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Increased rodenticide exposure rate and risk of toxicosis in barn owls (*Tyto alba*) from southwestern Canada and linkage with demographic but not genetic factors

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Abstract Among many anthropogenic drivers of population decline, continual rapid urbanization and industrialization pose major challenges for the survival of wildlife species. Barn owls (*Tyto alba*) in southwestern British Columbia (BC) face a multitude of threats ranging from habitat fragmentation to vehicle strikes. They are also at risk from secondary poisoning of second-generation anticoagulant rodenticides (SGARs), a suite of toxic compounds which at high doses results in a depletion of blood clotting factors leading to internal bleeding and death. Here, using long-term data (N = 119) for the hepatic residue levels of SGAR, we assessed the risk of toxicosis from SGAR for the BC barn owl population over the past two decades. We also investigated whether sensitivity to SGAR is associated with genetic factors, namely Single Nucleotide Polymorphisms (SNPs) found in the CYP2C45 gene of barn owls. We found that residue concentration for total SGAR was significantly higher in 2006–2013 (141 ng/g) relative to 1992–2003 (57 ng/g). The proportion of owls exposed to multiple SGAR types was also significantly higher in 2006–2013. Those measures accordingly translate directly into an increase in toxicosis risk level. We also detected demographic differences, where adult females showed on average lower concentration of total SGAR (64 ng/g) when compared to adult males (106 ng/g). Juveniles were overall more likely to show signs of toxicosis than adults (33.3 and 6.9 %, respectively), and those symptoms were positively predicted by SGAR concentrations. We found no evidence that SNPs in the CYP2C45 gene of barn owls were associated with intraspecific variation in SGAR sensitivity. We recommend several preventative measures be taken to minimize wildlife exposure to SGAR.

Keywords Barn owl · Second-generation anticoagulant rodenticide (SGAR) · Demographic differences · Temporal differences · British Columbia · Cytochrome P450 gene

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Introduction

Through the spread of urbanization and the intensive agriculture needed to support a rapidly growing population, humans have occupied many high diversity habitats in the world, principally estuaries, riverine valley bottoms and wetlands (McKinney 2002). Many species are unable to adapt to the changing environment and become locally displaced when loss of native habitat is large enough in scale. In the Lower Mainland of British Columbia (BC), Canada, the focus of the present study, many avian species

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such as the yellow-billed cuckoo (*Coccyzus americanus*), western screech-owl (*Megascops kennicottii*), and western bluebird (*Sialia mexicana*) have been extirpated over the last century as a result of the myriad stressors associated with human activities and land-use. Nonetheless, some resilient bird species—for instance the barn owl (*Tyto alba*)—have successfully adapted to a human modified environment. Barn owls take advantage of man-made structures for nesting such as tall barns and silos, and hunt in agricultural areas and remaining fragments of grassy habitats in urban landscapes. However, their persistence is fraught with a variety of threats. In the past two decades, suitable hunting and nesting habitats have diminished by 53 and 30 %, respectively, in the Lower Mainland as a result of the destruction of old barns, transitions to large-scale industrial agriculture, and the building of modern sealed barns (Hindmarch et al. 2012). In addition, large numbers of barn owls are killed annually by vehicle strikes (Preston and Powers 2006; Bishop and Brogan 2013). As a rodent specialist, barn owls are also vulnerable to poisoning by rodent control agents, principally second-generation anticoagulant rodenticides (SGARs).

SGARs are chemicals widely employed in agricultural and urban settings worldwide to reduce rodent infestations (Corrigan 2001). They are more potent and acutely toxic than their first-generation counterparts, such as warfarin (Vandenbroucke et al. 2008; Fisher et al. 2004). Anticoagulant rodenticide (AR) compounds disrupt the recycling of vitamin K, a key component required for blood coagulation, and as a result, ingestion at lethal doses causes internal hemorrhage and death (Berny 2007; Webster et al. 2015). Not surprisingly, many owl species that hunt extensively for small rodents are at risk of secondary poisoning from AR (Albert et al. 2010; Christensen et al. 2012; Murray 2011; Lopez-Perea et al. 2015). In Britain, the proportion of adult barn owls with detectable hepatic SGAR residues escalated to almost 60 % in the recent years, an approximate 6-fold increase since 1986 (Walker et al. 2014). In addition, the percentage of adults containing multiple SGARs has also increased significantly over the same time period (Walker et al. 2014). Likewise in BC, 62 % of barn owls tested between 1992 and 2003 were found to contain detectable AR residues (Albert et al. 2010). However, there is a clear need to assess the exposure rate in more recent years, in particular prior to 2013 when regulations on rodenticide application in Canada were less restrictive. Since then, Health Canada's Pesticide Management Regulatory Agency (PMRA) has placed new prohibitions on SGAR usage, such as restricting brodifacoum and difethialone to indoor application only (PMRA 2010, 2013).

