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Assessment of toxicity and coagulopathy of brodifacoum in Japanese quail and testing in wild owls

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Abstract Based on detection of hepatic residues, scavenging and predatory non-target raptors are widely exposed to second generation anticoagulant rodenticides (SGARs). A small proportion, generally <10 %, of tested birds are diagnosed as acutely poisoned. Little is known, however, of sub-lethal effects of SGARs, such as interaction of clotting capacity with traumatic injury. Assessment of coagulation function of birds submitted live to wildlife rehabilitators or veterinarians may provide a means of establishing the proportion of animals suffering sub-lethal coagulopathies, as well as identifying individuals requiring treatment. As a first step in exploring the potential of this approach, we dosed Japanese quail (*Coturnix japonica*) with the SGAR, brodifacoum, at 0, 0.8, 1.4, 1.9, and 2.5 mg/kg and sampled birds at 1, 3, 5 and 7 days post-dosing. Prothrombin time (PT), which measures the extrinsic coagulation pathway, was significantly prolonged in 98 % of brodifacoum-exposed quail in a dose- and time-dependent manner. 50-fold prolongation of PT occurred at higher brodifacoum dosages and correlated to hemorrhage found at necropsy. Activated clotting time (ACT), a measure of the intrinsic pathway also increased with dose and time. Hemoglobin (Hb) and hematocrit (Hct) decreased dose- and time-dependently at doses ≥1.4 mg/kg with no significant change at 0.8 mg/kg. Reference intervals for PT (10.0–16.2 s), ACT (30–180 s), Hb (9.6–18.4 g/dl), and Hct (34–55 %) were established in Japanese quail. Species-specific reference intervals are required as barn owl PT (17–29 s) and quail PT were different. The proportion of brodifacoum-exposed quail with hemorrhage was not correlated with liver residues, but was correlated with PT, suggesting that this assay is a useful indicator of avian anticoagulant rodenticide exposure. PTs measured in free-living barn owls sampled between April 2009 and August 2010 in the lower Fraser Valley of BC do not suggest significant exposure to SGARs.

Keywords Brodifacoum · Rodenticide · Prothrombin time · Japanese quail · Biomarker · Barn owl

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Introduction

Use of anticoagulant rodenticides (ARs) has become the dominant option worldwide to control rodent infestations, which are estimated to cause as much as $ 50 billion damage annually to food supplies and public health (Eason et al. 2010). First generation compounds (FGARs) such as warfarin were widely used, but became ineffective in some locations as Norway rats (*Rattus norvegicus*) developed genetic resistance. In response, second generation compounds (SGARs), typified by the compound, brodifacoum, were developed and extensively deployed. Due to

persistence and capacity to bioaccumulate, combined with extensive and often regular prophylactic use in agricultural, commercial, domestic, public and wildlife conservation applications, SGAR compounds are now widespread contaminants of non-target scavengers and predators of rodents (e.g. Newton et al. 1990; Stone et al. 1999, 2003; Albert et al. 2010; Murray 2011; Christensen et al. 2012; Gabriel et al. 2012; Rattner et al. 2014).

Although high proportions of tested birds of prey are exposed to SGARs, a relatively low proportion, usually in the range of 5–10 % are diagnosed at necropsy as having been acutely poisoned (Albert et al. 2010; Murray 2011). Determination of coagulopathy, particularly in the presence of a concurrent traumatic injury, may be problematic and requires examination by an experienced veterinary pathologist. Cost efficient, effective diagnostic tools, analogous to the measurement of blood cholinesterase (Hill and Fleming 1982; Elliott et al. 2008) or blood lead (Franson and Pain 2011), for example, would be valuable for wildlife rehabilitators, biologists and veterinarians to diagnose coagulopathies in suspected cases.

Compared to chemical analysis of rodenticide residues in tissues or other media, coagulation dysfunction tests are a rapid and inexpensive tool that may provide evidence of individual exposure and potentially indicate rodenticide impacts to a population. Although standardized coagulation test methods have been developed in humans and domestic mammalian species to provide a rapid indication of coagulation dysfunction (e.g. Duxbury and Poller 2001; Tseng et al. 2001) and may be modified for birds, stabilized reagents and standardized test methods specific to avian species are not available. Currently, avian coagulation tests are not commercially available at most veterinary laboratories in Canada. The prothrombin time (PT) test is a laboratory assay that has been reliably modified for domestic mammals and has been used to detect a hemostatic response to anticoagulant exposure in research (e.g. Rattner et al. 2011; Bachman and Sullivan 1983). The activated clotting time (ACT) test uses whole blood samples to detect a change in hemostasis (Chang et al. 1998) and has the benefit that it can be modified for use outside of a laboratory.

We had the following objectives: firstly to develop optimized PT and ACT test protocols (including storage conditions) to monitor avian species in British Columbia; secondly to measure changes in PT and ACT upon exposure to varying doses of brodifacoum over time in a model avian species, Japanese quail (*Coturnix japonica*), quantify hepatic residues relative to brodifacoum doses, and correlate residues to PT, ACT, and necropsy findings; and finally to measure PT in plasma from wild barn owls collected during the breeding season in agricultural areas of the Fraser River Delta, British Columbia.

Materials and methods

Study species and animal housing

Japanese quail were obtained from the Agassiz Poultry Research Centre (Agriculture and Agri-Food Canada, Agassiz, BC, Canada) at 5 weeks of age and were acclimated for 17 weeks at the University of British Columbia (UBC) Animal Care Centre (Vancouver, BC). Upon arrival, each bird was banded with plastic split-ring leg bands (AC Hughes, UK) and randomly assigned to one of 5 large plastic bins (0.91 m × 1.6 m × 61 m—W × L × H) containing wood shavings to allow for dust bathing and pecking.

Birds in each bin were physically and visually, but not acoustically, isolated from other groups. Room temperature was 21 ± 1 °C and day length was limited to 8L/16D h. Quail were provided with water and food ad libitum, containing 26 % protein and 1 mg of vitamin K per kg feed and prepared according to the NRC nutrient requirement standards recommended for quail (http://www.nap.edu/catalog/2114.html).

Animal husbandry and experimentation were conducted under UBC and Simon Fraser University (Burnaby, BC) animal care permits following guidelines of the Canadian Council on Animal Care.

Experimental design

A total of 120 adult quail were divided into five treatment groups (n = 24: 12 male, 12 female). Each bird received a single oral dose of corn oil vehicle (0.5 ml corn oil/100 g body weight) or this vehicle containing brodifacoum (98.9 % purity, Syngenta Inc.). Doses were analytically verified to contain 0, 0.8, 1.4, 1.9 and 2.5 mg brodifacoum/kg body weight. Measured concentrations of brodifacoum were determined in dosing solutions at the Pacific Environment Science Centre (Environment Canada, North Vancouver, BC). Solutions were dissolved in hexane, liquid/liquid extracted into acetonitrile, and analyzed by LC–MS (1100 Series, Agilent Technologies) in negative electrospray ionization, ion monitoring mode.

Body weight (±0.1 g) was recorded immediately prior to dosing (day 0) and prior to blood sampling and euthanasia. During the exposure period, birds were monitored for signs of distress or external hemorrhage, and were euthanized if symptoms were severe. After 1, 3, 5 and 7 days of exposure, 6 birds from each dosing group were bled by sterile, atraumatic venipuncture, immediately euthanized with isoflurane gas, and frozen until necropsy (Carpenter 2000). Necropsy was performed to record evidence of gross pathology including internal or external hemorrhage and pallor of internal organs. Although every

effort was made to accurately identify these, it should be noted that freezing of carcasses may cause artifacts that could be incorrectly interpreted as hemorrhage (Stroud 2012). Microscopic evaluation of tissues was not conducted due to the inaccuracies of identifying petechial hemorrhages after freezing. Livers were removed, weighed in order to calculate liver somatic indices (LSI = liver weight/body weight), and immediately frozen for brodifacoum residue analysis.

