oiled birds may be a particularly valuable line of research that could both increase the sample size of birds potentially affected during spill events, and reduce the time and cost of thorough damage estimates. Achieving these goals requires transdisciplinary research targeting both field and laboratory applications.

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