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# Use of blood clotting assays to assess potential anticoagulant rodenticide exposure and effects in free-ranging birds of prey

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## HIGHLIGHTS

- Birds of prey are exposed to anticoagulant rodenticides (ARs) and can be poisoned.
- Exposure to ARs can prolong blood clotting time.
- Blood clotting measured in 61 raptors admitted to rehabilitation facility in Canada.
- Significant proportion of those admitted (23%) exhibited prolonged clotting time.
- Clotting time shows promise as a biomarker of AR exposure in free-ranging birds.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Non-target wildlife, particularly birds of prey, are widely exposed to and acutely poisoned by anticoagulant rodenticides (ARs). An unresolved issue surrounding such exposure, however, is the potential for sublethal effects. In particular, the consequences of AR exposure and resulting coagulopathy on health and survival of unintentionally exposed animals, which often encounter a multitude of anthropogenic stressors, are understudied. In a wildlife rehabilitation setting, AR intoxication may be masked by more obvious injuries related to collision with vehicles or electrocution, thereby obfuscating proximate from ultimate cause of mortality. An assessment of coagulation function of admitted wildlife may provide a means of identifying animals exhibiting sublethal coagulopathy, and ultimately ensuring provision of appropriate and swift treatment. In conjunction with routine diagnostics for injury and disease, we performed two blood clotting assays (prothrombin time, Russell's viper venom time) affected by vitamin K-dependent coagulopathy of samples from six species of live raptors admitted to a rehabilitation facility. We also measured clotting time in pre-fledgling barn owl chicks (*Tyto furcata*) from 10 nest sites in Lower Mainland Canada. Prolonged clotting time or failure to form a clot altogether was observed in 23.0% of 61 sampled raptors admitted to the rehabilitation facility. This is a biologically significant proportion of individuals given the fortuitous and likely biased nature by which raptors are found and admitted to rehabilitation facilities. In contrast, there was little evidence of coagulopathy in 19 pre-fledgling barn owl chicks. The utility of avian coagulation tests for diagnosing AR exposure is promising, yet there remains a need to establish species specific reference values and standardize assay methodologies among testing facilities.



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## 1. Introduction

Baiting with anticoagulant rodenticides (ARs) is the primary method employed worldwide to suppress commensal rodent populations in both urban and agricultural settings (Jacob and Buckle, 2018). From a regulatory perspective, many ARs are persistent, bioaccumulative and toxic, and thus consistently exceed levels of concern in ecological risk assessments (Elliott et al., 2016; Buckle and Prescott, 2018). However, because of the demand for rodent control (Corrigan, 2001; Jacob and Buckle, 2018), and the lack of effective alternatives, ARs remain the dominant control agent worldwide (Witmer, 2018). In terrestrial habitats, ARs have become widespread contaminants of both avian and mammalian scavengers and predators (López-Perea and Mateo, 2018), and have resulted in acute and chronic poisoning of many exposed animals (Albert et al., 2010; Thomas et al., 2011; Gabriel et al., 2012; Huang et al., 2016; Murray, 2017).

Measurement of residue levels of first- and second-generation ARs (FGARs, SGARs) in the liver of deceased animals provides a valuable tool for monitoring exposure and developing diagnostic thresholds to interpret toxicity of such residues (Newton et al., 1999a, 1999b; Thomas et al., 2011). Largely unknown, however, is the potential for subtle effects, in particular the effect of sublethal coagulopathy on health and survival of exposed animals (Rattner et al., 2014a). While liver testing provides AR concentrations in deceased raptors at the time of death, residues are less useful when determining the timing and frequency of exposure (current or weeks/months ago) and the degree to which an individual may have suffered sublethal consequences. Diagnosis of impaired hemostasis during examination (e.g., clotting time assays) or necropsy (e.g., hemorrhage, internal bleeding) may be particularly difficult in the presence of concurrent traumatic injury (Murray, 2011; Rattner et al., 2014a).

Annually, some 50,000 raptors are admitted to rehabilitation facilities across North America (The University of Minnesota, 2017). Admitted individuals exhibit various injuries including trauma from vehicle and window strikes, electrocution, entanglement, falling from nests, gunshot and contaminant intoxication. In many instances, poisoning is more challenging to detect than other causes of mortality, requiring specific diagnostic testing that is expensive and not always readily available (Murray, 2018). Exposure to contaminants may also cause an individual to be more susceptible to other risks, thereby obfuscating proximate from ultimate causes of mortality. Rattner et al. (2010, 2011, 2012b, 2014b, 2015) demonstrated the utility of blood clotting time as a biomarker to assess AR exposure in captive avian wildlife, and Murray and Tseng (2008) used delayed clotting of whole blood as a diagnostic tool in free-ranging raptors. Compared to liver AR residue testing, blood clotting assays are more cost effective, and if conducted at the rehabilitation facility, would assist in diagnosing AR exposure and development of a treatment plan. However, only preliminary research assessing the feasibility of blood clotting time in free-ranging wildlife has been conducted (Shlosberg and Booth, 2006; Murray and Tseng, 2008; Webster et al., 2015), and although promising, more rigorous evaluation is needed.

Our objectives were: 1) to assess the degree to which free-ranging raptors exhibit coagulopathy, 2) to examine the feasibility of blood clotting time assays as a tool to evaluate AR exposure and effects in birds admitted to wildlife rehabilitation facilities, and 3) to evaluate AR exposure in pre-fledgling barn owls (*Tyto furcata*) when monitoring productivity and collecting morphometric data at nest sites. Specifically, we used citrated blood plasma in three assays (prothrombin time and Russell's viper venom time to determine vitamin K-dependent coagulopathies, fibrinogen concentration to rule out nonspecific influences on clotting time), with assessment of other factors (e.g., diagnosis of injury and disease) to estimate the proportion of raptors that are exposed to ARs and potentially at risk.

## 2. Materials and methods

### 2.1. Raptors and blood sample collection

Sample collection was conducted from January to June 2015 at the principal raptor rehabilitator facility in British Columbia, Canada [Orphaned Wildlife Rehabilitation Society (OWL), 3800 – 72nd Street, Delta, BC, Canada V4K 3N2]. Annually ~500 injured or deceased raptors are admitted to this facility with birds coming predominantly from the Lower Mainland and the Fraser Valley (5000 $km^2$) regions of south-western British Columbia, which have been intensely developed for urban and agricultural uses. The region is important both for resident birds of prey and wintering migrants (Butler and Campbell, 1987). The OWL facility is formally licensed, and rehabilitation activities are carried out by qualified staff, with treatment of severe injuries conducted by or under the guidance of a licensed veterinarian. Our study procedures were reviewed and approved by the British Columbia Ministry of Environment and the Institutional Animal Care and Use Committee of the USGS-Patuxent Wildlife Research Center, and conducted under all the appropriate wildlife and animal ethics permits (Provincial wildlife permit SU12-76336), and sample import and export permits (CITES export permit 15CA01790/CWHQ and U.S. Fish and Wildlife Service Importation Permits MB8414B-0 and MB55205B-1). For this study, a blood sample was collected by a trained individual from 62 birds (comprising six raptorial species) within 48 h of admission. Only raptors weighing ≥280 g were sampled, and the blood volume drawn was below the 1% body mass maximum volume recommended by Fair et al. (2010). Blood was not obtained from raptors that were in critical condition, or had both wings broken if venipuncture was considered to exacerbate their condition. In addition, a blood sample was similarly collected from 19 pre-fledge barn owl chicks (35–50 days old) from 10 nest sites in the Lower Mainland between May and June of 2015.

Standard raptor handling techniques were used to maintain comfort and safety for the bird including hooding it with a cotton bag or towel and firmly holding talons. A 0.9 mL blood sample from the brachial vein was drawn into a 1 mL syringe containing 0.1 mL of 3.2% sodium citrate using a 26 or 27 gauge hypodermic needle. Immediately after sample collection, the needle was removed from the syringe and the sample was transferred to a 1.8 mL microcentrifuge tube, which was then inverted several times. The blood sample was then centrifuged within 15 min at 2000*g* for 5 min. The resultant citrated plasma was transferred into 3 cryovials (with no red blood cell residue) as follows: ~250 μL for prothrombin time, ~200 μL for Russell's viper venom time and ~50 μL for thrombin clotting time. Samples were temporarily stored in liquid nitrogen before being transferred to a −80 °C freezer.

In order to examine temporal variation in clotting time that occurs following AR exposure, blood was collected three times from each raptor over a 7 day period following admission. There is a 1–4 day lag between exposure and the onset of coagulopathy which is related to the clearance of fully-functional clotting factors and the appearance of *des*-γ carboxy dysfunctional factors that do not support hemostasis (Mount and Feldman, 1983; Furie et al., 1999; Rattner et al., 2011, 2014b). The short plasma half-life of some ARs also affect the time course of coagulopathy (Bachman and Sullivan, 1983; Vandenbroucke et al., 2008). Once AR exposure is terminated, recovery of clotting function can occur within a week (Mount and Feldman, 1983; Furie et al., 1999; Rattner et al., 2011, 2014b). Thus, the first sample was taken within 2 days of the raptor being admitted to the rehabilitation facility; a second sample was collected 2 days later, and the final sample was collected 5 days later (i.e., 7 days after the initial blood sample). Some individuals succumbed to their injuries before the second or third blood draw. Raptors that died during the 7-day sampling period were necropsied and liver tissue was collected for quantification of ARs.