With the increasing prevalence of SGAR usage in urbanized habitats (Lopez-Perea et al. 2015), there is

concomitantly a greater risk of mortality from intoxication for raptor species residing close to human habitation. However, diagnosis of actual toxicosis is problematic in salvaged carcasses, as it must rely on qualitative symptoms such as generalized carcass pallor and internal hemorrhaging in the absence of trauma (Murray 2011). Diagnostic liver residues are also problematic, although Thomas et al. (2011) have made an initial attempt using logistic regression analyses to estimate the probability of toxicosis as predicted by the liver SGAR residues for three species of owls. They showed that barn owls have an 11–22 % chance of exhibiting toxicosis symptoms based on a previously proposed "potential lethal range" of 100–200 ng/g (Newton et al. 1998, 1999), suggesting that SGAR poisoning poses a considerable risk for barn owls (Thomas et al. 2011). Given that barn owls are federally listed as a Species at Risk in Canada (COSEWIC 2010), it is essential to evaluate the potential toxicosis risks associated with SGAR exposure in BC.

While 100–200 ng/g of SGAR is oftentimes used as a reference for "potential lethal range" (Newton et al. 1998, 1999), tolerance to AR appears to be highly variable among individuals for any given avian species (Newton et al. 1998; Rattner et al. 2014; Webster et al. 2015). Barn owls found dead from AR poisoning have been reported to contain hepatic concentrations ranging from 60 ng/g (Walker et al. 2014) to 1720 ng/g (Newton et al. 1999), which could indicate more than an order of magnitude variation in individual sensitivity to anticoagulants. Although studies have compared interspecific differences in rodenticide tolerance (Watanabe et al. 2010; Thomas et al. 2011), little is known about the basis for individual variation, whether genetic or otherwise. From a pharmacokinetics perspective, the variation could be attributed to different factors, including differences in the enzymatic efficiencies of VKOR (vitamin K epoxide reductase; Watanabe et al. 2010), warfarin-binding capacity of albumin (Watanabe et al. 2015), and CYP-mediated (cytochrome P450) metabolic ability (Ishizuka et al. 2007; Watanabe et al. 2010). The CYP2C9 is a human-specific gene that is involved in warfarin detoxification (Aithal et al. 1999; Sconce et al. 2005; Higashi et al. 2002). Dosage requirement for patients with thrombosis—where coagulation occurs locally inside a vessel—varies considerably depending on the individual's ability to metabolize warfarin, which is predominantly determined by particular SNPs (Single Nucleotide Polymorphisms) in the CYP2C9 gene. Many SNPs in the gene have been identified to be associated with reduced warfarin metabolism (Aithal et al. 1999, Higashi et al. 2002). In comparison, the CYP2C45 gene found exclusively in avian species shares considerable protein and DNA sequence identity with other genes in the CYP2C subfamily of non-avian animals, and has

been designated as a homolog to the human CYP2C9 gene (Pruitt et al. 2002). Whether this gene plays an important role in metabolizing AR compounds in birds is currently unknown.

The objective of the current study was to assess the levels and impacts of SGAR poisoning in the remnant Canadian population of barn owls by evaluating temporal trends (1992–2003 vs. 2006–2013) and demographic differences. Specifically, we examined SGAR exposure rates, residue concentrations, and toxicosis risk levels. In addition, we evaluated whether presence of toxicosis symptoms can be explained by corresponding SGAR residue concentrations. Finally, we explored the potential associations between the CYP2C45 gene and intraspecific differences in susceptibility to SGAR in barn owls.

Materials and methods

Sample collection

We collected, autopsied and analyzed for hepatic AR residues in 78 and 41 (total N = 119) dead barn owl samples in 1992–2003 and 2006–2013, respectively. The samples were from the Lower Mainland and Fraser Valley regions of BC, in addition to 3 birds from Vancouver Island. Owl carcasses were received from the BC Ministry of Environment, Canadian Wildlife Service (Delta, BC), Orphaned Wildlife Rehabilitation Society (Delta, BC), Wildlife Rescue Association (Burnaby, BC), Mountainaire Avian Rescue Society (Courtenay, BC), as well as from the general public. Owls were either found dead, or if brought to rehabilitation facilities died upon arrival, died in care, or were euthanized due to the severity of their injuries. All post-mortem examinations (including sex and age) were conducted by doctors of veterinary medicine with a specialization in avian pathology. Carcasses were examined for exterior and interior signs of blunt force trauma, including fractures and bruising. Pathophysiological symptoms of toxicosis were also documented, including evidence of extensive hemorrhage from organs (e.g. heart, lungs, liver, stomach, intestines, and/or subcutaneous areas), bleeding from the mouth or beak, and/or marked generalized pallor in the absence of severe traumatic injuries (Albert et al. 2010; Murray 2011; Thomas et al. 2011). Owls showing both the foregoing symptoms and severe trauma were not categorized as having toxicosis, as it was not possible to determine, for instance, whether internal hemorrhaging resulted from vehicle collision or rodenticide poisoning. In addition, owls that only exhibited intestinal hemorrhage and had poor body conditions were not classified as having toxicosis, as those symptoms were likely associated with severe starvation. Livers were then

extracted from carcasses and sent to the National Wildlife Research Center in Ottawa, Ontario, Canada for AR residue analysis.

Chemical analysis

Chemical analysis was conducted following methods described in Albert et al. (2010), and thus the methods were the same for both the earlier dataset reported in Albert et al. (2010) and the later dataset presented here. In brief, 0.50 g of liver sample was ground in a mortar with 5 g of anhydrous sodium sulfate. The liver powder was extracted with acetonitrile (EMD Omnisolv, AX0142-1, HPLC Grade). The extract was then shaken vigorously for 15 min and 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 Quadrupole Mass Spectrometer with the TurboSpray ion source in negative polarity using multiple reaction monitoring). The minimum detectable amount was 5 ng/g for difethialone, and 2 ng/g for brodifacoum and bromadiolone. 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 carryover.