Blood collection and processing

The right jugular vein was used to collect inactivated whole blood for PT and ACT assays using a 23–27 gauge needle. Whole blood (0.5 ml) was added to the ACT tube (containing 3 mg of diatomaceous earth) and to 3.8 % (w/v) sodium citrate (1:9 ratio) for PT testing. Plasma was separated at 13,400 g for 3 min, transferred to a cryogenic vial, immediately frozen in liquid nitrogen and transferred to a −80 °C freezer until assayed. Heparinized microcapillary tubes of blood were collected to measure hemoglobin and hematocrit.

PT assay

Thromboplastin extract was prepared by incubating 50 mg of acetone-dried chicken brain powder (Lot #16529; Innovative Research, Michigan, USA) in 2.5 ml of 0.025 M $CaCl_2$ at 42 °C for 15 min, vortexing every 3 min (Doerr et al. 1975). The suspension was then centrifuged at 856*g* for 10 min, the supernatant was stored on ice, and an equal volume of 0.025 M calcium chloride was added. The thromboplastin extract was stored on ice when not in use, and all PTs were measured within 4 h of thromboplastin extraction as variability of PTs tended to increase substantially after this time.

PT was measured using the STart 4® Hemostasis Analyzer (Diagnostica Stago Inc.) at Idexx Reference Laboratories, Ltd. (Delta, BC, Canada) which detects increased viscosity when a clot is formed and reports this duration as the PT. Frozen plasma samples were thawed in a 37 °C waterbath for 3 min. Plasma and thromboplastin were then incubated individually at 37 °C for an additional 3 min in the STart 4® analyzer prior to adding 100 µl of thromboplastin extract to activate 50 µl of plasma. PT was run in duplicate with a coefficient of variation (CV) of <20 %. To aid in data interpretation, PT was measured in a diluted plasma pool to produce a standard curve for coagulation protein concentration. The plasma pool was diluted with phosphate buffer (pH 7.4, 8.3 mM) and PTs were measured at 10, 20, 40, 60, 80, and 100 % plasma (Bailey et al. 2005).

In order to control for variation associated with different batches of chicken thromboplastin, PT was measured in control plasma standards for normal and abnormal mammalian plasma (Pacific Hemostasis® Coagulation Controls, Level 1 and 2). Resulting mean PTs were 107.1 and 182.3 s for normal and abnormal controls, respectively. While use of mammalian plasma with avian thromboplastin does not produce accurate results, it did provide a means of quality control for reagent degradation.

To determine the effect of sample storage on PT, blood was collected from untreated Japanese quail (n = 14) to create a plasma pool, which was divided into 0.6 ml aliquots. The PT was measured in one fresh aliquot and all remaining aliquots were immediately frozen in liquid nitrogen at −80 °C. PT was measured in this frozen plasma pool after 3, 5, 7, 9 and 28 days, then every 4 weeks for up to 7 months.

ACT assay

A single blood sample for each bird was analyzed in an ACT tube containing 3 mg of diatomaceous earth and warmed to 37 °C prior to the addition of 0.5 ml blood (Bateman and Mathews 1999). The tube was inverted several times to mix, incubated for 1 min at 37 °C and visually examined for microclot formation.

Hemoglobin and hematocrit

Hemoglobin (Hb; g/dl whole blood) was measured using the cyanmethemoglobin method of Drabkin and Austin (1935) modified for use with a microplate spectrophotometer (BioTek Powerwave 340, BioTek Instruments, Ltd.) using 5 µl of whole blood and 1.25 ml of Drabkin's reagent (D5941, Sigma Aldrich Canada Inc.) with absorbance read at 540 nm. Standard curves were used to calculate Hb concentration using a cyanmethemoglobin standard (StanBio Laboratory, Texas, USA). Hematocrit (Hct; % packed red blood cells) was measured in heparinized microcapillary tubes following centrifugation of whole blood for 3 min at 13,400*g* (Campbell and Ellis 2007). Both hematocrit and hemoglobin were analyzed in triplicate with CVs <25 and 21 %, respectively.

Liver residue analysis

Whole quail livers were sent to the National Wildlife Research Center in Ottawa, Ontario, Canada for brodifacoum residue analysis. Quail liver residues were analyzed individually for birds in the 0.8 and 1.4 mg/kg dose groups. For all controls, and each time period of the 1.9 and 2.5 mg/kg dose groups, liver residue analysis was conducted on pooled samples. Individual liver residue analysis was also conducted for birds that succumbed to rodenticide toxicity or were euthanized prior to the assigned time group.

Chemical analysis was conducted following methods described in Albert et al. (2010). In brief, 0.50 g of liver sample was ground in a mortar with 5 g of anhydrous sodium sulfate (Fisher no. S420-3). The liver powder was extracted with acetonitrile (EMD Omnisolv, AX0142-1, HPLC Grade). The extract was then shaken vigorously for 15 min and then centrifuged. The supernatant was evaporated under nitrogen gas in a water bath to 10 ml. Then a 1 ml aliquot was transferred into a test tube and evaporated to dryness. This sample was reconstituted in 1 ml of methanol, and filtered through a Millex® HV 4 mm syringe filter with a 0.45 µm PVDF membrane. Samples were analyzed by liquid chromatography mass spectrometry (LC-MSMS; Agilent 1200 HPLC; AB Sciex API 5000 Triple Quadropole Mass Spectrometer with the TurboSpray ion source in negative polarity using multiple reaction monitoring). The method detection limit for brodifacoum was 0.002 µg/g (sample diluted 10×). Recoveries of brodifacoum in liver tissues were 90 ± 5 %. The addition of a known amount of coumatetralyl (5 pg/µL—transition 291.00 > 140.90) and flocoumafen (1 pg/µL—transition 541.40 > 382.00) to each sample prior to the injection allowed monitoring for ion suppression. A blank containing 100 % methanol was injected between each sample to monitor for any possible contamination due to carry over. For precision, quality control was assessed by extracting and analyzing 8 of the 31 samples in duplicate resulting in coefficients of variation <15 %. For quality assurance, accuracy was verified using a second source standard of brodifacoum.

Barn owl PT

Blood samples were collected from barn owls (53 rural chicks, 4 urban chicks, 4 urban adults and 1 rescue adult) in the Lower Fraser Valley during the breeding season between April 2008 and October 2010. For baseline PT evaluation, blood was collected from two barn owls which lived in captivity for at least 1 year and were assumed to have limited rodenticide exposure. Blood was collected by venipuncture from the brachial vein as described above for Japanese quail. Blood was centrifuged within 4 h of sampling and plasma was frozen in liquid nitrogen until storage at −80 °C. PTs were measured as described for Japanese quail and adjusted using a plasma storage adjustment factor as determined by the freezing study.

Statistical analysis

Statistical analyses were carried out using JMP software version 7.0.2 (SAS Institute 2007). To confirm that data met the assumptions for a parametric ANOVA, it was first assessed for equal variance and normality using the Shapiro–Wilk test. Data from the hemostatic assays and liver somatic index were found to be nonparametric. PT and ACT data were analyzed using multiple linear regression. The Hb, Hct, and weight data, which met the assumptions for parametric analysis, was analyzed by two-way ANOVA. A significant dose × time interaction for both Hb and Hct resulted in further one-way ANOVAs being conducted for each dose group and time period were conducted along with the conservative Tukey–Kramer's HSD post-test ($\langle = 0.05$) to determine differences between groups.

PT assay validations for freezing and dilution studies were analyzed using bivariate linear and nonlinear regression models, respectively. Potential correlations were investigated between individual brodifacoum liver residues and LSI, Hb, Hct, ACT, and PT using linear and non-linear regression models. A one-phase exponential decay model was used to assess the accumulation of brodifacoum in the liver of 0.8 and 1.4 mg/kg quail dose groups assuming maximum dose absorption into liver at time 0 (average of 40 and 76 µg/g w/w, respectively). Diagnostic sensitivity and specificity were calculated for both PT and ACT. Sensitivity is the number of true positive test results divided by the sum of true positive and false negative test results. Specificity was calculated as the number of true negative test results divided by the sum of true negative and false positive test results. Reference intervals were calculated according to American Society of Veterinary Clinical Pathology Guidelines (Friedrichs et al. 2012) using the Reference Value Advisor (Geffré et al. 2011).