### 2.2. Clotting time assays

Citrated plasma samples were analyzed for prothrombin time, Russell's viper venom time and fibrinogen concentration at USGS-Patuxent Wildlife Research Center in Beltsville, MD (Rattner et al., 2010, 2011, 2012a, 2014b, 2015). Anticoagulant rodenticides inhibit vitamin K epoxide reductase, an enzyme required for the formation of vitamin K hydroquinone that is necessary for the γ-glutamyl carboxylation (activation) of clotting factors II, VII, IX and X (Rattner et al., 2014a). Prothrombin time (PT; thromboplastin activation of Factor VII, resulting in Factor X activation, conversion of prothrombin to thrombin, and subsequently fibrinogen to fibrin) and Russell's viper venom time (RVVT; directly activates Factor X, resulting in conversion of prothrombin to thrombin, and subsequently fibrinogen to fibrin) were used to examine potential effects of AR exposure on the post-translational processing and activation of clotting factors. While fibrinogen concentration in blood is insensitive to vitamin K-dependent clotting factor deficiency, reduced levels in blood can occur from improper sample collection technique, and can result in prolonged clotting time independent of AR exposure (i.e., potential false-positive response; Rattner et al., 2010). Thus, in order to monitor sample integrity, fibrinogen concentration was first determined in all of the citrated raptor samples using a thrombin clotting time (TCT) assay, followed by assays of RVVT and finally PT. The TCT, RVVT and PT assays used in various species of birds have been described (Rattner et al., 2010, 2012a, 2014b, 2015), and are briefly presented below.

A heterologous TCT assay was used to measure the time for conversion of fibrinogen to fibrin using commercially available reagents (TCoag TriniCLOT Fibrinogen Kit, Diagnostic Stago Inc., Parsippany, New Jersey). To generate a 7 point standard curve (65.5 to 524 mg/dL), human reference material included in the kit was diluted with imidazole buffered saline (IBS; 0.0125 M imidazole-0.109 M sodium chloride, pH 7.4). Each raptor plasma sample was thawed in a 37 °C water bath, and 20 μL was diluted 1:10 with IBS directly in a sample cup of a BBL fibrometer (Becton Dickson & Co., Baltimore, MD) (Miale, 1965) with measurements to 0.1 s. The diluted human reference material or raptor sample (200 μL) was incubated for 2 min at 37 °C, and the reaction was then initiated by the addition of 100 μL of bovine thrombin reagent supplied with the assay kit. Using the standard curve, fibrinogen concentration (mg/dL) was determined from the clotting time of each raptor sample. Using this assay, it is our experience that fibrinogen concentration in excess of 50 mg/dL is adequate to support clot formation of citrated plasma from bobwhite (*Colinus virginianus*), American kestrels (*Falco sparverius*) and Eastern screech-owls (*Megascops asio*) (Rattner et al., 2010, 2011, 2012a, 2015). Over a 4-day period, a total of 167 raptor samples, specifically stored as separate aliquots in vials for fibrinogen determination, were successfully analyzed in batches that included a standard curve. Sample volume was adequate for duplicate determinations of 154 samples, which exhibited intra-assay precision (coefficient of variation ± standard deviation) of 3.81 ± 8.57%. A human reference sample analyzed each day yielded an estimated inter-day precision of 3.59%.

Russell's viper venom directly activates Factor X (but not Factor VII). Reconstituted RVV Factor X activator (5-Diagnostics US Corp, Newton, Connecticut) was diluted 1:10 with IBS and maintained at room temperature. Citrated raptor plasma (65 μL) was mixed with phosphate buffer (35 μL of 8.3 mM phosphate buffer, pH 7.2) incubated at 37 °C in a sample cup for 2 min, and 100 μL of diluted RVV was added to the sample cup and incubated for 15 s. The clotting reaction was initiated with 100 μL 25 mM $CaCl_2$, and time to clot was measured with the BBL fibrometer to 0.1 s. A total of 171 raptor samples were analyzed in batches over a 4 day period. Adequate sample volume permitted duplicate analysis of 164 of these samples, with an intra-assay precision of 2.81 ± 6.30%. For samples with clotting times <50 s, the intra-assay variation was 2.19 ± 3.15% (precision decreases with prolonged clotting time). A human reference sample was analyzed at various intervals each day, and when RVVT of the reference material was averaged by day, inter-day assay precision was 0.98%.

For the PT assay, crude chicken hatchling thromboplastin (CHT) was prepared by the method of Quick, as modified and described by Rattner et al. (2010). Raptor plasma samples were diluted (80 μL plasma +20 μL 8.3 mM phosphate buffer, pH 7.2), incubated at 37 °C in a sample cup for 2 min, and the reaction was initiated by the addition of 200 μL CHT in 25 mM $CaCl_2$ (1:6 or 1:8 dilution of CHT working solution depending on species) with clotting time measured to 0.1 s. A subset of 138 raptor samples was analyzed in batches over a 4 day period. Adequate sample volume permitted duplicate analyses of 131 of these samples, with an intra-assay precision of 6.49 ± 5.82%. Intra-assay variation of samples with clotting times of <50 s was 6.36 ± 5.09%. A pool of citrated plasma from American kestrels was analyzed at various intervals each day, and when PT of this pool was averaged by day, inter-day assay precision was 6.14%.

### 2.3. Liver residue analysis

Chemical analysis generally followed methods described in Albert et al. (2010), with some procedural and instrumentation changes which are described here. Liver samples were initially prepared by mincing smaller samples with scissors, while larger mass samples were homogenized with an Omni homogenizer (Kennesaw, GA). Samples were then frozen until chemical analysis. At that time, approximately 0.5 g of a thawed and homogenized liver sample was accurately weighed and transferred into a 15 mL polyethylene centrifuge tube, spiked with an internal standard solution, and 3 mL of acetonitrile added. The liver sample was then further homogenized using a VWR BIO-GEN 200 homogenizer (Mississauga, ON) equipped with the metal shaft for small sample size (~1–2 min). The homogenizer probe was then rinsed with a 1 mL aliquot of acetonitrile. The homogenizer was activated for 30 s and the rinse combined to the first homogenate for a total of 4 mL. A 0.2 g aliquot of the pre-QuEChERs salt (NaCl) (BDH9286) was added to the 4 mL homogenate and the tube was vortexed to ensure salts were well dispersed. The tube was then shaken and centrifuged. The resulting supernatant was transferred into a UCT QuEChERs tube (Chromatographic Specialties ECQUUS1215CT) to which 50 mg of SupelClean ENVI-18 had previously been added. The extract was subsequently shaken and centrifuged again. The final supernatant was transferred in a glass tube and evaporated to dryness before being reconstituted in 1 mL of methanol. The final extract was filtered through a Life Sciences Acrodisc 13 mm 0.45 μm Nylon filter using a 1 mL disposable Luer-Lok syringe directly into a 2 mL PTFE/Silicone septa auto sampler vial prior to injection. Samples were then injected onto a liquid chromatography system (Agilent 1200 HPLC system, Agilent Technologies, Santa Clara, CA).

Mass spectrometric analysis of target rodenticide compounds first generation compounds, pindone, warfarin, diphacinone and chlorophacinone, and second generation compounds, brodifacoum, bromadiolone, and difethialone, was done using an AB Sciex API 5000 Triple Quadropole Mass Spectrometer (Framingham, MA) with the TurboSpray ion source in negative polarity using MRM (multiple reaction monitoring) scan type. Quality assurance and control methods included the use of quantification rodenticide standards (obtained from Sigma Aldrich, ChemService, Toronto Research Chemicals, CDN isotopes and BOC Sciences) to generate calibration curves at 6 concentrations ranging from 1.0 to 100 ng/mL and were prepared on each day of injection. The calculations were done by linear regression ($R^2 > 0.995$; no weighting) using the peak area and the internal standard (IS) method. Each liver sample was spiked with the IS standard solution (containing 5 IS). Five of the compounds were corrected for the recoveries of their respective deuterated standard. Pindone was corrected with diphacinone-d4 and difethialone with brodifacoum-d4. One or two blanks (methanol) were injected at the beginning and the end of each set of samples and before and after the calibration standards to monitor

**Table 1**
Citrated blood plasma samples collected from various species of raptors.

| Species | Individuals | Sampled 3 times | Sampled 2 times | Sampled 1 time |
|---|---|---|---|---|
| Bald eagle, *Haliaeetus leucocephalus* | 10 | 5 | 1 | 4 |
| Cooper's hawk, *Accipiter cooperii* | 6 | 3 | 2 | 1 |
| Red-tailed hawk, *Buteo jamaicensis* | 7 | 4 | 1 | 2 |
| Barred owl, *Strix varia* | 13 | 10 | 1 | 2 |
| Great horned owl, *Bubo virginianus* | 7 | 5 | 1 | 1 |
| Barn owl, *Tyto furcata* | 19 | 15 | 4 | 0 |
| Barn owl (pre-fledgling) | 19 | | | 19 |

injection cross-contamination. An aliquot of an in-house avian liver pool (double-crested cormorant Phalacrocorax auritus) (pool11-30351-01) containing no rodenticides was spiked with IS solutions and extracted with each set (9 samples) to monitor possible contamination from the procedure. To validate our calibration accuracy we determined the recoveries of 5 s source standards against our daily calibration. Since there is no certified reference material containing rodenticides, a positive quality control liver pool (from various raptor species) was prepared at NWRC. This in-house reference material contains brodifacoum and bromadiolone, and a sample was included with every batch of nine extractions, and analyzed along with the samples to monitor day to day variability. The method detection limit was 2 ng/g liver for warfarin, brodifacoum, chlorophacinone and bromadiolone, and 5 ng/g for diphacinone and difethialone on a wet weight (ww) basis.

### 2.4. Statistical methods

For clotting time samples analyzed in duplicate, RVVT and PT values were averaged, and are presented in Supplemental Tables S2 and S4 to the 0.1 s. Data were tested for homogeneity of variance and normality. Repeated measures ANOVA was used to evaluate the differences in clotting time for the three sampling periods with species as a factor, excluding individuals that had samples that did not clot for either RVVT or PT. We also evaluated differences in clotting time by sex and age (Adult versus Juvenile – fledgling), including their interaction, using a repeated measures ANOVA.