Statistical analysis

The residue data published by Albert et al. (2010) were collected prior to 2003. In order to determine changes in recent years, we categorized the samples into two time periods: 1992–2003 (N = 78) and 2006–2013 (N = 41). We used Fisher's exact test to compare between the two time periods for differences in the proportion of owls with detectable SGAR residues, the number of compounds detected (≥ 1, ≥ 2, and 3), and proportion of owls with toxicosis symptoms. We used Mann–Whitney U test to evaluate differences in measured residue concentrations. To assess risk of toxicosis, we grouped the 119 barn owl samples into four categories of probability risk levels of toxicosis based on liver SGAR residues (Thomas et al. 2011): <5 % (<50 ng/g), 5–10 % (50–90 ng/kg), 10–15 % (90–130 ng/kg), 15–20 % (130–180 ng/g), and 20 % (>180 ng/g). Fisher's Exact Test was also used to evaluate for differences in toxicosis risk levels between the two time periods.

Samples were also separated into three demographic groups: adult male (N = 36), adult female (N = 51), and first-year juveniles (N = 27). All juveniles were grouped together regardless of sex due to low sample size and many unsexed individuals. We used the Kruskal–Wallis test to evaluate for differences in residue concentration among the three demographic groups, followed by post hoc Wilcoxon. Since Fisher's exact test can only be used for one independent and one dependent variable, we grouped female and male adults together and compared with juveniles when testing for differences in the proportion of individuals with detectable SGAR residues, the number of compounds detected (≥ 1, ≥ 2, and 3), and proportion of owls with toxicosis symptoms.

We performed multivariate generalized linear models (GLM) to examine how SGAR residues and their presence/absence are influenced by two predictor variables: demographic group and time period. We also used GLM to examine how presence (N = 15) and absence (N = 104) of toxicosis symptoms were affected by the following variables of interest: demographic group, time period, and residue concentrations of bromadiolone, brodifacoum, and difethialone. We used Akaike's second order information criterion (AIC_C) to select the most supported models ($\Delta AIC_C < 2$) among all possible combinations of variables and their interactions. All covariates were kept in the regression model as recommended by Harrell (2001). All statistical analyses were conducted using the program R (R Development Core Team 2011).

Genetic analysis

We extracted total DNA from 13 barn owl samples using 5 mg of muscle tissue based on a modified protocol of Meulenbelt et al. (1995). The 13 samples were from carcasses collected in 2012, and included 5 individuals that appeared to show low SGAR tolerance (toxicosis symptoms present with residues <100 ng/g) and 3 with high SGAR tolerance (toxicosis symptoms absent with residues ≥ 100 ng/g). DNA sequences from the CYP2C45 gene of chicken (*Gallus gallus*), wild turkey (*Meleagris gallopavo*), zebra finch (*Taeniopygia guttata*) were obtained from genome browsers: NCBI and Ensembl. We aligned the exon regions from the three avian species and identified conserved regions of approximately 20-30 base pairs. Based on those conserved sequences, we designed 6 sets of primer and amplified 6 segments of the CYP2C45 gene from the extracted barn owl DNA (see Supplementary Material 1). PCRs were performed in a final volume of 20μL containing 10–25 ng of template DNA, 1xPCR buffer, 0.2 μm of dNTPs, 0.3 U of *Taq*, and 0.1 μm of each forward and reverse primer. PCR products were sequenced at the NAPS Unit, University of British Columbia, using

Big Dye Terminator chemistry version 3.1 (Applied Biosystems, Ontario, Canada) and were resolved on Applied Biosystems 3730S 48-capillary DNA analyzer. We compared barn owl DNA sequences and the translated protein sequences with chicken, zebra finch, and wild turkey to confirm that the correct ortholog gene segment was amplified. We conducted all sequence alignment, editing of chromatograms, and identification of SNPs using SeqMan Pro 8.1.

Results

Proportion of owls showing toxicosis symptoms in 2006–2013 (29.3 %) was significantly higher than in 1992–2003 (3.8 %; Table 1). For instance, in 2006–2013 all of the toxicosis cases showed generalized pallor and/or hemorrhaging in various internal organs (e.g. gastric, intestinal, and pulmonary) in the absence of physical trauma, whereas in 1992–2003 most mortalities were due to vehicle collision. The mean concentration of total SGAR residues detected from barn owl livers was significantly higher in 2006–2013 (141 ng/g) than in 1992-2003 (57 ng/g). Percentages of barn owls with at least two SGARs and with all 3 SGARs detected were significantly higher in 2006–2013 (65.9 % and 26.8 %, respectively) compared to 1992–2003 (34.6 and 9 %, respectively). Individuals with all 3 SGAR types detected had significantly higher total hepatic SGAR concentration than those that had only 1 or 2 SGAR types (t = 2.580, $p = 0.012$). Similarly, the mean concentration of difethialone and the proportion of individuals exposed to it were both significantly higher in 2006–2013 (47 ng/g and 43.9 %, respectively) when compared to 1992–2003 (17 ng/g and 12.8 %, respectively). Brodifacoum and bromadiolone concentrations were also higher in 2006–2013, but the differences were not significant.