Results

Japanese quail body condition and gross pathology

There was no significant change in body weight (mean = 128.7 g) or liver somatic index (median = 1.9 %) of controls, among dose groups, or over time. Antemortem signs included lethargy, ataxia, and minor bleeding at sites of new feather growth (rump). During the seven day study, 2 birds (8.3 %) at 0.8 mg/kg, 4 (16.7 %) at 1.4 mg/kg, 3 (15 %) at 1.9 mg/g and 4 (16.7 %) at 2.5 mg/kg quail either died or were euthanized due to severe hemorrhage. Gross pathologies observed during necropsy included: subdermal hemorrhage of the cranium and limbs, red-tinged vitellogenic follicles, greyish testis, hemorrhage in the coelem and orbital cavities, and dark brown contents in the gastrointestinal system (crop, gizzard and intestines), bleeding at the site of new feather growth and pallor of limbs, organs, and cranium. Coagulopathy was observed in all brodifacoum dose groups, but not in

Table 1 Symptoms of anticoagulant rodenticide poisoning observed during necropsy for each group. No symptoms of coagulaopathy were observed in the control group at any time point (n = 24)

Days exposed	Brodifacoum dose							
	0.8 mg/kg		1.4 mg/kg		1.9 mg/kg		2.5 mg/kg	
1	Hematoma on breast muscle	(1 of 6)	Hematoma on limb(s)	(2 of 6)	Clot at base of gizzard	(1 of 10)	Hemorrhage near testis	(1 of 3)
	No obvious AC symptoms	(5 of 6)	Cranial hemorrhage	(1 of 6)	Hematoma on limb(s)	(1 of 10)	No obvious AC symptoms	(5 of 6)
			No obvious AC symptoms	(4 of 6)	No obvious AC symptoms	(8 of 10)		
3	No obvious AC symptoms	(6 of 6)	Hematoma/hemorrhage on breast muscle	(2 of 6)	Hemorrhage on wing	(1 of 6)	Blood in digestive system	(5 of 6)
			Hematoma on limb(s) or wing	(3 of 6)	Hematoma on breast muscle	(1 of 6)	Blood surrounding two eggs	(1 of 3)
			Blood in digestive system	(2 of 6)	Blood in abdominal cavity	(1 of 6)	Hematoma on breast muscle	(1 of 6)
			Cranial hemorrhage	(1 of 6)	Palor of skull	(1 of 6)	Skull paler than controls	(1 of 6)
			No obvious AC symptoms	(2 of 6)	Bleeding at sites of new feather growth (rump)	(1 of 6)	Hematoma at base of skull	(1 of 6)
					No obvious AC symptoms	(2 of 6)	Bruise on limb(s)	(1 of 6)
							Hemorrhage in abdominal cavity	(1 of 6)
							Bleeding at sites of new feather growth (rump)	(3 of 6)
5	Hematoma on limb(s)	(1 of 6)	Hematoma on limb(s)	(4 of 6)	Hematoma/hemorrhage on breast muscle	(3 of 5)	Hematoma on limb(s)	(4 of 8)
	Hemorrage on breast muscle	(1 of 6)	Hemorrhage near ear	(1 of 6)	Cranial hemorrhage	(1 of 5)	Hemorrage at shoulder joint	(3 of 8)
	Cranial hemorrage	(1 of 6)	Bruise on breast muscle	(1 of 6)	Hematoma/hemorrhage on limb(s)	(2 of 5)	Eggs paler than controls	(1 of 4)
	Orbital hemorrage	(1 of 6)	Blood in digestive system	(1 of 6)	Bleeding at sites of new feather growth on back	(1 of 5)	Hematoma on torso	(1 of 8)
	Blood in digestive system	(1 of 6)	Palor of lungs	(2 of 6)	Grey testis	(1 of 2)	Palor of skull	(3 of 8)
	Blood surrounging egg	(1 of 3)	Palor of skull	(4 of 6)	Palor of lungs	(1 of 5)	Hemorrhage on breast muscle	(2 of 8)
	No obvious AC symptoms	(1 of 6)	Palor of intestines and feet	(1 of 6)	Palor of skull	(2 of 5)	Cranial hemorrhage	(2 of 8)
			Hemorrhage near cloaca	(1 of 6)			Hemorrhage in abdominal cavity	(1 of 8)
							Hemorrhage/blood in digestive system	(4 of 8)
							No obvious AC symptoms	(1 of 8)
7	Hematoma on limb(s)	(2 of 4)	Hematoma on limb(s)	(1 of 2)				
	Possibly hemorrhage on lung	(1 of 4)	Hemorrhage associated with digestive system	(1 of 2)				
	Dark grey/black testis	(1 of 1)	Hemorrhage in shoulder joint	(1 of 2)				
	No obvious AC symptoms	(1 of 4)	Hemorrhage on breast muscle	(1 of 2)				
			Blood clot near heart	(1 of 2)				
			Palor of skull	(1 of 2)				
Deceased	Death (2 on Day 2)	(2 of 24)	Death (2 on Day 2, 1 on Day 4, 1 on Day 6)	(4 of 24)	Death (2 on Day 2, 1 on Day 4)	(3 of 24)	Death (4 on Day 4)	(4 of 24)
	External bleeding on back	(1 of 6)	Hematoma on leg	(3 of 4)	Hematoma on leg or shoulder	(2 of 3)	Blood around egg	(1 of 4)
	Blood in digestive system	(1 of 6)	Palor of lungs or kidney	(2 of 4)	Hemorrage on breast muscle	(1 of 3)	Hematoma on leg or shoulder	(2 of 4)
	Palor of skull	(2 of 6)	Blood in abdomen	(2 of 4)	Dark grey testis	(1 of 3)	Hemorrage on breast muscle	(1 of 4)
			Hemorrhage around neck	(1 of 4)	Blood in digestive system	(3 of 3)	Hemorrhage in abdomen	(1 of 4)
			Blood surrounding egg	(1 of 4)	Cranial hemorrage	(2 of 3)	Blood in digestive system	(4 of 4)
			Blood in digestive system	(4 of 4)	Palor of skull	(1 of 3)	Cranial hemorrage	(1 of 4)
			Palor of skull	(3 of 4)			Palor of skull	(3 of 4)
							Bleeding at sites of new feather growth (rump)	(1 of 4)
							Blood in orbital socket	(1 of 4)

AC *anticoagulant*

any control birds. Symptoms of AR poisoning observed during necropsy are summarized by group in Table 1, with more detailed data provided in Supplement 1 (S1).

PT assay performance

The standard curve for PT, produced to simulate a decrease in coagulation protein concentration in the blood, demonstrated the threshold response of reduced coagulation factors: for example, a 60 % dilution resulted in a 22 % increase in PT, and a 90 % dilution resulted in a 220 % increase in PT (Fig. 1).

PT measurements in the pooled quail plasma frozen at −80 °C resulted in a slight decrease of 0.0078 s/day ($p = 0.053$) and an intra-day coefficient of variation of <8 % (Fig. 2). The mean monthly PTs for this frozen plasma pool ranged from 10.6 to 13.4 s over 7 months. The inconsistency demonstrated by this freezing study is similar to the analytical variation found in PT assays and is not likely to affect the diagnostic outcome of the PT results for the Japanese quail or Barn owl study.

Japanese quail PT

Brodifacoum exposure resulted in a clear dose–response with PT increasing at a rate significantly greater than zero ($p < 0.001$) (see Table 2 for median PTs for each dose group). PT measured in control birds did not statistically change over the study period (median = 12.7 s; reference interval = 10.0–16.2 s for all controls combined). As shown in Fig. 3, inter-individual variation also increased with exposure to brodifacoum. Due to mortalities at the two highest doses, no PT data could be obtained for day 7. For the 0.8 and 1.4 mg/kg dose groups, 4 (60.0, 50.9, 28.1, and 26.5 s) and 2 (58.6 and 22.2 s) PT values were obtained 7 days after exposure, respectively. By day 7 coagulopathy appeared to be resolved based on PT times decreasing towards to baseline values (Fig. 3). The diagnostic specificity and sensitivity of the PT assay were calculated as 100 and 99 %, respectively. A positive correlation was shown between mean PT and the proportion of quail with gross pathology in each dose group ($p = 0.037$; Fig. 4).