Reference intervals, which provide the range of values of a measured quantity in healthy unexposed individuals, were calculated using samples from captive raptor species (American kestrel, Eastern screech-owl) that had not been exposed to ARs (Rattner et al., 2014b, 2015) as a guide to identify samples with seemingly prolonged clotting time (outlier, response to clinically significant AR exposure). We used the statistical software program MedCalc© and followed the clinical laboratory guidelines C28-A2 and C28-A3 for estimating percentiles and their 90% confidence intervals, including upper reference limits (NCCLS, 2000; CLSI, 2010). All other statistical analyses were conducted using the software JMP© or SAS (version 9.3).

## 3. Results

In 2015, a total of 62 individual raptors were blood sampled at OWL and 19 pre-fledgling barn owls were blood sampled in the field (Table 1). The majority of the birds admitted to OWL were sampled three times (64.5%). However, some individuals died shortly

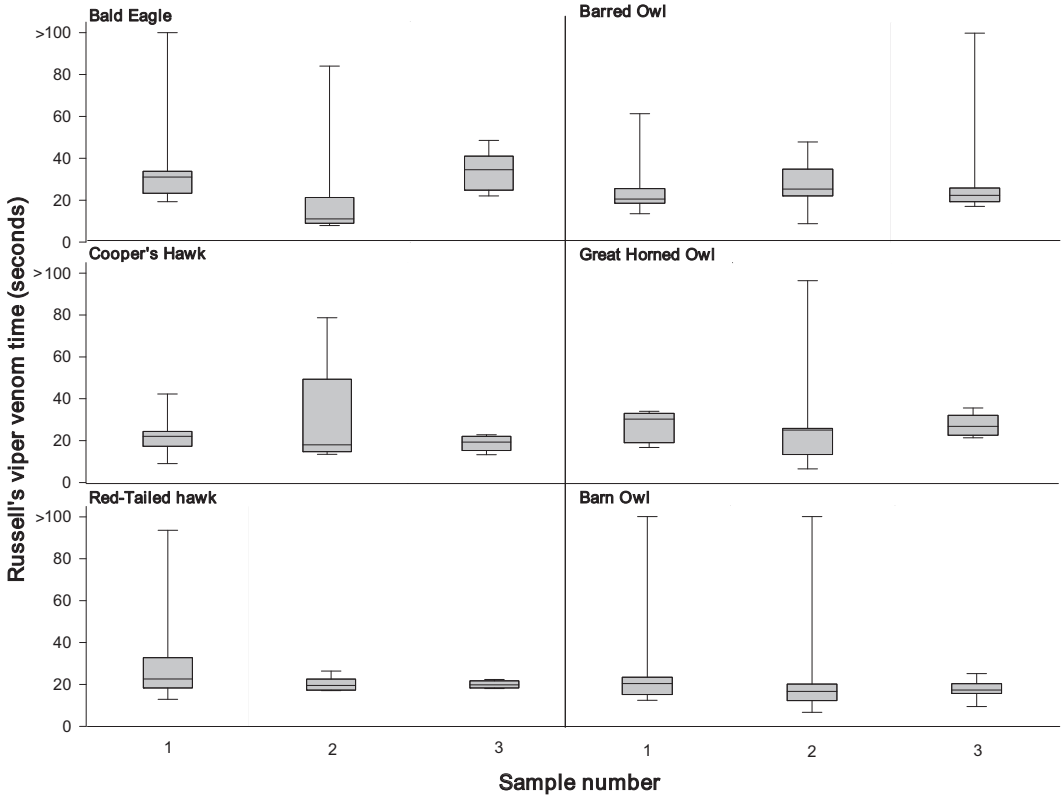


**Fig. 1.** Quantile box plot (minimum value, 25% quartile, median, 75% quartile and maximum value) of Russell's viper venom time (seconds) for sample number and species (excluding bird L15-031). Sample numbers 1, 2 and 3 correspond to collections at 2, 4 and 7 days post-admission. Species (sample size for collections 1, 2 and 3) are: bald eagle (9, 6, 5), Cooper's hawk (6, 4, 4), red-tailed hawk (7, 5, 4), barred owl (13, 9, 9), great horned owl (7, 6, 5), barn owl (18, 16, 18).

after admission, while others were undergoing treatment and not available for blood sampling or injuries were too severe to permit subsequent collection of blood samples at day 4 or day 7. Plasma from sampled raptors was assayed for fibrinogen concentration (TCT) and RVVT. All raptor samples were also assayed for PT, except for barn owls for which we only had capacity to run assays from 10 individuals (juvenile or adult) admitted to OWL and from 11 prefledge barn owls sampled in the field. We selected a subset of barn owl samples for PT testing based on their RVVT, assaying samples from some individuals with longer RVVTs and an equal number with shorter RVVTs.

### 3.1. Fibrinogen concentration

A total of 167 citrated plasma samples from 81 individuals were successfully analyzed in the TCT assay. Fibrinogen concentration exceeded 50 mg/dL in 166 of these samples (range: 56.2 to 576.3 mg/dL; see average concentration for each species at each sampling time, Supplemental Table S1). The initial sample collected from barn owl L15-013 had low fibrinogen concentration (40.3 mg/dL) but did produce a clot in the RVVT assay. In addition to these 167 samples that were successfully assayed, three other samples were problematic. The initial citrated plasma sample for barn owl LC15-027 appeared to contain a clot in its

**Table 2**
Case record of individual raptors that were released following treatment or humanely euthanized that had one or more RVVT or PT values exceeding the upper bound of the 90% confidence interval of the upper limit for American kestrels or Eastern screech-owls that were not necropsied and screened for ARs.

| Species | Identification | Sex, age | Russell's viper venom time (s) | | | Prothrombin time (s) | | | History | Clinical signs | Final disposition |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Time 1 | Time 2 | Time 3 | Time 1 | Time 2 | Time 3 | | | E = euthanized R = released |
| Bald eagle | L15-018 | F, Adult | 19.6 | 28.6 | 41.0 | 11.9 | 11.8 | 13.6 | Found in ditch | Open wound on right wing at radius/ulna, excessive bleeding, given baytril and metacam | R |
| Bald eagle | L15-019 | M, adult | 23.3 | 28.8 | 48.6 | 14.8 | 15.4 | 18.8 | Found buried at the landfill | Dirty, thin, given baytril, metacam and lactated ringers | E |
| Bald eagle | L15-091 | F, adult | 32.3 | 40.3 | 34.6 | 12.3 | 15.0 | 16.8 | Found at landfill | Emaciated, foot infection, mites, given baytril, metacam and lactated ringers | E |
| Bald eagle | L15-103 | F, adult | 19.3 | >100 | 24.8 | 9.3 | >100 | 18.0 | Interspecies fighting | Bruising on chest, swollen lreft wing, given baytril and ivomec | E |
| Bald eagle | L15-105 | M, adult | 31.1 | 32.3 | 22.0 | 11.3 | 15.3 | 12.6 | Found on roadside | Left shoulder dislocated, emaciated and dehydrated, given lactated ringers | R |
| Cooper's hawk | L15-033 | M, adult | 42.3 | 79.0 | 22.8 | 12.8 | 88.2 | 11.2 | Hit window | Thin, stunned | R |
| Red-tailed hawk | L15-025 | F, juvenile | 12.9 | 26.4 | 22.4 | 9.4 | NA | 12.8 | Found on ground | Emaciated, feet and wrist swollen, given metacam, | R |
| Red-tailed hawk | L15-092 | F, adult | 28.9 | NA | NA | 23.3 | NA | NA | Found on ground | Emaciated, no meds | R |
| Red-tailed hawk | L15-094 | F, juvenile | 32.8 | 22.6 | NA | 12.3 | 10.8 | NA | Head trauma | Swollen eyes, metacam, released | R |
| Barred owl | L15-037 | F, adult | 24.8 | 35.1 | 17.8 | 11.4 | NA | 10.60 | Hit by vehicle | Right wing fracture, given meloxicam | R |
| Barred owl | L15-108 | F, juvenile | 47.1 | 45.8 | >100 | 41.6 | 24.8 | 31.3 | Young on ground | Abdominal puncture wounds, covered in fly eggs and dried blood, given baytril and ivermectin | R |
| Barred owl | L15-118 | M, adult | 32.6 | 25.3 | 27.3 | 9.4 | 8.40 | 11.6 | Caught in netting | Left eye injured, few tail feathers broken | R |
| Great horned owl | L15-102 | F, juvenile | 30.3 | 83.8 | 36.0 | 11.3 | >100 | 8.9 | Young owlet found in the middle of a field | No apparent injuries, a thin | R |
| Great horned owl | L15-104 | M, juvenile | 16.8 | 15.3 | 23.0 | 9.6 | 10.6 | 15.3 | Young on ground | No apparent injuries | R |
| Great horned owl | L15-107 | F, juvenile | 34.1 | 29.0 | 27.3 | 8.3 | 9.8 | 9.8 | Young on ground | No injuries, fostered | R |
| Great horned owl | L15-109 | F, juvenile | 20.6 | 30.0 | 21.8 | 9.6 | 12.4 | 8.8 | Young on ground | Skinny, fostered | R |
| Great horned owl | L15-110 | F, juvenile | 30.8 | 29.8 | NA | 7.6 | 8.1 | NA | Young on ground | Skinny, fostered | R |
| Great horned owl | L15-112 | F, juvenile | 33.1 | 20.6 | 32.6 | 8.6 | 9.9 | 9.3 | Young on ground | No injuries, fostered | R |
| Barn owl | L15-024 | M, adult | >100 | 9.6 | 20.6 | 14.8 | 7.4 | 8.4 | Found inside boathouse on ground | No apparent injuries | R |
| Barn owl | L15-028 | M, juvenile | 17.1 | >100 | 9.3 | 7.6 | 21.1 | 6.4 | Found on ground below nest | No apparent injuries | R |
| Barn owl | L15-098 | M, adult | >100 | 22.8 | NA | 28.2 | 9.2 | NA | Hit by vehicle | Right eye bloody and retina looks damaged, stunned, given metacam | R |
| Barn owl | L15-100 | M, juvenile | 28.6 | 22.8 | 15.0 | 11.1 | 8.3 | 7.0 | Young on ground | Dehydrated, thin, given lactated ringers and vitamin B-12 in food, fostered | R |
| Barn owl | L15-117 | F, juvenile | 28.3 | 12.3 | 17.6 | 9.9 | 7.4 | 8.8 | Young on ground | Fostered | R |

NA = The raptor was not available for sampling due to veterinary appointment or further blood sampling would compromise its condition.
Fostered = Placed in care with a conspecific adult at the rehabiliation facility.