The proportion of barn owls with 10–15 % (90–130 ng/g), 15–20 % (130–180 ng/g), and >20 % (>180 ng/g) probability risk levels of toxicosis all increased from 1999–2003 to 2006–2013 (Fig. 1). The most dramatic increase was at the 20 % of toxicosis risk level, which increased from 9 to 22 %. Proportions of owls having >10 and >15 % probability risk levels were significantly higher in 2006–2013 (39 and 29.3 %, respectively) than in 1992–2003 (14.1 and 11.5 %, respectively), using Fisher's exact test ($p = 0.003$ and $p = 0.022$, respectively).

In terms of demographic differences, total SGAR was found to be significantly different among the three demographic groups (Table 2). Post-hoc Wilcoxon test revealed that total residue detected in adult males (111 ng/g) was significantly higher than females (64 ng/g; W = 642, $p = 0.016$). Adult males also contained significantly higher

Table 1 Percentage of barn owls (*Tyto alba*) in British Columbia with toxicosis symptoms, detectable residues of three types of SGARs and their mean (max) concentrations (ng/g), compared between 1992–2003 and 2006–2013

	1992–2003 (n = 78)	2006–2013 (n = 41)	Fisher's Exact test *p* value	Mann–Whitney U test *p* value
SGAR type detected				
Brodifacoum	47.4 %	41.5 %	0.566	0.501
	22 (358) ng/g	47 (472) ng/g		
Bromadiolone	42.3 %	39.0 %	0.845	0.976
	17 (385) ng/g	46 (688) ng/g		
Difethialone	12.8 %	43.9 %	**<0.001**	**0.020**
	17 (720) ng/g	47 (857) ng/g		
Total SGAR	57 (797) ng/g	141 (1020) ng/g	–	**0.047**
# of SGAR types detected				
≥1 SGAR	59.0 %	68.3 %	0.192	–
≥2 SGAR	34.6 %	65.9 %	**0.004**	–
All SGAR	9.0 %	26.8 %	**0.029**	–
Toxicosis symptoms				
Present	3.8 %	29.3 %	**0.001**	–
Absent	96.2 %	70.7 %	**0.001**	–

P values from Fisher's Exact test and Mann–Whitney U test indicate significant differences (bold) in percentages and mean concentrations, respectively
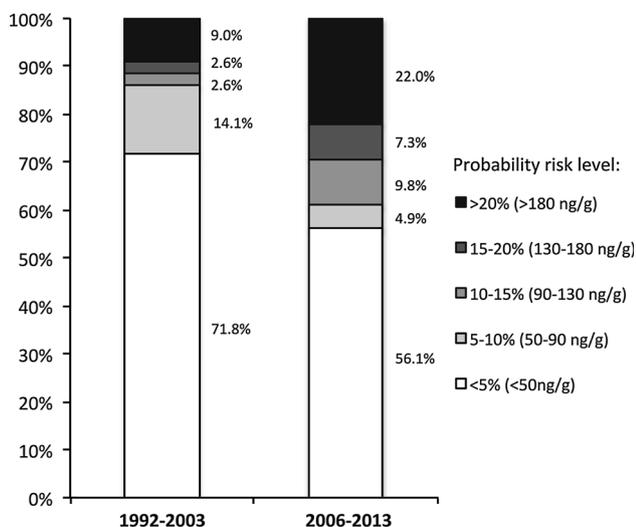
Fig. 1 Proportion of barn owls (*Tyto alba*) in British Columbia with different probability risk levels of toxicosis (<5, 5–10, 10–15, and >20 %) and the associated threshold for SGAR residue values, compared between 1999-2003 (N = 78) and 2006-2013 (N = 41). Probability risk levels were based on a logistic regression estimation method by Thomas et al. (2011)

residues of bromadiolone (45 ng/g), compared to adult females (16 ng/g; W = 697, *p* = 0.043) and juveniles (30 ng/g; W = 708, *p* = 0.001). Bromadiolone concentration in juveniles was also significantly less than female adults (W = 862, *p* = 0.031). Fisher's exact test shows that the proportion of juveniles exposed to bromadiolone was significantly lower than all adults combined (*p* = 0.028). However, there was no significant difference in the number of SGAR types detected between the demographic groups. Lastly, a higher proportion of juveniles exhibited toxicosis symptoms (33.3 %) than all adults combined (6.9 %, *p* = 0.006).

GLM models were significant for three measures of SGARs and presence/absence of toxicosis symptoms in barn owls (Table 3; see Supplementary Material 2 for AIC_C statistics). Total residue concentration and exposure to any SGAR were both significantly higher in 2006–2013 than in 1992–2003. Adult males were more likely to be exposed to at least one SGAR compound; however, this was just above the significant level (*p* = 0.052). Adult males were also more likely to be exposed to bromadiolone, whereas juveniles were less likely. Owls were more likely to exhibit signs of toxicosis when bromadiolone residue was higher. The significant interaction term between [brodifacoum] × demographic group and [difethialone] × demographic group indicate that juvenile owls were more likely to exhibit toxicosis symptoms when brodifacoum and difethialone concentrations were higher.