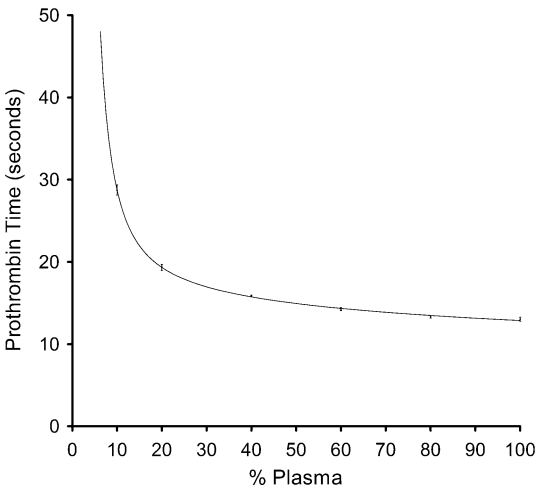

Fig. 1 Prothrombin time standard curve. PT measured in diluted plasma pool to estimate the % normal clotting activity of a given sample based on prothrombin time ($r^2 = 0.9996$). *Error bars* represent the standard error of the mean (SEM) PT for each dilution

Japanese quail ACT

The ACT test assesses the overall functional ability of blood to clot using diatomaceous earth as a charged surface to trigger the intrinsic coagulation cascade. Brodifacoum exposure resulted in a dose–response with ACT increasing at a rate significantly greater than zero ($p < 0.02$) (see Table 2 for median ACTs for each dose group). ACT measured in control birds did not statistically change over the study period (median = 103.8 s; reference interval = 30–180 s; n = 20 for all controls combined). Inter-individual variation in ACT appeared to increase with exposure to brodifacoum (Fig. 5). Due to mortalities at the two highest doses, no ACT data could be obtained for day 7. For the 0.8 and 1.4 mg/kg dose groups, 4 (9.3, 5.6, 5.6, and 3.4 min) and 1 (7.9 min) ACT values were obtained

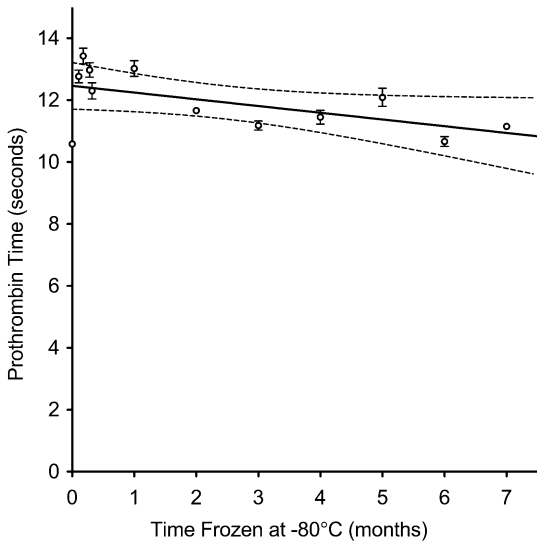

Fig. 2 Prothrombin time response to Japanese quail (*Coturnix japonica*) plasma pool after freezing ay −80 °C for up to 7 months (slope = −0.0078 s/day; $R^2 = 0.33$; $p = 0.053$). One pool was used for each time point with 8–12 replicates per pool (12 replicates for 0, 3, 5, and 7 days; 11 for 56 and 168 days; 10 for 28, 84, 112, and 140 days; and 8 for 9 and 196 days). Error bars represent the standard error of the mean (SEM) PT at each time point

Table 2 Median (5–95th percentile) results for PT and ACT data by treatment group

Treatment group	PT (s)	ACT (s)
0 mg/kg		
Day 1	12.4 (11.3–13.1, n = 5)	106.2 (66.6–115.8, n = 6)
Day 3	14.93 (12–15.4, n = 6)	130.8 (78.6–166.2, n = 6)
Day 5	12.2 (10.8–14.25, n = 4)	82.5 (51.6–154.2, n = 4)
Day 7	13.05 (12.05–14.6, n = 6)	101.4 (49.8–147, n = 5)
All controls	12.7 (10.85–15.39, n = 21)	103.8 (49.89–165.6, n = 21)
0.8 mg/kg		
Day 1	39.4 (32.05–29.7, n = 6)	193.8 (141–249, n = 3)
Day 3	158.6 (79.18–59.4, n = 6)	395.1 (229.8–595.2, n = 6)
Day 5	121.2 (55.28–41.6, n = 6)	661.2 (444–745.8, n = 5)
Day 7	60.05 (39.48–26.45, n = 4)	336 (204–555, n = 4)
1.4 mg/kg		
Day 1	24.5 (21.9–56.5, n = 6)	307.2 (96–1812.6, n = 6)
Day 3	147.6 (99.75–322.7, n = 6)	165 (124.8–1234.8, n = 5)
Day 5	136.4 (90.6–859.8, n = 6)	447 (166.8–1215, n = 5)
Day 7	40.4 (22.2–58.6, n = 6)	471 (n = 1)
1.9 mg/kg		
Day 1	25.7 (21.5–55.3, n = 6)	451.2 (319.2–5209.8, n = 5)
Day 3	174.5 (117.6–344.1, n = 4)	239.4 (229.8–345, n = 3)
Day 5	247.5 (155.6–999, n = 5)	597 (141–864.6, n = 3)
2.5 mg/kg		
Day 1	29.7 (16.7–38.1, n = 5)	112.98 (49.998–193.8, n = 5)
Day 3	157.7 (112.4–202.7, n = 5)	468 (331.8–526.98, n = 5)
Day 5	192.8 (152.3–999, n = 5)	543 (347.52–1902, n = 4)

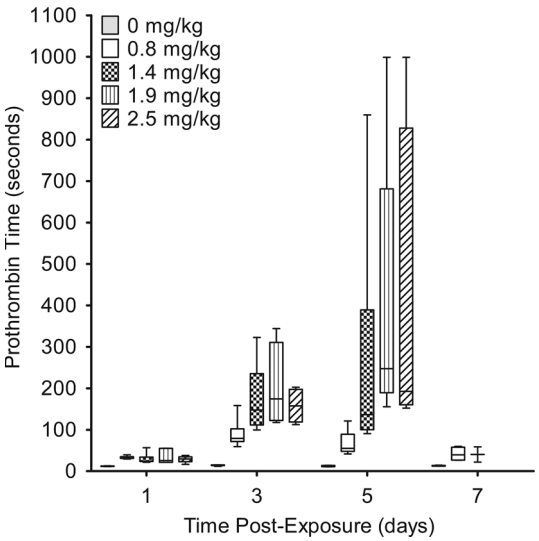

Fig. 3 Prothrombin time measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Median presented with *error bars* showing the 5th to 95th percentile. See Table 2 for sample sizes for all 20 dose groups

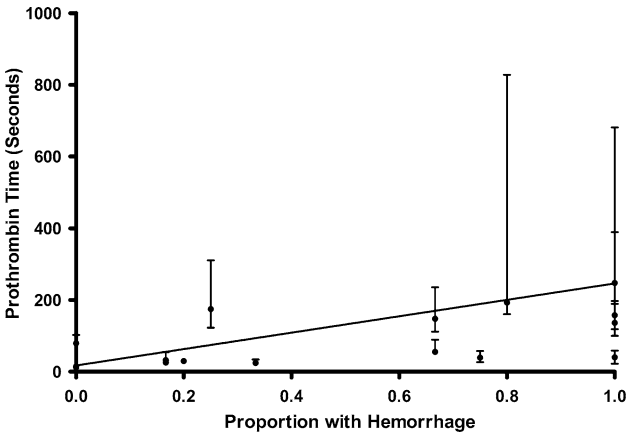

Fig. 4 Median prothrombin time (median ± interquartile range) for each treatment group (dose × time) and the corresponding proportion of these quail with hemorrhage observed during necropsy ($R^2 = 0.2341$, $p < 0.0001$)

7 days after exposure, respectively. The diagnostic specificity and sensitivity of the ACT test were calculated as 100 and 75 %, respectively.