**Table 3**
Case records of raptors that died or were humanely euthanized and necropsied, with AR residue values and clotting times.

| Species | Identification | Sex, Age, Body Condition | Brodifacoum (μg/g ww) | Bromadiolone (μg/g ww) | Difethialone (μg/g ww) | ΣSGARs (μg/g ww) | RVVT (s) | | | PT (s) | | | History | Clinical Signs | Necropsy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Time 1 | Time 2 | Time 3 | Time 1 | Time 2 | Time 3 | | | |
| Bald eagle | L15-008 | F, adult, fair | <MDL | 0.006 | <MDL | 0.006 | 33.8 | 27.6 | | 13.9 | 15.4 | | Found in ditch, euthanized 4 days after admission | Left wing and right foot electrocuted | Bilateral pulmonary hemorrhage, moist necrosis and green discoloration of the left wing. Button laceration on the right shank and red discloroation of the right foot. Diagnosis:electrocution |
| Bald eagle | L15-031 | M, adult, good | <MDL | 0.003 | 0.005 | 0.009 | >100 | | | >100 | | | On ground with blood in mouth, died 2 days after admission | Given calcium EDTA | Bilateral pulmonary hemorrhage, ribs fractured on the right side and the pelvis is fractured. Diagnosis: trauma |
| Bald eagle | L15-032 | M, adult, fair | 0.026 | 0.009 | 0.016 | 0.051 | >100 | | | >100 | | | Found on ground not moving. | Slow deep “honking” breathing, feet curled, given lactated ringers, metacam, baytril and activated charcoal after blood sampling. | Brain cholinesterase levels are normal. Liver is negative for barbiturates. Specific cause of death not determined. |
| Bald eagle | L15-034 | M, adult, fair | <MDL | <MDL | <MDL | <MDL | 25.6 | | | 18.0 | | | Found on trail, died 5 days after admission | Open fracture of left radius/ulna | Trauma |
| Bald eagle | L15-035 | M, adult, good | NA | NA | NA | NA | 35.8 | | | 22.6 | | | Found on ground, unable to fly | Given activated charcoal after blood sampling, died next day | Severe freezing artifact and autolysis makes difficult to interpret results. Multifocal caseation necrosis of liver. Did not liver test ind. |
| Cooper's hawk | L15-005 | F, juvenile, poor | 0.017 | 0.004 | <MDL | 0.021 | 9.0 | | | 4.4 | | | No details | Left leg partial paralysis | Aspergillosis |
| Red-tailed hawk | L15-130 | M, Nestling, Poor | 0.105 | <MDL | <MDL | 0.105 | 93.6 | | | 77.1 | | | Found on ground, suspected fell out of nest, died same day. | Lethargic and thin | No palpable fractures, a large blood clot adjacent to a tear in the liver capsule and hemorrhage at the thoracic inlet. Diagnosis: trauma |
| Barred owl | L15-001 | F, adult, good | 0.014 | 0.086 | <MDL | 0.100 | 13.8 | | | 6.8 | | | Hit by car | Nerve damage, not standing | No palpable fractures, extensive bruising and hemorrhage on the right flank. Diagnosis: trauma |
| Barred owl | L15-020 | M, Adult, Poor | 0.005 | 0.011 | 0.018 | 0.034 | 18.8 | | | 7.3 | | | Found under beaver dam in poor condition, died two days later. | Emaciated, eyes cloudy, beak cracked | Bilateral pulmonary hemorrhage. Diagnosis: trauma |
| Barred owl | L15-021 | M, Adult, Fair | 0.006 | 0.321 | 0.016 | 0.343 | 61.6 | 48.0 | | 30.3 | 41.8 | | Found being attacked by crows. | Small peck at back of neck, right side near shoulder. | Mild bilateral pulmonary edema. Specific cause of death could not be determined. |
| Great horned owl | L15-113 | M, Adult, Fair | 0.004 | 0.009 | <MDL | 0.013 | 19.0 | | | 10.3 | | | Suspected electrocution or hit by car. Euthanized five days after admission. | Right wing has a moist necrotic laceration. There is a button ulceration in the right foot. | Electrocution |

NA = not analyzed.

storage vial; this sample did not clot in the TCT assay (presumably fibrinogen depleted), and thus was not analyzed for RVVT or PT. The second sample from barn owl L15-095 exhibited extremely short clotting time, likely due to debris in the sample. The clot endpoint was not detected in the sample from barn owl pre-fledgling 1947-19559, although a clot formed and was visibly present in the fibrometer assay cup, suggesting an instrument malfunction; the companion sample for this bird did produce a clot in the RVVT assay.

### 3.2. Russell's viper venom time

A total of 171 citrated plasma samples from 81 raptors were assayed using RVV. Eight of 62 raptors admitted to OWL had one sample that did not clot (i.e., one or more replicate analyses >100 s). One of these samples was from a bald eagle (L15-031) given chelating agent Calcium EDTA two to three hours before venipuncture due to suspected lead poisoning. This treatment can affect blood clotting (Zucker, 1954); hence the individual was excluded from statistical analyses. The remaining seven samples had fibrinogen levels judged to be adequate to produce a clot in this assay (range: 81.0–266.1 mg/dL) and medication was not a factor.

Russell's viper venom time was relatively similar among species, with the exception of seemingly shorter RVVTs in barn owls (Supplemental Table S2). Repeated measures ANOVA revealed no significant difference in RVVT among the three sampling periods ($F_{(2,29)} = 1.00$, $p = 0.38$; Supplemental Table S2; Fig. 1). A second repeated measures ANOVA indicated no significant trends for RVVT in relation to sex ($F_{(2,31)} = 0.47$, $p = 0.62$), age (adult versus juvenile) ($F_{(2,31)} = 0.06$, $p = 0.94$) or the interaction of these factors ($F_{(2,31)} = 0.85$, $p = 0.44$).

We were unable to obtain reference blood samples from the raptor species being studied which we could be certain had not been exposed to ARs. The rehabilitation facility only had one or two permanent captive individuals of each species, which was insufficient to establish a baseline or reference interval. Thus, there was no species-specific reference data against which to compare our results. Although use of interspecific reference values has limitations, we compared our data to reference values calculated for captive adult American kestrels (n = 49) and Eastern screech-owls (n = 40) (Rattner et al., 2014b, 2015) (Supplemental Table S3). We compared the bald eagle and Cooper's and red-tailed hawks (order Accipitriformes) with baseline data for the American kestrel (order Falconiformes), and barred, great horned and barn owls with baseline data for Eastern screech-owls (all from order Strigiformes). For bald eagles (excluding L15-031) and hawks, 22 of 50 samples had clotting times greater than the upper bound of the 90% confidence interval of the upper reference limit for captive American kestrels (>24.7 s). Nine of these 22 samples (samples from 6 bald eagles excluding L15-031, 1 Cooper's hawk, 1 red-tailed hawk) had clotting times >1.5 times the upper reference limit (>35.4 s; a rigorous value to identify outliers) (Tables 2 and 3; Fig. 2). With respect to the owl species undergoing rehabilitation, 24 of 101 samples had clotting times above the upper bound of 90% confidence interval of the upper reference limit for captive Eastern screech-owls (>26.5 s); 9 samples (samples from 2 barred owls, 1 great-horned owl, 3 barn owls) had clotting times longer than 1.5 times the upper reference limit (>37.6 s) (Tables 2 and 3; Fig. 3).

### 3.3. Prothrombin time

Because of limited capacity, only 138 plasma samples from 64 of the 81 individuals were assayed for PT. In addition to the sample from the bald eagle (L15-031) that was given Calcium EDTA, four other samples did not clot (i.e., one or more replicates >100 s). These four samples included two bald eagles, one Cooper's hawk, and one great horned owl, none of which had low fibrinogen concentrations (256.0 to 337.3 mg/dL) nor had received any medication that might affect blood clotting.

Visual inspection suggested a narrower range for PT values among species, and there was no significant difference in PT among the three sampling periods ($F_{(2,\ 21)} = 0.05$, $p = 0.95$; Supplemental Table S4; Fig. 4). The repeated measures ANOVA showed no significant trends between PT and sex ($F_{(2,\ 23)} = 1.4$, $p = 0.26$), age ($F_{(2,\ 23)} = 0.19$, $p = 0.83$) or the interaction of sex and age ($F_{(2,\ 23)} = 0.93$, $p = 0.41$). For the subset of samples analyzed for both RVVT and PT, values were correlated ($r = 0.76$, $p < 0.001$, n = 135, Pearson correlation; Supplemental Fig. S1).