Exons 2, 3, 4, 5 and 6 of the CYP2C45 gene were fully sequenced from all 13 barn owls. Primers were designed 30 base pairs upstream from the start codon, and hence 30 base pairs from the 5′ end of exon 1 were not sequenced. Similarly, the primers only sequenced a portion of exon 7 (104 base pairs missing). Exons 8 and 9 were not sequenced successfully. Amino acid sequence alignment between barn owl, chicken, finch, and cormorant showed high sequence similarity (83.1–93.4 %, Fig. 2). Long stretches of identical regions (i.e. more than 10 continuous

Table 2 Percentage of barn owls (*Tyto alba*) in British Columbia with toxicosis symptoms, detectable residues of three types of SGARs and their mean concentrations (ng/g), compared between adult female, adult male, and first-year juveniles

	Adult female (n = 51)	Adult male (n = 36)	Juvenile (n = 27)	Kruskal–Wallis *p* value
SGAR type detected				
Brodifacoum	45.1 %	61.1 %	33.3 %	>0.05
	26 ng/g	42 ng/g	30 ng/g	
Bromadiolone	43.1 %	63.9 %	14.8 %*	**0.002**
	16 ng/g[a]	45 ng/g[b]	30 ng/g[c]	
Difethialone	19.6 %	25.0 %	33.3 %	>0.05
	23 ng/g	23 ng/g	46 ng/g	
Total SGAR	64 ng/g[a]	111 ng/g[b]	106 ng/g[ab]	**0.040**
# of SGAR types detected				
≥1 SGAR	60.8 %	80.1 %	56.5 %	–
≥2 SGAR	31.4 %	55.6 %	30.4 %	–
All SGAR	15.7 %	13.9 %	4.3 %	–
Toxicosis symptoms				
Present	5.9 %	8.3 %	33.3 %*	–
Absent	94.1 %	91.7 %	66.6 %*	–

Superscript letters on the concentration values denote significant differences among the 3 demographic groups (<0.05) using post hoc Wilcoxon

Values in bold and with asterisk indicate significant differences between juveniles and all adults combined using Fishers exact test

amino acids) are found in exons 2, 3, 4, 6 and 7. No stop codons were detected in the translated barn owl amino acid sequence, suggesting that the amplified sequence is a functional gene and not a pseudogene.

Sequencing of the CYP2C45 gene in barn owls revealed that one juvenile individual had a heterozygous nucleotide change at position 1011 of the transcriptome, located on exon 7. Wild-type individuals were homozygous (C/C) at this position, whereas this particular mutant individual was heterozygous (C/T). This C1011T transversion leads to an Ala344Val substitution in the encoded protein, and is located in one of the conserved regions (Fig. 2). Necropsy results indicated that the bird exhibited signs of secondary poisoning (generalized pallor and hemorrhage in stomach and intestines), while toxicology analysis showed that only 11 ng/g of SGAR residue was found in its liver. Nine individual SNP sites among the 13 genotyped barn owls in the non-coding regions (i.e. introns) were also identified. These SNPs have no apparent correlation with increased or decreased tolerance to any of the 3 SGARs (see Supplementary Material 3). No other SNPs in the exon region were detected in the other 12 individuals.

Discussion

Temporal differences in exposure and risk

Like many species residing in human modified landscapes, barn owls in British Columbia (BC) face an array of anthropogenic stressors, namely degradation of their breeding and hunting habitats (Hindmarch et al. 2012, 2014; Hindmarch and Elliott 2015), vehicle collisions (Preston and Powers 2006; Bishop and Brogan 2013), and secondary poisoning from anticoagulant rodenticides (Albert et al. 2010). Our study demonstrated that the extent of secondary poisoning from SGARs poses a considerable and increasing threat to barn owls in BC. We found that the mean concentration of total SGAR detected in their livers has more than doubled in the past decade. The increase is predominantly driven by difethialone, as mean difethialone concentration almost tripled within the same time period. Similarly, the proportion of owls exposed to multiple types of SGAR compounds was also higher in recent years, and again with difethialone exposure rate showing the most marked change. The increase in those different SGAR measures translates directly into a greater risk from secondary poisoning in recent years.

Widespread SGAR exposure in non-target raptor species has been documented globally, including the USA (Murray 2011), Norway (Langford et al. 2013), Denmark (Christensen et al. 2012), Great Britain (Walker et al. 2014), Spain (Lopez-Perea et al. 2015), Malaysia (Salim et al. 2015), and New Zealand (Eason et al. 2010). However, with the exception of a long-term study from Britain (Walker et al. 2014), few have explicitly demonstrated an increasing trend in exposure incidence and residue concentration over time in raptor species. Similar to our study, in Britain the proportion of adult barn owls with detectable residues of SGARs in the liver has progressively increased from none in 1980 to approximately 60 % in 2012 (Walker et al. 2014). Walker et al. (2014) took into account the improved sensitivity of

Table 3 Summary of the best-supported generalized linear models with statistically different outcome of interest. For categorized variables (i.e. demographic group and time period), the results for SGAR measures and presence/absence of toxicosis symptoms. Positive/negative group being affected are shown in brackets parameter estimates indicate a positive/negative relation between the variable and the