Japanese Quail Hb and Hct

The concentration of Hb and the Hct varied significantly with dose and time ($p < 0.001$; Fig. 6a, b, respectively; Table 3). The mean Hb and Hct of all controls combined were 14.0 ± 0.4 g/dl and 44.6 ± 1.1 %, respectively, with

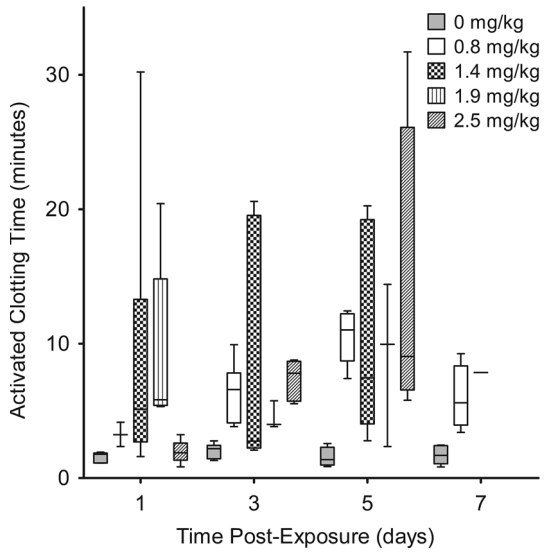

Fig. 5 Activated clotting time (min) measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Median presented with *error bars* showing the 5th to 95th percentile. See Table 2 for sample sizes for all 20 dose groups

reference intervals of 9.6–18.4 g/dl for Hb and 34–55 % for Hct. There were no significant differences in Hct or Hb over time within the control or 0.8 mg/kg dose groups after any exposure period, or 1 day after dose administration between dose groups. In contrast, there were significant decreases in Hct and Hb over time within the 1.9 and 2.5 mg/kg dose groups on days 3 and 5, and within the 1.4 mg/kg dose group on days 5 and 7 when compared to same-dose group results on day 1. At 1.9 mg/kg, both Hct and Hb were significantly reduced 5 days post exposure, and at 1.4 mg/kg, Hct and Hb remained significantly reduced 7 days post-exposure.

Japanese quail liver residues

A maximum liver concentration of 0.84 µg/g wet weight was observed in the 1.4 mg/kg dose group, 3 days post-exposure, and the 97.5 % upper confidence limit of the mean (UCLM) for all dose groups was 0.51 µg/g wet weight. Liver residues are presented in Table 3 by treatment group. As shown in Fig. 7, liver residue data for individual birds in the 0.8 and 1.4 mg/kg dose groups corresponds to a one-phase exponential decay model ($R^2 = 0.141$ and $R^2 = 0.558$, respectively). However, sampling intervals were not prolonged enough to calculate a tissue half-life. Brodifacoum residues in the liver were not correlated with liver somatic index, Hb concentration, Hct, ACT, PT, or sex. No significant difference in brodifacoum liver residue was determined between quail

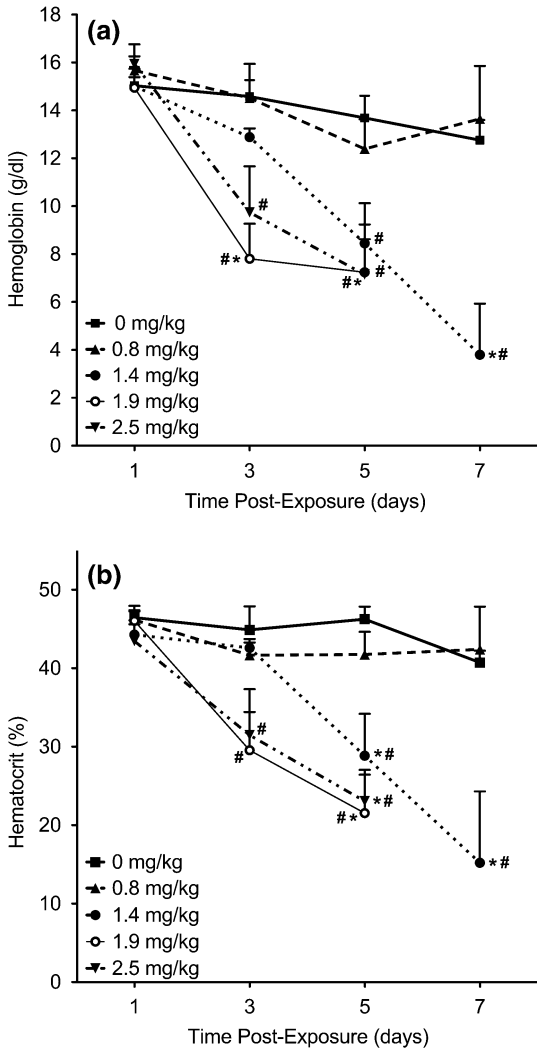

Fig. 6 **a** Hemoglobin (g/dl) measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Significant differences ($p < 0.05$) occurred between dose groups in comparison to same-day control 1, 3, 5, and 7 days post-exposure (*), and within each dose group after 3, 5, and 7 days post-exposure when compared with 1 day post-exposure (#). b) Hematocrit measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Significant differences ($p < 0.05$) occurred between dose groups in comparison to same-day control 1, 3, 5, and 7 days post-exposure (*), and within each dose group after 3, 5, and 7 days post-exposure when compared with 1 day post-exposure (#). See Table 3 for sample sizes for all 20 dose groups

with or without gross pathological signs of toxicosis; nor was a correlation determined between the mean hepatic residue and the proportion of quail with these signs in each treatment group ($R^2 = 0.0006$, $p = 0.925$; Fig. 8).

Barn owl PT

All Barn Owl PTs were adjusted using the freezing storage factor based on data for quail. After adjustment for storage

Table 3 Mean (SEM) results for hemoglobin, hematocrit and liver residues for each treatment group

Treatment group	Hb (g/dl)	Hct	Liver residue (μg/g)
0 mg/kg			
Day 1	15.0 (1.17, n = 6)	0.465 (0.032, n = 6)	–
Day 3	14.6 (1.17, n = 6)	0.449 (0.032, n = 6)	–
Day 5	13.7 (1.17, n = 6)	0.462 (0.032, n = 6)	–
Day 7	12.8 (1.17, n = 6)	0.407 (0.032, n = 6)	–
All controls	14.0 (0.42, n = 6)	0.446 (0.011, n = 6)	0 (pooled, n = 24)
0.8 mg/kg			
Day 1	15.7 (1.17, n = 6)	0.462 (0.032, n = 6)	0.35 (0.089, n = 6) range 0.24–0.5
Day 3	14.5 (1.17, n = 6)	0.417 (0.032, n = 6)	0.31 (0.093, n = 6) range 0.21–0.43
Day 5	12.4 (1.17, n = 6)	0.418 (0.032, n = 6)	0.27 (0.073, n = 6) range 0.19–0.38
Day 7	13.7 (1.43, n = 4)	0.424 (0.040, n = 4)	0.24 (0.074, n = 4) range 0.15–0.33
1.4 mg/kg			
Day 1	15.0 (1.17, n = 6)	0.443 (0.032, n = 6)	0.50 (0.17, n = 6) range 0.4–0.84
Day 3	12.9 (1.17, n = 6)	0.426 (0.032, n = 6)	0.27 (0.080, n = 5) range 0.17–0.37
Day 5	8.5 (1.28, n = 5)	0.289 (0.035, n = 5)	0.25 (0.065, n = 6) range 0.16–0.34
Day 7	3.8 (2.02, n = 2)	0.152 (0.056, n = 2)	0.23 (0.064, n = 2) range 0.18, 0.27
1.9 mg/kg			
Day 1	15.0 (1.17, n = 6)	0.458 (0.035, n = 5)	0.32 (pooled, n = 6)
Day 3	7.8 (1.28, n = 5)	0.296 (0.032, n = 6)	0.51 (pooled, n = 6)
Day 5	7.2 (1.43, n = 4)	0.216 (0.035, n = 5)	0.30 (pooled, n = 5)
2.5 mg/kg			
Day 1	15.9 (1.17, n = 6)	0.441 (0.032, n = 6)	0.48 (pooled, n = 6)
Day 3	9.7 (1.17, n = 6)	0.315 (0.032, n = 6)	0.29 (pooled, n = 6)
Day 5	7.2 (1.08, n = 7)	0.231 (0.030, n = 7)	0.48 (pooled, n = 8)