When PTs for raptors undergoing rehabilitation (excluding the Calcium EDTA-treated bald eagle) were compared to values from captive American kestrels and Eastern screech-owls, fewer values ($p = 0.003$, Fisher's Exact Test) exceeded the upper bound of the 90% confidence

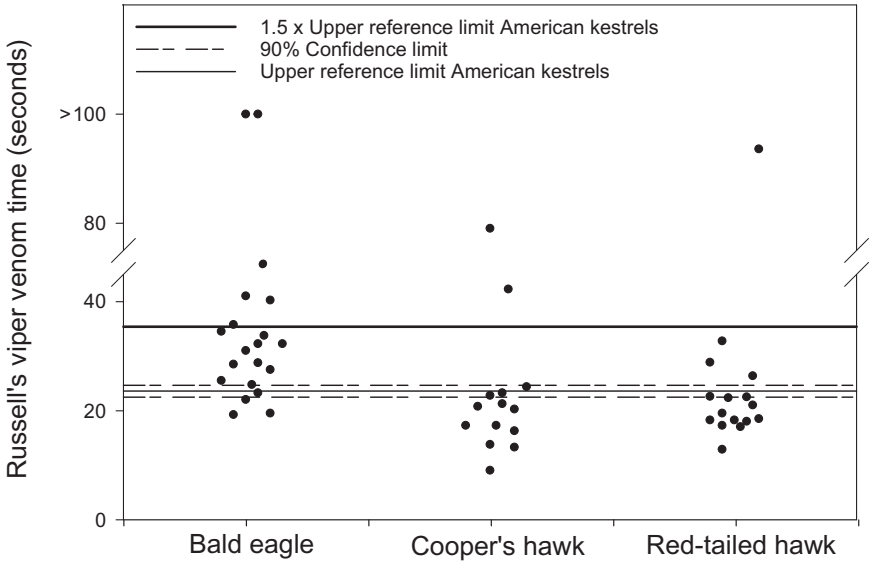


**Fig. 2.** Russell's viper venom time for bald eagle (excluding bird L15-031), Cooper's hawk and red-tailed hawk compared to the upper bound of the 90% confidence interval of the upper reference limit (24.7 s) and the 1.5 x the upper reference limit (35.4 s) derived from samples obtained from captive adult American Kestrels.

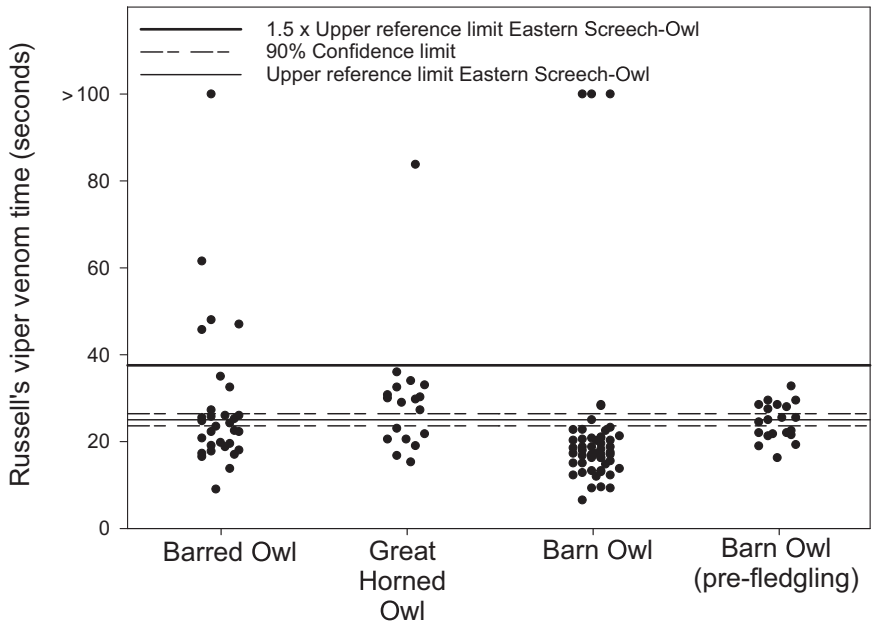


**Fig. 3.** Russell's viper venom times for the barred owl, great horned owl, and barn owl compared to the upper bound of the 90% confidence interval of the upper reference limit (26.5 s) and the 1.5 × upper reference limit (37.6 s) derived from samples obtained from captive adult Eastern screech-owls.

interval of the upper reference limit compared to the RVVTs (i.e., 19 of 126 samples versus 46 of 151 samples). Of the 19 samples exhibiting prolonged PT, nearly all (18 of 19 samples) had prolonged RVVT. For the bald eagles (excluding L15-031) and hawks, nine samples had clotting times longer than the upper bound of the 90% confidence interval of the upper reference limit for captive American kestrels (>17.3 s) and four of these samples (2 bald eagles, 1 Cooper's hawk, 1 red-tailed hawk) had clotting times longer than 1.5 times the upper reference

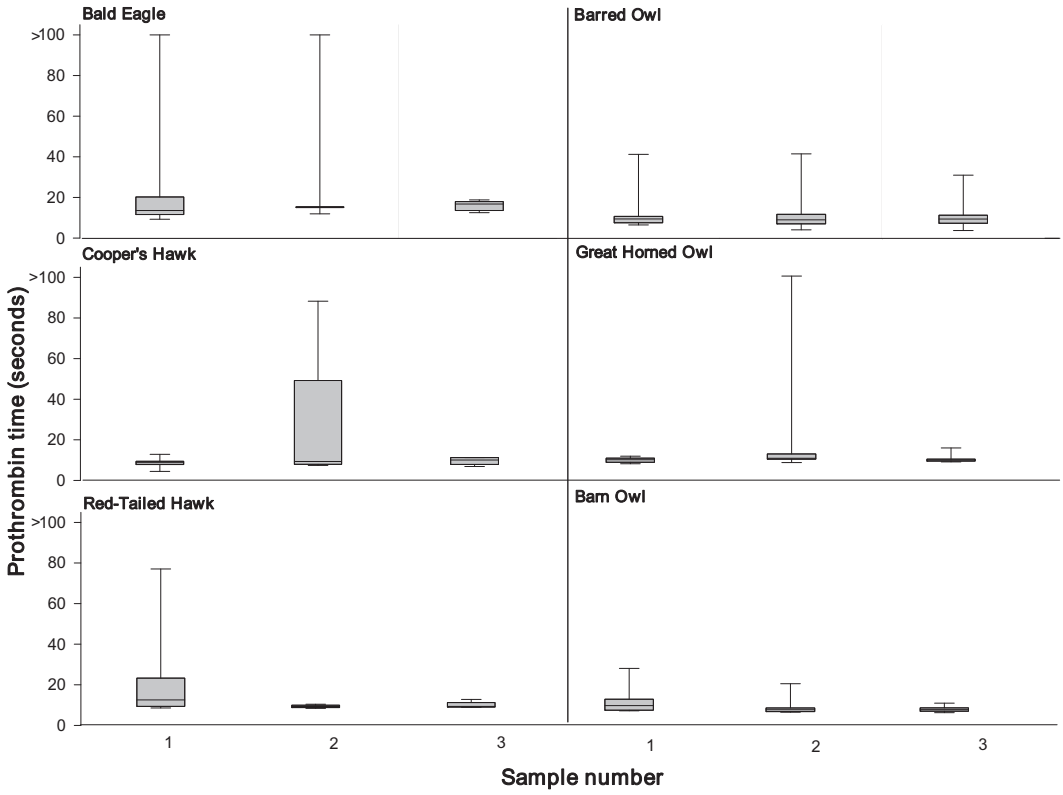


**Fig. 4.** Quantile box plot (minimum value, 25% quartile, median, 75% quartile and maximum value) of prothrombin time (seconds) for sample number and species (excluding bird L15-031). Sample numbers 1, 2 and 3 correspond to blood samples collected at 2, 4 and 7 days post-admission. Species (sample size for collections 1, 2 and 3) are: bald eagle (9, 6, 5), Cooper's hawk (6, 4, 4), red-tailed hawk (7, 4, 4), barred owl (13, 9, 10), great horned owl (7, 6, 5), barn owl (9, 9, 9).

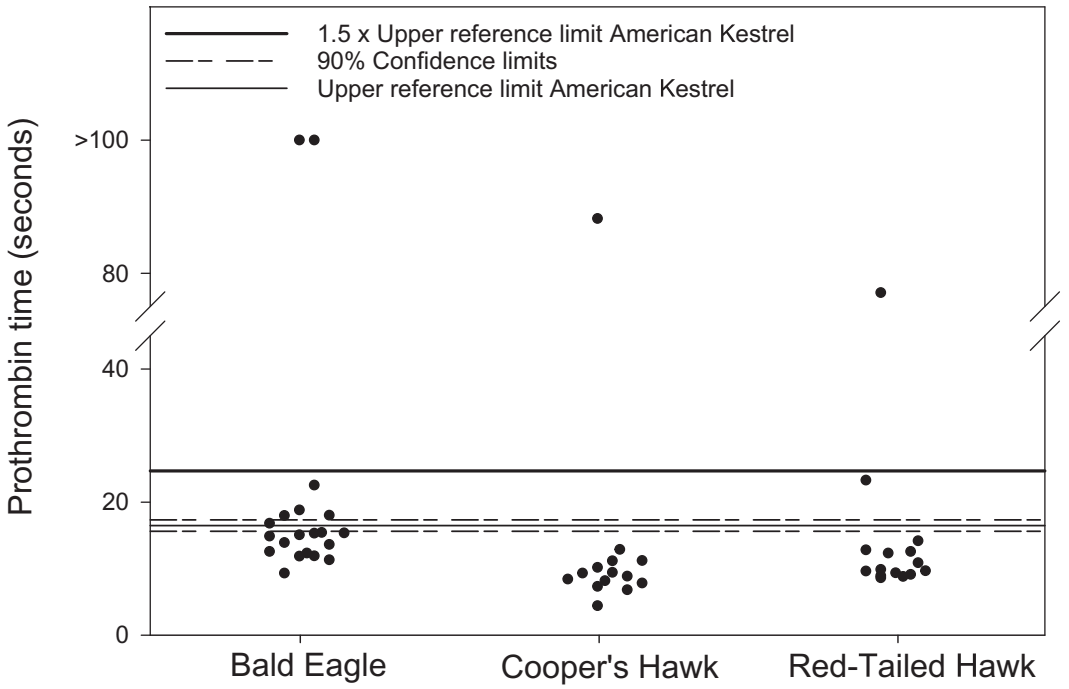


**Fig. 5.** Prothrombin times for the bald eagle, Cooper's hawk and red-tailed hawk compared to the upper bound of the 90% confidence interval of the upper reference limit (17.3 s) and the 1.5 × upper reference limit (24.7 s) derived from samples obtained from captive adult American kestrels.

limit (>24.7 s) (Fig. 5). For owls, ten samples had clotting times longer than the upper bound of the 90% confidence interval of the upper reference limit for captive Eastern screech-owls (>13.8 s); eight samples from five birds (2 barred owls, great horned owl, 2 barn owls) were above 1.5 times the upper reference limit (>19.5 s) (Fig. 6).