Outcome	Best supported model	Predictor variables	Estimate	Std. Error	t-value	P
Total residue concentration	Demographic + time period	Demographic (adult male)	0.0469	0.0363	1.291	0.200
		Demographic (juvenile)	−0.0342	0.0489	−0.698	0.487
		Time.period (2006–2013)	0.1096	0.0413	2.656	**0.009***
Presence/absence of any SGAR	Demographic + time period	Demographic (adult male)	0.1981	0.10090	1.963	0.052
		Demographic (juvenile)	−0.2520	0.13594	−1.854	0.066
		Time.period (2006–2013)	0.23483	0.1167	2.048	**0.043***
Bromadiolone presence/absence	Demographic + time period	Demographic (adult male)	0.2079	0.1004	2.070	**0.041***
		Demographic (juvenile)	−0.4383	0.1353	−3.240	**0.002***
		Time.period (2006-2013)	0.2238	0.1141	1.962	0.052
Toxicosis symptoms presence/absence	[Bromadiolone] + [brodifacoum] × [difethialone] × [demographic] + time period	[Bromadiolone]	0.8952	0.2858	3.133	**0.002***
		[Brodifacoum]	−0.0781	0.5939	−0.131	0.896
		[Difethialone]	0.3077	0.9119	0.337	0.736
		Demographic (adult male)	−0.0658	0.0685	−0.961	0.339
		Demographic (juvenile)	0.0288	0.0886	0.325	0.746
		Time.period (2006-2013)	0.0464	0.0729	0.637	0.526
		[Brodifacoum]: [Difethialone]	−9.3201	19.8423	−0.470	0.640
		[Brodifacoum]: Demographic (adult male)	0.0932	0.9576	0.097	0.923
		[Brodifacoum]: Demographic (juvenile)	1.8343	0.8264	2.220	**0.029***
		[Difethialone]: Demographic (adult male)	0.8736	1.2857	0.679	0.498
		[Difethialone]: Demographic (juvenile)	8.2512	1.9384	4.257	**<0.001***
		[Brodifacoum]: [Difethialone]: Demographic (adult male)	37.7406	25.0196	1.508	0.135
		[Brodifacoum]: [Difethialone]: Demographic (juvenile)	−38.8826	22.4365	−1.733	0.086

Values in bold and with asterisk indicate significant difference for the predictor variable ($p < 0.05$)

Fig. 2 Alignment of amino acid sequences of the CYP2C45 gene between barn owl (*TA Tyto alba*), chicken (*GG Gallus gallus*), zebra finch (*TG Taeniopygia guttata*) and great cormorant (*PC Phalacrocorax carbo*). The numbers on the right indicate the amino acid position at the end of each exon. Regions that are highlighted in *red* are long stretches (>10) of conserved amino acid sequences. Highlighted in *yellow* indicates the position where the mutant individual exhibited a C1011T transversion, which led to an Ala344Val substitution. Chicken sequence was retrieved from Baader et al. (2002). Zebra finch and great cormorant sequences were retrieved from the Ensembl Genome Browser (Color figure online)

Exon	Species	Amino acid sequence	
1	TA	----------LLVCIACLLSFAAWKGRSGKGKMPPGPAPLPILGNVLQVKPKNLAKTFQK	60
	GG	MLLLGAASVVLLVCVACLLSIVQWRKRTGKGKMPEGPTPLPIVGNILEVKPKNLAKTLEK	
	TG	MELLGGVTVVLLVCIACLLSFAAWKGRSGKGKMPPGPAPLPILGNLLQVKPSNMTKTLQK	
	PC	MELLGAGTVVLLVCIACLLSVAAWRRRSGKGKMPPGPAPLPILGNVLQVKPKHLAKTLQK	
2	TA	LSEEYGPVFTVHLGSDPVVVLHGHDVVKEALVERADEFAARGHMPIGDRANNGL	114
	GG	LAEKYGPVFSVQLGSTPVVVLSGYEAVKEALIDRADEFAARGHMPIGDRANKGL	
	TG	LSEEYGPVFTVHLGSDPVVVLYGHDVVKEALVDRADEFAARGHMPIGDRTNKGL	
	PC	LSEEYGPVFTVHLGSDPVVVLHGHDVVKEALVDRADEFAARGHMPIGDRANNGL	
3	TA	GIIFSNNEEWLQVRRFALSTLRSFGMGKRSIEERIQEESDYLLEEINKTKG	165
	GG	GIIFSNNEGWLHVRRFALSTLRNFGMGKRSIEERIQEEAEHLLEEITKTKR	
	TG	GIIFSNNELWLQGRRFSLTTLRNFGMGKRSIEERIQEESDYLLEEINKTKS	
	PC	GIIFSNNKEWLEVRRFALSTLRNFGMGKRSIEERIQEETEYLLEEINKTKG	
4	TA	TPFDPTFILSCAISNVVCSIVFGKRYDYKDKKFLALMNNMNNIFEMVNSHWGQ	218
	GG	LPFDPTFKLSCAVSNVICSIVFGKRYDYKDKKFLSLMNNMNNTFEMMNSRWGQ	
	TG	TPFDPTFMLSCAVSNVICSIVFGKRYDYKDKKFLALMNNMNNIFEMMNSRWGQ	
	PC	TPFDPTFTLGCAVSNVICSIVFGKRYDYKDKKFLALMTNMNNIFEMMNSHWGQ	
5	TA	LYQMFSNILDYLPGPHNKIFTEFDALKAFVSEEVKMHQDSLDPSSPQDFIDCFLSKMQE	277
	GG	LYQMFSYVLDYLPGPHNNIFKEIDAVKAFVAEEVKLHQASLDPSAPQDFIDCFLSKMQE	
	TG	LYQMFSNILDYLPGPHNNIFAEFDALKAFVAEEVKLHQASLDPSSPQDFIDCFLCKMQE	
	PC	LYQMFSRILDYLPGPHNKIFDEFDALKAFVSEEVKIHQASLDPSSPQDFIDCFLSKMQE	
6	TA	EKEHPNSSFHMKNLITSTFDLFIAGTETTSTTIRYGLLLLLLKYPKIQ	324
	GG	EKDNPKSHFHMTNLITSTFDLFIAGTETTSTTTRYGLLLLLLKYPKIQ	
	TG	EKDRPNSSFYMKNLITSTFDLFLAGTETTSTTLRYGLLLLLLKYPKIQ	
	PC	EKEHPNSSFHMKNLITSTFDLFIAGTESTSTTIRYGFLLLLLKYPKIQ	
7	TA	EKVQEEIDQVVGRSRRPCVADRTQMPYT......	352
	GG	EKVQEEIDRVVGRSRRPCVADRTQMPYT......	
	TG	EKIQEEIDQVVGQSRKPCVADRTQMPYT......	
	PC	EKVQEEIDWVVGRSRRPCVADRTQMPYT......	