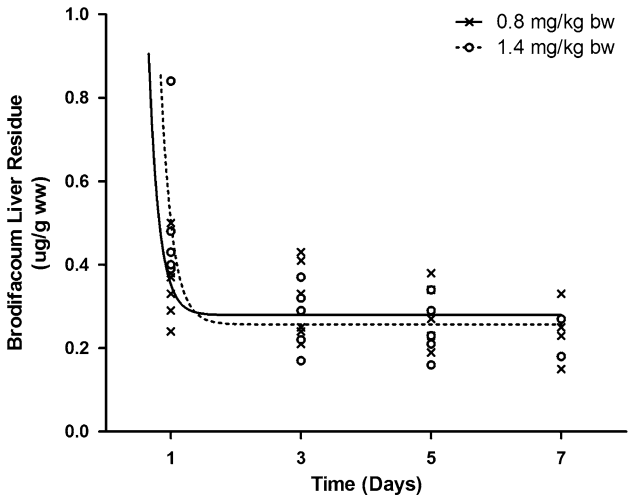

Fig. 7 Hepatic brodifacoum residue for individual Japanese quail (*Coturnix japonica*) in the 0.8 and 1.4 mg/kg dose groups over 7 days of exposure. A one-phase exponential decay model was used after the first 24 h and maximum dose absorbed in the liver of 40 and 76 μg/g in the 0.8 and 1.4 mg/kg dose groups respectively ($Y = 39.72e^{6.31x} + 0.28$ and $Y = 75.74e^{5.73x} + 0.26$, respectively)

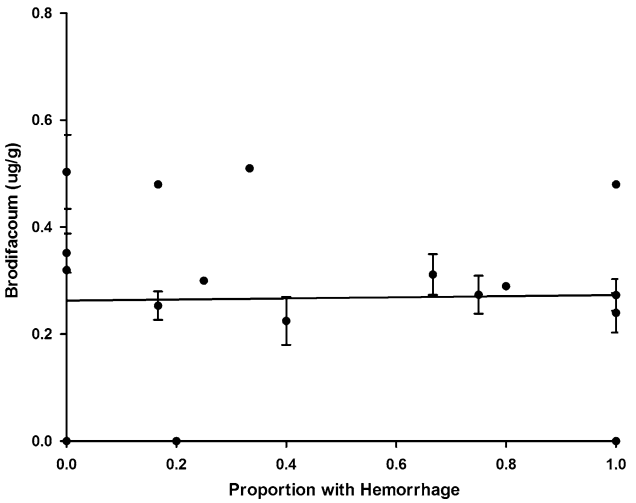

Fig. 8 Mean brodifacoum liver residue (Mean ± SEM) for all treatment groups (dose × time) and the proportion of quail in each treatment group with hemorrhage observed during necropsy ($R^2 = 0.0006$, $p = 0.925$)

time, the median PT of barn owl chicks (aged 40–55 days) sampled directly from nest sites, and adults was 22.1 s (n = 61, reference interval = 17–29 s; Fig. 9). The mean PT of two captive barn owls was 22.6 s, and the PT of one Barn Owl admitted to a local wildlife rehabilitation centre was 103.6 s (Fig. 9).

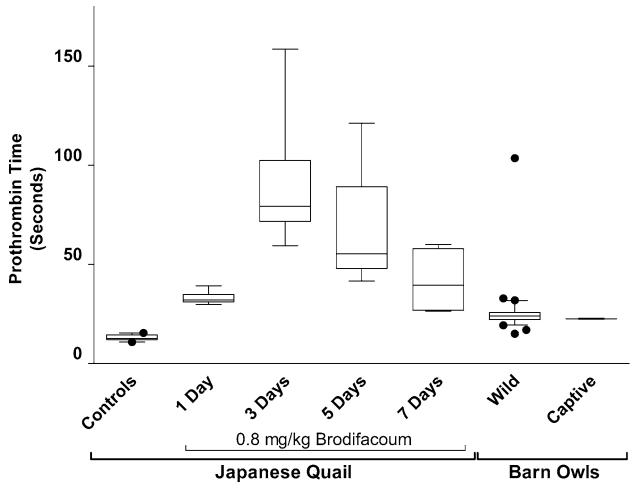

Fig. 9 Prothrombin times measured in wild (n = 61, rescue adult n = 1) and captive (n = 2) barn owls (*Tyto alba*) and in Japanese quail (*Coturnix japonica*) dosed with corn oil (n = 21), or brodifacoum at 0.8 mg/kg, 1, 3, 5 and 7 days post-exposure (n = 6). Median presented with *error bars* showing the 5th to 95th percentile

Discussion

Acute toxicity to Japanese quail

Our study clearly shows that brodifacoum is a potent disruptor of coagulation in Japanese quail at 0.8 mg/kg or greater. Acute toxicity was observed in Japanese quail exposed to 0.8 mg/kg brodifacoum within 3 days of exposure, and severe coagulopathy was observed 5 days post-exposure. This dose is environmentally relevant for both primary consumption of brodifacoum in bait and secondary poisoning by consumption of brodifacoum-contaminated prey animals (US EPA 2011). Estimated daily intakes of brodifacoum by Passeriforms from bait was 7–13 mg/kg, and by secondary consumer birds was 0.82–19.14 mg/kg (US EPA 2011). In this study at brodifacoum doses of 1.9 and 2.5 mg/kg, coagulopathy resulted in severe hemorrhage only 1 day after exposure. Overt signs of toxicity included ataxia and immobility similar to that described in other AR studies (e.g. Rattner et al. 2011, 2012a, b), and may contribute to increased predation and incidences of secondary poisoning in the wild (Cox and Smith 1990; US EPA 2004).

Necropsy revealed hemorrhages in 10 of 24 quail at the 0.8 mg/kg bw dose group, 18 of 24 birds at the 1.4 mg/kg dose, 13 of 24 birds at the 1.9 mg/kg dose, and 18 of 24 birds treated with a 2.5 mg/kg dose. However, this study found no obvious signs of coagulopathy (e.g. hematoma, macroscopic hemorrhage) in 45 % of quail with prolonged PT. Non-remarkable signs of AR exposure were also reported in 43 % of American Kestrels exposed to diphacinone, where histology showed signs of SGAR exposure in 83 % of birds (Rattner et al. 2011). Hemorrhage frequency in treated Japanese quail may have been partially caused by social interactions, e.g. mating behaviors, which naturally occur in group housing conditions. External hemorrhages appeared to be associated with routine minor injuries and growth (i.e. as a result of pecking and new feather growth) and likely represent the superficial physical points of weakness in each individual. It is possible that wild raptors with similar SGAR exposure may experience increased blood loss due to natural exertion (e.g. hunting or predator avoidance) combined with apparent higher sensitivity to anticoagulants in comparison to Japanese Quail (Rattner et al. 2011).