### 3.4. Pre-fledgling barn owls

All of the pre-fledgling barn owl plasma samples produced clots in the RVVT and PT assays. Seven individuals had RVVT values above the upper bound of the 90% confidence interval of the upper reference

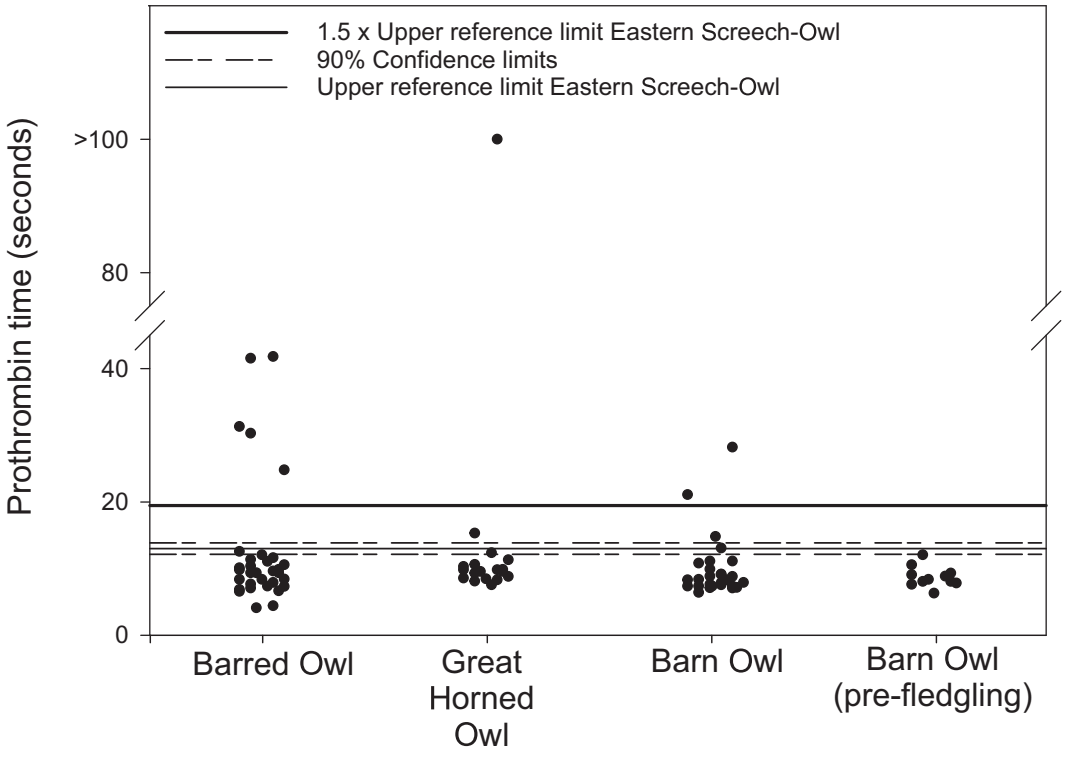


**Fig. 6.** Prothrombin times for the barred owl, great horned owl and barn owl compared to the upper bound of the 90% confidence interval of the upper reference limit (13.8 s) and the 1.5 × upper reference limit (19.5 s) derived from samples obtained from captive adult Eastern screech-owls.

limit of captive Eastern screech-owls, but well below 1.5 times the upper reference limit (Fig. 3). All barn owl PT values were below the Eastern screech-owl upper bound of the 90% confidence interval of the upper reference limit (Fig. 6), suggesting little to no exposure in the sampled pre-fledglings.

### 3.5. Liver anticoagulant rodenticide residues

Eleven raptors were necropsied, of which 10 had liver samples tested for ARs (one bald eagle liver was too decomposed to permit analysis) (Table 2). Nine of the ten raptors tested positive for SGARs of which seven had two or more SGARs. No FGARs were detected. Hepatic SGAR concentrations in the nine positive samples ranged from 0.006–0.343 μg/g ww (mean ± SD 0.076 ± 0.107 μg/g ww). Visual inspection of these data were suggestive of longer clotting times in raptors exposed to ARs. However, there was no significant correlation between the sum of SGARs and RVVT ($p = 0.43$) or PT ($p = 0.46$) when the longest running assay time for each individual species was compared with total SGAR concentration in liver (Supplemental Figs. S2 and S3).

Ante-mortem observations associated with AR intoxication (e.g., delayed whole blood clotting time, anemia, excessive hemorrhage in absence of traumatic injuries, Murray, 2011, 2017) and post-mortem results associated with AR toxicosis (e.g., $\sum$SGARs >0.1 μg/g ww liver + hemorrhage in organs or subcutaneous tissue, Newton et al., 1999a, 1999b, Thomas et al., 2011) did not *definitively* indicate AR poisoning as the cause of death for any of the deceased raptors (Table 3). Trauma was the primary cause of death (n = 5), followed by electrocution (n = 2), and Aspergillosis (n = 1) (Table 3). For three individuals, cause of death could not be determined based on necropsy results (bald eagles L15-032 and L15-035, and barred owl L15-021). However, there were raptors that were diagnosed as having died of trauma that also had elevated hepatic concentrations of one or more SGARs (0.105 μg/g ww for red-tailed hawk L15-130 and 0.100 μg/g ww for barred owl L15-001). Notably, barred owl L15-021 (specific cause of death not determined, $\Sigma$SGARs = 0.343 μg/g ww) and red-tailed hawk L15-130 (cause of death trauma), both had elevated hepatic SGAR residues and exhibited prolonged clotting times, and thus it seems likely that these two birds were AR-intoxicated.

## 4. Discussion

For free-ranging raptors, diagnosis of AR intoxication is often made only on overt clinical signs and simple diagnostic tests (e.g., hematocrit) (Redig and Arent, 2008). Our study is one of the few attempts (Murray and Tseng, 2008; Webster et al., 2015) to use whole blood clotting or more specific clotting time assays to evaluate AR exposure in free-ranging raptors admitted to rehabilitation facilities. Citrated plasma samples from admitted raptors were used to assess the utility of blood clotting assays to detect sublethal exposure to ARs, which might also be present in birds being treated for traumatic injury. In total, blood samples were collected from 62 admitted raptors and 19 pre-fledgling barn owls at nest sites. Results of the RVVT assay indicated that seven raptors (11.5% of admitted birds excluding a Calcium EDTA-treated bald eagle) had samples that did not clot and many others had prolonged RVVT. Fewer birds had prolonged PT values; four admitted individuals (6.6%) had samples that did not clot, and others had lengthy clotting times. Based upon adequate fibrinogen concentration in samples that failed to clot in the RVVT and PT assays, and evidence of long-term stability of PT in samples frozen for extended periods (Webster et al., 2015; Zhao et al., 2017), we have no reason to suspect that the results are an artifact. Interestingly, even though AR effects on blood clotting can be short-lived (Mount and Feldman, 1983; Furie et al., 1999; Rattner et al., 2011), and we designed the study with that in mind, there were no significant temporal trends in clotting time observed over the course of rehabilitation. In addition, there was no apparent effect of sex or age on clotting times.

A surprisingly large number of individuals exhibited prolonged clotting time. From the complete dataset (excluding Calcium EDTA-treated bald eagle L15-031), 35 of 80 raptors (43.8%) had one or more RVVTs classified as prolonged or the sample failed to clot; 15 of 63 raptors (23.8%) had one or more PTs classified as prolonged or the sample failed to clot. Using a more rigorous threshold (i.e., 1.5 times the RVVT and PT upper limit for captive American kestrels and Eastern screech-owls), 14 of 80 raptors (17.5%) had one or more RVVTs classified as prolonged or the sample failed to clot, and 9 of 63 raptors (14.3%) had one or more PTs classified as prolonged or the sample failed to clot. Of the raptors admitted to OWL (excludes L15-031 Ca EDTA-treated bald eagle), 14 of 61 (23.0%) individuals had one or more sample RVVT or PT value elevated above 1.5 times the upper reference limit. None of the pre-fledgling barn owls had RVVT or PT values that exceeded 1.5 times the upper reference limit. Notably, two of the study species exhibiting high incidence of coagulopathy, namely the bald eagle and barred owl, are both experiencing population growth and expansion into urbanized environments (Elliott et al., 2011; Hindmarch and Elliott, 2015).

Of the 11 raptors necropsied, trauma was diagnosed as the principal cause of death, which is in line with the main reason raptors are admitted to OWL, and indeed the primary cause of death of urban raptors across North America (Dwyer et al., 2018). Nine of the ten tested raptors had detectable SGAR residues in their liver. Long-term regional and global trends show that the proportion of raptors exposed to individual or multiple ARs has been increasing over the past two decades (Walker et al., 2014; Huang et al., 2016; Murray, 2017). Our results suggest that raptors in British Columbia are being exposed to multiple ARs (Table 3). Three of the exposed raptors, a red-tailed hawk and two barred owls, had liver SGAR concentrations ≥0.1 μg/g ww, which has been suggested as a concentration above which there is a significant likelihood of toxicosis (Newton et al., 1999a, 1999b). Two of the birds in that category, a red-tailed hawk and a barred owl, had prolonged clotting times, and it seems likely that these birds were AR-intoxicated. While the present dataset of raptors tested for SGAR residues was small, the number of affected individuals (2 of 9, 22%) is not unlike that observed in a larger contemporary study in northeastern US for which 18% of 90 SGAR-positive raptors were symptomatic (Murray, 2017). In addition to these necropsied birds, 23 other admitted individuals exhibited prolonged RVVT and/or PT (Table 1). Some may have been exposed to ARs at levels that caused transient coagulopathy, perhaps adversely affecting birds on a temporary basis, but recovery occurred and they were released. We do not know the ultimate fate of the released birds, but it is possible that there were some effects on fitness of some individuals (reviewed in Rattner et al., 2014a; Rattner and Mastrota, 2018). Finally, in line with Webster et al. (2015), clotting time values for 19 pre-fledgling barn owls in the Lower Mainland of British Columbia do not indicate marked AR exposure.