analytical methods when measuring temporal changes. In California, both AR exposure prevalence and residue concentration increased in bobcats (*Lynx rufus*) from 2002 to 2012 (Serieys et al. 2015). Not surprisingly, AR exposure in these rodent-eating mammals was found to be more common in areas with increased commercial, residential and agricultural developments, where rodenticide bait usage is typically more intensive (Serieys et al. 2015). Barn owls in BC occupying more urbanized habitats were more likely to consume rats (*Rattus spp*), which are the primary targets of rodenticide baiting (Hindmarch and Elliott 2015). The increase in SGAR prevalence and residues found in our study is therefore likely due to the intensifying development in the Lower Mainland (Hindmarch et al. 2012), leading to an escalating usage of rodenticide bait as reported elsewhere (Lopez-Perea et al. 2015). An alternative possibility would be illegal placement of bait stations containing SGARs (e.g. in the centre of cropfields instead of along fence lines), resulting in more rats and non-target rodents becoming exposed. Additional studies investigating the relationship between SGAR exposure and land-use are warranted in order to elucidate the underlying factors driving the uptake of SGAR in barn owls and other raptor species.

Interestingly, while both liver residue and exposure to difethialone in BC barn owls have increased by about 3 fold,

this does not match up with the sales trend of rodenticide products to professional pest control operators in BC, where difethialone continuously had the lowest sales out of all three compounds and no increasing trends in usage (Elliott et al. 2014). Sales in bromadiolone and brodifacoum from 2001 to 2009 were on average 8.9 and 4.9 times higher, respectively, than difethialone (Elliott et al. 2014). According to our study however, percentage of owls exposed do not differ markedly among the three SGAR compounds. One possibility of this mismatch is that these residue levels were a result of unlawful application of products containing difethialone. It is also possible that difethialone was heavily applied through domestic usage in BC, for which data are unavailable from the provincial regulator (Elliott et al. 2014).

While our study showed that 29.3 % of owls were found with signs of toxicosis, another study showed that none of the 61 barn owls sampled in the Lower Mainland of BC had delayed prothrombin time (PT), a test used to detect hemostasis response to anticoagulant exposure (Webster et al. 2015). Owls examined in our study died upon arrival, died in care, or were euthanized due to severe injuries, whereas Webster et al. (2015) sampled live owls from the wild. Furthermore, ARs accumulated in the liver are very poorly metabolized in owl species (Watanabe et al. 2010); in contrast, the toxic effects in blood are more difficult to

detect as clotting times restore to baseline levels within a few days after exposure to AR (Rattner et al. 2014). Our findings showed that owls were more likely to develop toxicosis symptoms at higher hepatic SGAR levels, and that barn owls in BC have become more at risk from SGAR over the past 23 years.

Demographic differences in exposure

We found that relative to male adults, female adult barn owls contained lower residues of total SGAR in their livers. The demographic difference could be attributed to the deposition of SGAR from females to their eggs. This carryover effect has been reported in wild birds for other toxicants, primarily highly lipid soluble organic compounds such as polychlorinated dioxins (Kubota et al. 2013) and PCBs (polychlorinated biphenyl; Traag et al. 2006), and also protein bound chemicals such as methyl-mercury (Robinson et al. 2012). Fetal and neonatal AR transfers have recently been documented in two mammalian species (Serieys et al. 2015; Gabriel et al. 2012). Detectable levels of AR compounds were found in bobcat fetal samples (Serieys et al. 2015) and in a fisher (Martes pennanti) kit still suckling (Gabriel et al. 2012). In Malaysia, bromadiolone and chlorophacinone were detected in 29.7 and 5.4 % of barn owl eggs, respectively, with mean residue concentration ranging from 9 to 31 ng/g (Salim et al. 2015). To our knowledge no other studies have investigated the carryover effect of anticoagulant residues into eggs in wild birds. However, past studies have shown that eggs laid by poultry exposed to ARs contained traces of these toxicants, namely warfarin, coumatetralyl, coumachlor, bromadiolone, difenacoum, and brodifacoum (Shimshoni et al. 2013; Giorgi and Mengozzi 2010; Kammerer et al. 1998). AR concentration was found to be higher and more persistent in yolk than albumen (Shimshoni et al. 2013; Giorgi and Mengozzi 2010; Kammerer et al. 1998). When blood vessels carry nutrients to a developing yolk inside the ovarian follicle, any AR compounds can also be deposited either in their free form, or bound to VKORC1 enzymes or plasma albumin (Watanabe et al. 2015). That could potentially increase exposure and risk to the survival of nestlings as our findings showed that 33.3 % of our sampled juveniles displayed signs of toxicosis during necropsies, whereas only 6.9 % were found in adults. However, the primary route of nestling exposure is still expected to be consumption of contaminated prey. Alternatively or concomitantly, greater residue concentrations in males may be caused by their higher energetic demand, and hence greater food intake, as they are responsible for provisioning females during incubation and chick rearing (Marti et al. 2005).