Prothrombin time

ARs inhibit coagulation by binding strongly to vitamin K epoxide reductase (VKOR) in the endoplasmic reticulum of hepatocytes, and blocking the conversion of vitamin K from its epoxide to its hydroquinone form (Hadler and Shadbolt 1975). Vitamin K hydroquinone is required as a cofactor for the γ-carboxylation of glutamic acid residues on several coagulation propeptides for conversion to active factor (Berkner 2000), specifically II (prothrombin), VII, IX and X and proteins C, S, and Z (Mann et al. 1990). Hemostasis is disrupted when functional coagulation factor concentrations decrease below a critical threshold, resulting in hemorrhage (Griminger 1986). The PT assay activates prothrombin to assess the ability of this protein to activate the coagulation cascade.

Brodifacoum has been shown to disrupt coagulation in Japanese quail and numerous other species (Bachmann and Sullivan 1983; Breckenridge et al. 1985; Mosterd and Thijssen 1991; Howald 1997; Bailey et al. 2005). Prolonged PT has been reported in response to the anticoagulant diphacinone in the American Kestrel (Rattner et al. 2011), Crow (*Corvus corax*; Massey et al. 1997) and Golden Eagle (*Aquila chrysaetos*; Savarie et al. 1979), and warfarin in the chicken (*Gallus gallus domesticus*; Stopforth 1970). However, few studies have examined the sublethal effect of brodifacoum on PT in avian species (Bailey et al. 2005; Howald 1997). Clinically, anticoagulant exposure is suspected when PT is increased by at least 25 % above high normal values (Shlosberg and Booth 2006). In our study, a 25 % increase in quail PT would be equivalent to approximately 19.3 s which was exceeded in 98.6 % of exposed quail for which PT was measured (71 of 72 quail).

Peak PT disruption, observed greater than 48 h after exposure, is likely due to time required for depletion of functional coagulation factors in the blood. At 0.8 mg/kg, PT was maximally disrupted 3 days after exposure with clotting ability reduced to about 4.5 % of initial values (89.3 s), while seven days post-exposure, the average PT

had increased to 41.1 s (8.2 % of control values), suggesting some hepatic recovery. At a comparable dose, Bailey et al. (2005) found that in chickens dosed with brodifacoum at 0.75 mg/kg, PT was maximally prolonged 7 days after exposure resulting in only 6.1 % of normal clotting activity, with PT recovery 28 days after exposure. The prolonged effect of brodifacoum was not visible in a similar study with Eastern Screech Owls exposed to diphacinone, where PTs were almost completely recovered by day 4 (Rattner et al. 2014). Brodifacoum metabolism is expected to be similar to that of warfarin, including glucuronidation and elimination into bile (Watt et al. 2005), a process which tends to increase its elimination rate from the body. However, the metabolic process for brodifacoum is expected to be substantially slower than for warfarin due to its substantially longer biological half-life (Watt et al. 2005). Enterohepatic circulation of brodifacoum was also suggested by Bachman and Sullivan (1983) when intestinal levels of brodifacoum increased between 24 and 72 h in rats administered a 0.2 mg/kg dose.

Coagulopathy resulting from SGAR exposure is due to an individual-specific coagulation factor threshold, thereby resulting in high inter-individual variation in toxic dose within a species (Stone et al. 2003; Walker et al. 2008). The PT data in the present study is consistent with the typical anticoagulant threshold behavior associated with a steep dose–response curve and narrow therapeutic index (Wadelius and Pirmohamed 2007). Brodifacoum doses $\geq$1.4 mg/kg resulted in three birds with extremely long PTs (>800 s), suggesting that the concentration of circulating clotting factors was under the threshold level and coagulation was unattainable. The PTs of the plasma dilution series similarly illustrates that effect, as PT increased to abnormal values when the concentration of plasma was reduced to approximately 30 %, with coagulation appearing unattainable at 10 % plasma. Coagulopathy resulting from brodifacoum exposure was effectively detected by the PT assay, and its positive correlation with the proportion of quail exhibiting hemorrhage at necropsy (Fig. 4).

Activated clotting time

The ACT test is used in veterinary medicine to assess coagulation dysfunction and the cellular component of coagulation in mammals when more targeted methods are unavailable, specifically deficiencies in factors (VIII) hemophilia factor, (IX) Christmas factor, (V) proaccelerin, (X) Stuart Prower factor, (II) prothrombin and (I) fibrinogen (Bateman and Mathews 1999; Middleton and Watson 1978; Harr 2011). Contact activation in mammals is widely accepted to involve factors (XII) Hageman factor, (XI) plasma thromboplastin, and IX (Harr 2011). A recent comparative genomic study has shown that chickens lack Factor XII and possess a "predecessor" combined gene for prekallikrein-Factor XI, rather than the two distinct genes possessed by mammals (Ponczek et al. 2008). Furthermore, this combined prekallikrein-factor XI protein was found to lack the binding site for factor IX that is typically present on factor XI. It was therefore suggested that this combined protein may have activity similar to prekallikrein. As clotting was initiated by contact activation using diatomaceous earth (silica) in the ACT assay with a measurable difference between control and anticoagulant treated quail, it is possible that only the serine protease prekallikrein participated in the initial stages of contact activation. This repudiates early literature which suggested that the intrinsic pathway was absent or of limited importance in avian species (Bigland and Triantaphyllopoulos 1961; Bigland 1964; Stopforth 1970). Our results support the presence of the contact activation system, although it likely plays a secondary role in maintaining hemostasis (Doerr et al. 1976; Harr 2011).

As the intrinsic pathway requires vitamin K-dependent coagulation factors, anticoagulant rodenticide exposure prolonged the ACT as expected (Fig. 5). Due to the complex nature of coagulation involving thrombocytes and numerous factors, some of which are not effected by Vitamin K deficiency, ACT results were more variable but still resulted in a dose- and time-dependent response. Although ACT cannot measure exposure level, the practicability (i.e. cost, time, convenience) of the ACT test would make it suitable for screening birds in the field or at wildlife rehabilitation centers to indicate SGAR exposure. However, species-specific reference intervals would be required for this use. In this study, the ACT test provided consistent results when birds were not exposed to brodifacoum, but provided occasional false-negative results in some exposed birds (75 % diagnostic sensitivity, 100 % diagnostic specificity). Consequently, the ACT test is a less reliable indicator of anticoagulant rodenticide exposure compared to the PT test (99 % sensitivity, 100 % specificity), but may be more indicative of whole body functional response.

Hct and Hb

Hct and Hb provide a reliable gross indication of overall hemostasis in the bird as significant blood loss can frequently be measured within 12–24 h. Both Hct and Hb remained within the reference interval for control quail (Coenen et al. 1994; Cheng et al. 2010) over the study period. At high brodifacoum doses, there were marked decreases in Hct and Hb, and increased evidence of hemorrhaging during post-mortem necropsy. However, at 0.8 mg/kg, Hct and Hb did not differ from same-day controls, even though PTs were significantly prolonged in this dose group, suggesting a recoverable, subclinical

pathologic effect. In contrast, 1.4 mg/kg of brodifacoum resulted in significant reductions in Hb and Hct even 7 days post-exposure, from 12.8 to 3.8 g/dl (Fig. 6a) and 40.7–15.2 % (Fig. 6b), respectively, when PTs decreased due to apparent hepatic recovery (Fig. 3). This discrepancy at day 7 reflects faster hepatic recovery as a minimum of 3–5 days are required for the avian bone marrow to begin producing increased numbers of new red blood cells (Weiss and Wardrop 2011).

The Hct and Hb levels measured in brodifacoum-treated Japanese quail are similar to those reported in response to incidences of SGAR poisoning. Hct in the Red-tailed hawk (*Buteo jamaicensis*) and White-winged wood duck (*Cairina scutulata*) was reported at 9 and 16 %, respectively, after exposure to brodifacoum (James et al. 1998; Murray and Tseng 2008). Hct values such as those reported above are indicative of anemia (hematocrit <34 % based on reference interval above) and correlated to acute vascular blood loss by necropsy findings.