While nine of ten raptors tested positive for AR residues, there was, however, no clear relation between clotting time and AR residues. This may be a reflection of small sample size and even restoration of hemostasis that can occur days to weeks following AR-induced coagulopathy. Previous studies have reported a wide range of responses in avian species to AR contamination in respect to clotting time, and toxicosis symptoms ante-mortem and post-mortem (Murray and Tseng, 2008; Murray, 2011, 2017; Webster et al., 2015). Consequently, determining a critical AR concentration threshold for coagulopathy in wild birds has been challenging (Rattner et al., 2014a, 2015). In addition, freezing and thawing of carcasses before necropsy is known to cause artefactual changes that could falsely be interpreted as toxicosis (Stroud, 2012).

The diagnosis of AR-induced mortality is complicated by determination of the ultimate from proximate causes of death (e.g., Murray, 2017, 2018). In our study, 90% of tested birds had detectable residues of one or more ARs; of those, seven were diagnosed initially with a non-toxicosis

cause of death and two individuals had an unknown cause of death. For example, a male nestling red-tailed hawk (L15-130) found "on ground" in an emaciated and lethargic state, that succumbed the same day as it was admitted to OWL, was diagnosed as having died from trauma, most likely from falling from the nest. However, its citrated plasma sample did not clot in the RVVT assay, its PT was more than three times the American kestrel upper reference limit, and its liver brodifacoum concentration was 0.105 μg/g ww, conclusive evidence of AR poisoning. Major trauma would in most cases obscure any AR-related lesions during necropsy that would not have been detected without further diagnostic testing, which is not typically performed at a rehabilitation facility. We also had a bald eagle (L15-032) that had a relatively low SGAR concentration (0.05 μg/g ww), but its plasma did not clot and cause of death could not be determined. Conversely, a barred owl (L15-001) that had low levels of SGARs in liver (0.01 μg/g ww) had normal clotting times, and diagnosis both at the rehabilitation facility and at necropsy was trauma.

Laboratory dose-response studies using both RVVT and PT have shown clear temporal trends between AR dose and clotting times for several species, including raptors such as the American kestrel and Eastern screech-owl (Rattner et al., 2011, 2014b; Webster et al., 2015). Peak coagulopathy typically occurs 1–4 days after exposure, but coagulopathy can be resolved within a matter of days or at the most weeks post-AR exposure (Mount and Feldman, 1983; Mosterd and Thijssen, 1991; Rattner et al., 2014a, 2014b). However, enzymes which synthesize vitamin K may remain partially inhibited for many weeks, thus rendering animals highly sensitive to subsequent AR exposure (Mosterd and Thijssen, 1991). In this context, our snapshot attempt to detect temporal trends in an individual raptor's clotting time was challenging and no consistent trends were detected in the six species studied. Unlike a controlled laboratory study, we did not know the timing, frequency or amount of AR (s) consumed by each wild raptor. Some of the birds undergoing rehabilitation developed coagulopathy several days after admission, which might reflect latent effects of recent exposure or depletion of clotting factors associated with an injury. Liver residue data from the present study and others (Albert et al., 2010; Huang et al., 2016) demonstrate that the majority of raptors ingest multiple AR compounds, which further confound dose-response relationships. To date, no laboratory study has examined the effect of ingesting multiple ARs over a specific time period in raptors.

In veterinary diagnostics, clotting times are suggestive of anticoagulant exposure if they are ≥125% of baseline values (Shlosberg and Booth, 2006). However, the absence of baseline clotting time data or reference values for companion, exotic and wild species of birds has been acknowledged by many (e.g., Tahira et al., 1977; Morrisey et al., 2003; Bailey et al., 2005; Webster et al., 2015), thus making such determinations difficult. Due to limited availability of captive raptors, we were unable to collect blood samples from non-AR exposed individuals to make statistically relevant comparisons. Instead our reference values were based on samples obtained from captive unexposed American kestrels and Eastern screech-owls (Rattner et al., 2014b, 2015). Despite the potentially confounding effect of inter-species variation, comparing the sampled bald eagles and hawks with the American kestrel reference values and owls with the Eastern screech-owl reference values assists in guiding identification of individuals with prolonged clotting time. The estimated upper reference limit for RVVT and PT corresponds well with two standard deviations above arithmetic mean clotting times for both the American kestrel and Eastern screech-owl. We also included a more rigorous threshold (1.5 times the upper reference limit) to identify prolonged clotting times, as we could not make direct species comparisons. This seems particularly warranted for the bald eagle and hawk samples (Accipitriformes) as the American kestrel reference value is probably more representative of a different phylogenetic order (i.e., Falconiformes). Nonetheless, within a species group it was relatively easy to classify individuals as having prolonged clotting times, particularly for PT which appeared to exhibit more of an "all-or-none" response.

## 5. Conclusion

Using the more rigorous thresholds to identify coagulopathy, 23.0% of raptors admitted to the OWL rehabilitation facility exhibited prolonged clotting time or failure to form a clot altogether in one or both in vitro coagulation assays. Depending on the assay, there was a slight difference in the proportion of individuals exhibiting coagulopathy, with PT seemingly being less responsive than RVVT. We argue this is a biologically significant proportion of individuals given the fortuitous nature by which raptors are found and admitted to rehabilitation facilities. Contrary to our hypothesis (i.e., declining clotting time during rehabilitation), there was no distinct clotting time pattern when measured repeatedly over the course of the rehabilitation period. In the broader context of detecting AR toxicosis in raptors admitted to rehabilitation facilities, we recommend simple visual monitoring and timing of whole blood clotting within a serum collection tube and pack cell volume (Murray and Tseng, 2008; Redig and Arent, 2008). While less sensitive than PT and RVVT assays, these are efficient initial screening methods to identify individuals that may require vitamin $K_1$ antidote administration. If coagulation assays are to be more widely used as a diagnostic tool at avian rehabilitation facilities, there is a need for more species specific reference data. Generating such data is challenging, given the difficulties of obtaining large numbers of quality samples from unexposed raptors. Furthermore, for comparisons to be valid, clotting time assay reagents and methodology would have to be consistent among facilities, or alternatively, all samples could be analyzed at a single laboratory. If those challenges could be overcome, the ability to diagnose and treat AR toxicosis in wild birds would be greatly improved. We would also obtain a better understanding of the effects of ARs on free-ranging raptors, including the degree to which sublethal coagulopathies might affect response to and recovery from trauma.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.485.

## Data availability statement

Data owned by the U.S. Geological Survey and described in this manuscript are publicly available; Rattner and Mastrota (2018), Use of blood clotting assays to assess potential anticoagulant rodenticide exposure and effects in free-ranging birds of prey: U.S. Geological Survey data release https://doi.org/10.5066/P9EXCLIT.

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## References

Albert, C.A., Wilson, L.K., Mineau, P., Trudeau, S., Elliott, J.E., 2010. Anticoagulant rodenticides in three owl species from Western Canada, 1988-2003. Arch. Environ. Contam. Toxicol. 58, 451–459.