As vitamin K antagonists, all three SGAR compounds share a similar mechanism of action in that they inhibit the production of vitamin K, a cofactor necessary for blood coagulation (Berny 2007). Depletion of vitamin K in the blood ultimately results in mortality by hemorrhage (Webster et al. 2015). Brodifacoum, with its greater toxicity (LD50 for mice = 0.4 mg/kg) compared to bromadiolone and difethialone (Vandenbroucke et al. 2008), is expected to be the strongest predictor for presence of toxicosis symptoms in barn owls. Our findings indicate that, rather, higher hepatic concentration of bromadiolone were associated with toxicosis symptoms across all demographic groups, despite its lower toxicity (LD50 for mice = 1.75 mg/kg; Vandenbroucke et al. 2008). The same was found for difethialone and brodifacoum in juveniles only, perhaps suggesting some age-related differences in sensitivity to those two SGAR compounds.

CYP2C45 gene and intraspecific variation in SGAR sensitivity

We identified a single point mutation in the CYP2C45 gene of one individual barn owl with apparent low tolerance to SGAR. The heterozygous change involving a C to T transversion resulted in an amino acid substitution from alanine to valine in a conserved region. Whether this substitution could potentially have downstream consequential and adverse effects on the activity and structure of the enzyme is unclear. Given that alanine and valine both share non-polar characteristics, it is possible that this amino acid substitution may not significantly change the protein's folding properties. Further, the same mutation was not identified in the other 4 individuals with apparent low SGAR tolerance. Thus, we cannot provide any evidence that SNPs in the CYP2C45 gene are associated with intraspecific variation in SGAR sensitivity in barn owls.

Apart from CYP-mediated metabolism, other genetic and/or physiological factors are known to determine SGAR sensitivity level in vertebrates. Previous studies have shown that variation in warfarin dosing in humans is due partially to polymorphisms in the gene encoding for VKORC1, the enzyme responsible for the regeneration of vitamin K (Li et al. 2006). Differences among bird species in their VKORC1 enzymatic efficiencies could also explain differential sensitivity to SGARs (Watanabe et al. 2010). In contrast, a recent study showed that the CYP isoforms may not have a critical role in warfarin metabolism in birds, and that the inhibition effect of warfarin on the VKORC1 enzyme becomes subdued when they are bound to plasma albumin instead (Watanabe et al. 2015). It could further be argued that because SGARs are very slowly metabolized in owl species (Watanabe et al. 2010), variation in individual capacity to metabolize warfarin may not be a relevant factor. Further investigations into the toxicology and pharmacokinetics of coumarin compounds in avian species

are needed to explain the apparent inter- and intraspecific variation in sensitivity to SGAR.

Conservation implications

SGAR exposure is increasingly recognized as a conservation concern due to the fatal and likely sublethal effects on non-target raptor (Christensen et al. 2012; Murray 2011; Walker et al. 2014; Lopez-Perea et al. 2015) and mammalian wildlife species (Elmeros et al. 2011; Sanchez-Barbudo et al. 2012; Gabriel et al. 2012). Even a sublethal dose of SGARs may cause prolonged clotting times, increasing the negative consequences for health and survival (Rattner et al. 2014). With the current small population number of barn owls in BC, additional conservation efforts are needed to minimize exposure to SGAR compounds. As part of the Recovery Strategy for the species under the Federal Species at Risk Act, we recommend enforcing regulations for rodenticide application at a regular basis and encouraging the use of snap-traps. Programs to reduce prophylactic bait placement in industrial and commercial settings, and improved education to agricultural users and the wider public on safe usage are also recommended (Elliott et al. 2016). Preventative measures can include steps such as removing potential food sources for rats, blocking access points enabling indoor exposure, and improved management of outdoor waste.

We also recommend that long-term monitoring studies are necessary to evaluate the health and status of wildlife populations in the urban environment. As of December 2012, new federal regulations require the phase out of SGAR products such as brodifacoum, difethialone and bromadiolone from the domestic retail market, and currently these products can only be used by licensed applicators (PMRA 2010, 2013). With the new regulatory restrictions in North America on the domestic use of SGARs, we anticipate some slowing and eventually a reversal of the increasing trend of exposure prevalence and concentration found in our study. Therefore, it is all the more important to continue long-term research on susceptible wildlife species and surveillance of their exposure to SGARs.

Compliance with ethical standards

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

Research involving human participants or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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