Japanese quail liver residues

Hepatic concentrations were not measured within the first 24 h in this study, however the decrease in hepatic brodifacoum concentration was assumed to be rapid during this period, followed by a slow decrease demonstrating brodifacoum's persistence in the liver (Fig. 7). In rats, a liver elimination half-life of up to 350 days has been reported (Batten and Bratt 1990). The persistence of brodifacoum in the liver and the delayed action of this toxin prior to death both contribute to secondary poisoning of wildlife which consume intoxicated prey animals. This delayed persistant action may be due to a reduced reserve capacity of VKOR synthesis after full or partial recovery of clotting time from brodifacoum exposure (Mosterd and Thijssen 1991).

The hepatic concentration of SGARs is a reliable indicator of exposure *incidence*, but does not provide adequate information about potential exposure *impacts* or *risk* to an individual or population. Although the present study found a liver saturation level of approximately 0.51 µg/g wet weight based on the 97.5 % UCLM across all treatment groups, death was observed at a hepatic concentration of 0.31 µg/g w/w, and severely prolonged PT associated with hemorrhagic effects occurred at a hepatic concentration of 0.23 µg/g wet weight. A recent study on raptors in Massachusetts discovered that although hepatic SGAR residues ranged from 0.012 to 0.269 µg/g wet weight (where 98 % contained brodifacoum as the only SGAR), only 6 % ($n = 164$) of all birds could be definitively related to SGAR toxicosis (Murray 2011). A recent probabilistic risk assessment proposed threshold liver residue concentrations which would result in signs of toxicosis at 5–20 % effects levels for four raptor species, including a 5 % effects level at 0.05 mg/kg wet weight for barn owls (Thomas et al. 2011). Our study found no correlation between liver residues and oral brodifacoum dose, similar to Gray et al. (1994), and no correlation between liver residues and the proportion of quail with hemorrhage or pallor (Fig. 8). However, a correlation was uncovered between increasing PT and the proportion of each treatment group experiencing AR signs ($p < 0.0001$; Fig. 4). Taken together, our study (1) supports the recommendation that liver residues be used to confirm SGAR exposure but not be relied upon as an indicator of toxicosis (Murray 2011) and (2) shows that PT is a useful tool to aid in monitoring and assessing risk of SGARs to a specific population of concern.

Evaluation of prothrombin times in barn owls

Barn Owls (*Tyto alba*) were used to evaluate PT as a biomonitoring assay due to a concurrent study that enabled access to both urban and agricultural chicks and adults during the breeding season. As barn owls are designated as Threatened by COSEWIC (Committee on the Status of Endangered Wildlife in Canada; Environment Canada 2011), and hunt for rodents in environments where AR use is known or suspected (Hindmarch and Elliott 2014; Hindmarch et al. 2014a, b), evaluation of SGAR exposure to this species was warranted. Prolonged coagulation may exacerbate minor traumatic injuries in any wild species including raptors. In addition, previous studies have shown barn owls to be more susceptible to brodifacoum through secondary poisoning compared to bromadiolone, difenacoum, diphacinone and chlorophacinone (Mendenhall and Pank 1980). The intent of this study was to include barn owls at wildlife rehabilitation centers that were exhibiting signs of AR toxicity, such as with testing for anticholinesterase poisoning (e.g. Elliott et al. 2008) or lead (Elliott et al. 1992). However, during the length of the study, a sample was obtained from only one individual presented to the partner rehabilitation centre with these symptoms (see Fig. 9). This low number of samples from potentially impacted individuals is likely due to either death of the exposed, or AR poisoning having subtle sublethal symptoms that may be difficult to separate from those caused by other trauma. In addition, due to typical barn owl and other raptor carcass discovery and handling (e.g. freezing upon discovery, delayed discovery), SGAR poisoning may only be diagnosed at high dose levels and injuries potentially caused by exposure-induced behavioral changes may not be revealed.

The PT for different species varies partly due to actual species variability in coagulation enzymes, but also due to laboratory method such as the type of thromboplastin used (Tahira et al. 1977; Morrisey et al. 2003, Bailey et al. 2005,

Doerr et al. 1975). As the same thromboplastin and laboratory method was used for both species in this study, it appears that the median PT measured in free-living barn owls (22.1 s; 17–29 s reference interval; n = 61) was higher ($p < 0.0005$) than that of undosed Japanese quail (12.7 s), but much lower than PTs measured in brodifacoum-exposed quail ($p < 0.0005$), even at the lowest dose level. The mean of two captive barn owl PTs was 22.6 s and, although not a robust sample size, provides evidence that barn owls in the Fraser Valley are likely not significantly exposed to brodifacoum. However, one adult barn owl with prolonged PT (PT = 103.6 s; Fig. 9) was presented to a rehabilitation facility suggesting potential SGAR exposure.

Variability in PT within a population of a particular species may also provide an indication of SGAR exposure. As demonstrated in our quail study, variability in PT increases significantly with exposure to brodifacoum likely due to inter-individual or inter-species differences in hepatic cytochrome P450 metabolism or sensitivity of VKOR (Godfrey 1985; WHO 1995; Eason et al. 2002; Watanabe et al. 2010; Rattner et al. 2011; 2012a, b). As a result, PT variability may be useful as an additional line of evidence to assess exposure. The mean Barn Owl PT had a coefficient of variation (CV) only slightly higher (14.1 %) than that measured for the control quail (10.5 %), which would be expected when laboratory variability is compared to that of free-living birds. In contrast, the CVs of brodifacoum-treated quail, with the exception of the lowest dose group 1 day post-exposure, varied from 25 to 115 %.

SGARs have been re-evaluated in Canada and the United States (US) after a thorough risk assessment which concluded that brodifacoum and difethialone, followed by bromadiolone and diphacinone, posed the greatest hazard to non-target mammals and birds from primary and secondary poisoning (US EPA 2004). New rodenticide labeling requirements regarding usage, and limitations on product registration are now being implemented in both the US and Canada (US EPA 2008, 2011; PMRA 2010). However, some permitted uses of SGARs and use violations (e.g. agricultural; Elliott et al. 2014) will likely continue to pose a risk to non-target wildlife such as barn owls. As extensive SGAR use will no doubt continue in the foreseeable future, PT provides a potential biomarker for evaluating risk to specific avian populations. Standardized blood sampling methods with precise venipuncture, handling, and storage is required for PT analysis when wildlife is brought to rehabilitation centers and veterinary clinics. Although most commercial laboratories do not have standard protocols for avian PT, in part due to a lack of commercially stabilized avian thromboplastin, most do have the equipment required to run the PT test for avian species. Two effective avian coagulation tests are now commercially available to monitor for coagulation impairment: (1) Avian ACT supplies are now available from URIKA LLC; and (2) Avian Russell viper venom time (RVVT) in the United States (Marshfield Labs). RVVT has been shown to detect diphacinone exposure in American Kestrels (*Falco sparverius*; Rattner et al. 2011). However, a partnership between those receiving injured wildlife, a commercial laboratory and government researchers would provide the most effective means to accurately monitor potential AR effects on a population using coagulation tests and contribute to enforcement of proper rodenticide use.

Conclusion

We confirmed brodifacoum as a potent disruptor of coagulation resulting in prolonged PT and ACT, and in reduced Hct and Hb in quail. The PT threshold response to SGAR exposure is largely dose-dependent and may be a reliable indicator of exposure in Japanese quail, while the ACT test is more sensitive to inter-individual variation and conveys a whole-organism response. Hepatic concentrations of brodifacoum were not correlated to oral dose, PT, or to the proportion of quail experiencing hemorrhage and anemia, and therefore could not be used to assess toxicity, but rather to confirm the chemical cause of coagulopathy. However, oral dose and the proportion of birds showing AR signs were correlated with PT, suggesting this assay, or the commercially available options such as the RVVT or ACT tests, have potential as valuable tools for assessing AR toxicity. Free-living barn owls sampled during the breeding season in the Fraser River Delta of BC were not exposed to SGARs at levels which significantly affect PT. Future assessment of clotting in wild birds brought into rehabilitation centers is recommended.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All field protocols were conducted under necessary permits acquired from provincial, federal and institutional authorities.

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