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Short Communication

A PRACTICAL QUANTIFICATION METHOD FOR HEINZ BODIES IN BIRDS APPLICABLE TO RAPID RESPONSE FIELD SCENARIOS

JESSE A. FALLON,*† WILLIAM A. HOPKINS,† and LEE FOX‡
†Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, Virginia, USA
‡Save Our Seabirds, Sarasota, Florida, USA

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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. Environ. Toxicol. Chem. 2013;32:401–405. © 2012 SETAC

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 [1–4]. 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 [2,4,5]. Sublethal injury from oil exposure spans an array of physiological effects, including inflammation, immunosuppression, and hemolytic anemia [6–9]. 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 [10,11].

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 [10]. When exposed to reactive oxygen compounds, the Hb molecule undergoes changes in sulfhydryl bonds resulting in denaturation of the oxygen-carrying protein [12]. 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 [10]. 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 [12]. Heinz body–induced hemolytic anemia promotes fatigue and a reduction in energy availability for metabolic processes, and ultimately can decrease fitness [13].

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 [14–18]. Oil-induced oxidative damage is believed to be mediated by metabolites of polycyclic aromatic hydrocarbons generated from the metabolic actions of cytochrome P450 enzymes [14]. Consequently, it has been suggested that Heinz bodies can be used as a marker for exposure to crude oil [18]. However, previous studies have often 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 [18]. 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 brown pelican (*Pelecanus occidentalis*, hereafter pelicans) blood following in vitro incubation with multiple concentrations of acetylphenylhydrazine, an oxidizing compound known to induce Heinz body formation [19–23]. 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 Diagnos-

* To whom correspondence may be addressed (jfallon@vt.edu).
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tics) 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) [24].

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 [25]. 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 (~25°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 techniques that differ in how blood cells are stained with new methylene blue (0.5% [w/v], RICCA Chemical), whereas technique C follows a clinically based wet-mount staining technique. 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 [26]. 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 [27–29]. Newman et al. reported an air-dried technique for identifying Heinz bodies in rhinoceros auklets (*Cerrorhinca monocerata*) but their study failed to detect any Heinz body formation [30]. 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 [26,28]. 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 [18].

For all three techniques, we counted the number of cells affected with Heinz bodies per 1,000 erythrocytes under 1,000 × light microscopy immediately after preparation [28]. 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 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. We used Wilcoxon tests with Bonferroni adjustment to assess differences between individual techniques and concentrations.

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 correction to account for violation of sphericity [31]. 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 [32,33]. 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

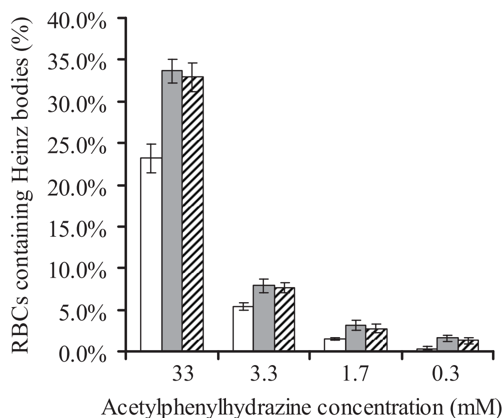

Fig. 1. In vitro Heinz body generation in brown pelican (*Pelecanus occidentalis*, mean ± 1 standard error, $n = 10$ per time/dose combination) blood treated with different concentrations of acetylphenylhydrazine (mM) quantified with three different slide preparation techniques. Open bars represent field-adapted 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 hatched bars represent traditional wet-mount technique C.

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).

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 affect on Heinz body formation ($F_{3,36} = 274$, $p < 0.001$; Fig. 2).

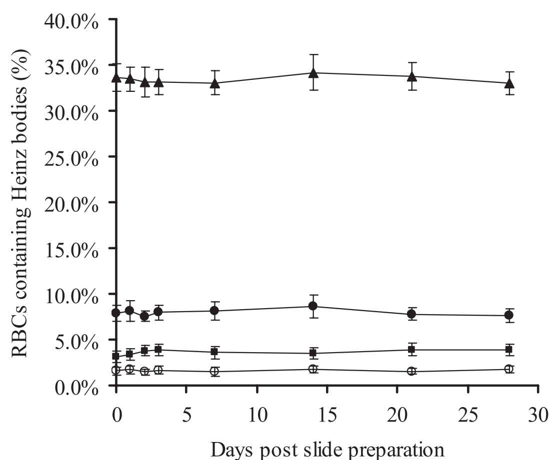

Fig. 2. The effect of time after preparation of blood on mean (mean ± 1 standard error, $n = 10$ per time/dose combination) Heinz body counts in brown pelican (*Pelecanus occidentalis*) red blood cells exposed in vitro to different concentrations of acetylphenylhydrazine (33 mM▲, 3.3 mM●, 1.7 mM ■, 0.33 mM ○) using field-adapted staining technique B.

DISCUSSION

Oil spills can have dramatic consequences to wildlife, particularly seabirds (e.g., Piatt et al. [1]). 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, this is the first demonstration of Heinz body formation in vitro in the class Aves [34]. 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 [36], white phosphorus [37], phenylhydrazine [38], and phenylhydrazine-hydrochloride [34]. However, the most frequently reported chemical cause of Heinz body formation in birds is exposure to crude oil [14–18].

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 and specifically defined [14–18]. Staining techniques for enumeration of Heinz bodies have often relied on clinical wet-mount preparations with fresh blood [18,39]. These wet-mount slide preparations (technique C), however, can be impractical in field situations, as they must be evaluated microscopically immediately after preparation [18,39]. 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 [27,29,30]. 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*) [27], black-crowned cranes (*Balearica pavonina*) [26], and laughing kookaburra (*Dacelo novaeguineae*) [26], and members of the Corvidae [39] and Megapodiidae [26] 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 [27].

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 [40,41]. 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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