Bachman, K., Sullivan, T., 1983. Dispositional and pharmacodynamics characteristics of brodifacoum in warfarin-sensitive rats. Pharmacology 27, 281–288.
Bailey, C., Fisher, P., Eason, C.T., 2005. Assessing Anticoagulant Resistance in Rats and Coagulation Effects in Birds Using Small-Volume Blood Samples. Sci Conserv. 249 (22 pp).
Buckle, A.P., Prescott, C., 2018. Anticoagulants and risk mitigation. In: van den Brink, N., Elliott, J.E., Shore, R.F., Rattner, B.A. (Eds.), Anticoagulant Rodenticides and Wildlife. Springer International Publishing, Cham, Switzerland, pp. 319–355.
Butler, R.W., Campbell, R., 1987. The birds of the Fraser River Delta: Populations, ecology, and international significance. Canadian Wildlife Service Occasional Paper Number 65. Ottawa, Canada, p. 73.
CLSI, 2010. Clinical and Laboratory Standards Institute's (CLSI) newly revised document Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline— Third Edition (EP28-A3). 3rd edition. Wayne, PA 72 pp. https://clsi.org/standards/products/method-evaluation/documents/ep28/, Accessed date: April 2018.
Corrigan, R., 2001. Rodent Control: A Practical Guide for Pest Management Professionals. 1st edition. GIE Inc, Cleveland, OH (355 pp).
Dwyer, J., Hindmarch, S., Kratz, G., 2018. Raptor mortality in urban landscapes. In: Raptors, Urban, Boal, C.W., Dykstra, C.R. (Eds.), Island Press. Washington, DC, pp. 199–213.
Elliott, K.H., Elliott, J.E., Wilson, L.K., Jones, I., Stenerson, K., 2011. Density-dependence in the survival and reproduciton of bald eagles: linkages to chum salmon. J. Wildl. Manag. 75, 1688–1699.
Elliott, J.E., Rattner, B.A., Shore, R.F., van den Brink, N.W., 2016. Paying the pipers: mitigating the impact of anticoagulant rodenticides on predators and scavengers. Bioscience 66, 401–407.
Fair, J.M., Paul, E., Jones, J., Barrett Clark, A., Davie, C., Kaiser, G., 2010. Guidelines to the Use of Wild Birds in Research. Ornithological Council, Washington DC, p. 215.
Furie, B., Bouchard, B.A., Furie, B.C., 1999. Vitamin K-dependent biosynthesis of gamma-carboxyglutamic acid. Blood 93, 1798–1808.
Gabriel, M.W., Woods, L.W., Poppenga, R., Sweitzer, R.A., Thompson, C., Matthews, S.M., Higley, J.M., Keller, S.M., Purcell, K., Barrett, R.H., et al., 2012. Anticoagulant rodenticides on our public and community lands: spatial distribution of exposure and poisoning of a rare forest carnivore. PLoS One 7, e40163.
Hindmarch, S., Elliott, J.E., 2015. When owls go to town: the diet of urban barred owls. J. Raptor Res. 49, 66–74.
Huang, A.C., Elliott, J.E., Hindmarch, S., Lee, S.L., Maisonneuve, F., Bowes, V., Cheng, K.M., Martin, K., 2016. Increased rodenticide exposure rate and risk of toxicosis in barn owls (*Tyto alba*) from southwestern Canada and linkage with demographic but not genetic factors. Ecotoxicology 25, 1061–1071.
Jacob, J., Buckle, A.P., 2018. Use of anticoagulant rodenticides in different applications around the world. In: van den Brink, N., Elliott, J.E., Shore, R.F., Rattner, B.A. (Eds.), Anticoagulant Rodenticides and Wildlife. Springer International Publishing, Cham, Switzerland, pp. 11–44.
López-Perea, L., Mateo, R., 2018. Secondary exposure of anticoagulant rodenticides and effects in predators. In: van den Brink, N., Elliott, J.E., Shore, R.F., Rattner, B.A. (Eds.), Anticoagulant Rodenticides and Wildlife. Springer International Publishing, Cham, Switzerland, pp. 159–193.
Miale, J.B., 1965. The fibrometer system for routine coagulation tests. Am. J. Clin. Pathol. 43, 475–480.
Morrisey, J.K., Paul-Murphy, J., Fialkowski, J.P., Hart, A., Darien, B.J., 2003. Estimation of prothrombin times of Hispaniolan amazon parrots (*Amazona ventralis*) and umbrella cockatoos (*Cacatua alba*). J. Avian Med. Surg. 17, 72–77.
Mosterd, J.J., Thijssen, H.H.W., 1991. The long-term effects of rodenticide, brodifacoum, on coagulation and vitamin K metabolism in rats. Br. J. Pharmacol. 535, 531–535.
Mount, M., Feldman, B., 1983. Mechanism of diphacinone rodenticide toxicosis in the dog and its therapeutic implications. Am. J. Vet. Res. 2009–2017.
Murray, M., 2011. Anticoagulant rodenticide exposure and toxicosis in four species of birds of prey presented to a wildlife clinic in Massachusetts, 2006–2010. J. Zoo Wildl. Med. 42, 88–97.
Murray, M., 2017. Anticoagulant rodenticide exposure and toxicosis in four species of birds of prey in Massachusetts, USA, 2012–2016, in relation to use of rodenticides by pest management professionals. Ecotoxicology 26, 1041–1050.
Murray, M., 2018. Ante-mortem and post-mortem signs of anticoagulant rodenticide toxicosis in birds of prey. In: van den Brink, N., Elliott, J.E., Rattner, B.A. (Eds.), Anticoagulant Rodenticides and Wildlife. Springer International Publishing, Cham, Switzerland, pp. 109–134.
Murray, M., Tseng, F., 2008. Diagnosis and treatment of secondary anticoagulant rodenticide toxicosis in a red-tailed hawk (*Buteo jamaicensis*). J. Avian Med. Surg. 22, 41–46.
NCCLS, 2000. How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline. Second edition. C28-A2. Wayne, PA. http://www.zxyjhjy.com/upload/attached/file/20170406/20170406120112_8797.pdf (Accessed 2018).
Newton, I., Dale, L., Finnie, J.K., Freestone, P., Wright, J., Wyatt, C., Wylie, I., 1999a. Wildlife and Pollution: 1997/98 Annual Report. Joint Nature Conservation Committee, Peterborough, UK 61 pp. http://jncc.defra.gov.uk/pdf/jncc285.pdf, Accessed date: October 2018.
Newton, I., Shore, R.F., Wyllie, I., JDS, Birks, Dale, L., 1999b. Empirical evidence of side-effects of rodenticides on some predatory birds and mammals. In: Cowan, D.P., Feare, C. (Eds.), Advances in Vertebrate Pest Management. Filander Verlag, Fürth, Germany, pp. 347–367.
Rattner, B.A., Mastrota, F., 2018. Anticoagulant rodenticide toxicity to non-target wildlife under controlled exposure conditions. In: Van Den Brink, N., Elliott, J.E., Shore, R.F., Rattner, B.A. (Eds.), Anticoagulant Rodenticides and Wildlife. Springer International Publishing, Cham, Switzerland, pp. 45–86.
Rattner, B.A., Horak, K.E., Warner, S.E., Johnston, J.J., 2010. Acute toxicity of diphacinone in northern bobwhite: effects on survival and blood clotting. Ecotoxicol. Environ. Saf. 73, 1159–1164.
Rattner, B.A., Horak, K.E., Warner, S.E., Day, D.D., Meteyer, C.U., Volker, S.F., Eisemann, J.D., Johnston, J.J., 2011. Acute toxicity, histopathology, and coagulopathy in American kestrels (*Falco sparverius*) following administration of the rodenticide diphacinone. Environ. Toxicol. Chem. 30, 1213–1222.
Rattner, B.A., Horak, K.E., Lazarus, R.S., Eisenreich, K.M., Meteyer, C.U., Volker, S.F., Campton, C.M., Eisemann, J.D., Johnston, J.J., 2012a. Assessment of toxicity and potential risk of the anticoagulant rodenticide diphacinone using eastern screech-owls (*Megascops asio*). Ecotoxicology 21, 832–846.
Rattner, B.A., Lazarus, R.S., Eisenreich, K.M., Horak, K.E., Volker, S.F., Campton, C.M., Eisemann, J.D., Meteyer, C.U., Johnston, J.J., 2012b. Comparative risk assessment of the first-generation anticoagulant rodenticide diphacinone to raptors. Proceeding 25th Vertebr Pest Conf, pp. 124–130.
Rattner, B.A., Lazarus, R.S., Elliott, J.E., Shore, R.F., Van Den Brink, N., 2014a. Adverse outcome pathway and risks of anticoagulant rodenticides to predatory wildlife. Environ. Sci. Technol. 48, 8433–8445.
Rattner, B.A., Horak, K.E., Lazarus, R.S., Goldade, D.A., Johnston, J.J., 2014b. Toxicokinetics and coagulopathy threshold of the rodenticide diphacinone in eastern screech-owls (*Megascops asio*). Environ. Toxicol. Chem. 33, 74–81.
Rattner, B.A., Horak, K.E., Lazarus, R.S., Schultz, S.L., Knowles, S., Abbo, B.G., Volker, S.F., 2015. Toxicity reference values for chlorophacinone and their application for assessing anticoagulant rodenticide risk to raptors. Ecotoxicology 24, 720–734.
Redig, P.T., Arent, L., 2008. Raptor toxicology. Vet. Clin. Exot. Anim. Pract. 11, 261–282.
Shlosberg, A., Booth, L., 2006. Veterinary and Clinical Treatment of Vertebrate Pesticide Poisoning – A Technical Review. Lincoln, NZ (101 pp).
Stroud, R.K., 2012. Bruising encounters in veterinary forensics. Vet. J. 194, 278–279.
Tahira, N., Dube, B., Agrawal, G., 1977. Blood coagulation studies in some wild Indian birds: effects of different tissue thromboplastins. J. Comp. Pathol. 87, 451–457.
The University of Minnesota, 2017. The Raptor Centre. https://www.raptor.umn.edu/our-research, Accessed date: December 2018.
Thomas, P.J., Mineau, P., Shore, R.F., Champoux, L., Martin, P.A., Wilson, L.K., Fitzgerald, G., Elliott, J.E., 2011. Second generation anticoagulant rodenticides in predatory birds: probabilistic characterisation of toxic liver concentrations and implications for predatory bird populations in Canada. Environ. Int. 37, 914–920 (and corrigendum 40: 256).
Vandenbroucke, V., Bousquet-Melou, A., De Backer, P., Croubels, S., 2008. Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration. J. Vet. Pharmacol. Ther. 31, 437–445.
Walker, L.A., Llewellyn, N.R., Pereira, M.G., Potter, E.D., Sainsbury, A.W., Shore, R.F., 2014. Anticoagulant Rodenticides in Predatory Birds 2007 & 2008: APredatory Bird Monitoring Scheme (PBMS) Report. Lancaster. 18 pp. http://nora.nerc.ac.uk/12976/, Accessed date: August 2018.
Webster, K.H., Harr, K.E., Bennett, D.C., Williams, T.D., Cheng, K.M., Maisonneuve, F., Elliott, J.E., 2015. Assessment of toxicity and coagulopathy of brodifacoum in Japanese quail and testing in wild owls. Ecotoxicology 24, 1087–1101.
Witmer, G.W., 2018. Perspectives on existing and potential new alternatives to anticoagulant rodenticides and the implications for intergrated pest management. In: van den Brink, N., Elliott, J.E., Rattner, B.A. (Eds.), Anticoagulant Rodenticides and Wildlife. Springer International Publishing, Cham, Switzerland, pp. 357–378.
Zhao, Y., Feng, G., Zhang, J., Gong, R., Cai, C., Feng, L., 2017. Effects of preanalytical frozen storage time and temperature on screening coagulation tests and factors VIII and IX activity. Sci. Rep. 7, 12179.
Zucker, M.B., 1954. Some effects of disodium ethylenediamine tetraacetate on blood coagulation. Am. J. Clin. Pathol. 24, 39–41.