Second generation anticoagulant rodenticide residues in barn owls 2017

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1 Executive Summary

A wide range of avian and mammalian predators and scavengers in rural Britain is known to be exposed to Second Generation Anticoagulant Rodenticides (SGARs). The barn owl *Tyto alba* is a sentinel for species that are generalist predators of small mammals in rural areas in the UK and monitoring of liver SGAR residues in barn owls has been adopted as an element of the monitoring undertaken as part of anticoagulant rodenticide stewardship. Monitoring of liver SGAR residues in some 100 barn owls per year is conducted in support of stewardship and annually collected data are compared with those from 395 barn owls that died between 2006 and 2012 (hereafter termed baseline years), prior to the 2016 changes in anticoagulant rodenticide (AR) authorisations and onset of stewardship.

The rationale for using data on SGAR residues in barn owls that died between 2006 and 2012 as a baseline was that all measurements had been made using the same analytical techniques, there had been little clear change in exposure over that time period, and the data were the most recent available.

The aim of the current study was to measure SGAR exposure in barn owls in 2017.

As in the baseline years, the compounds detected most frequently in barn owls that died in 2017 were bromadiolone, difenacoum and brodifacoum. Overall, 90% of the owls had detectable liver residues of one or more SGAR.

The metrics to be used for stewardship monitoring are reported below in terms of differences between owls that died in 2017 and in baseline years.

- *Numbers of barn owls containing detectable residues of flocoumafen and difethialone.* There was no significant difference in the proportion of barn owls with detectable liver residues of either flocoumafen or difethialone between the baseline years and 2017.

- *The ratio of birds with "low" (<100 ng/g wet wt.) vs "high" (>100 ng/g wet wt.) concentrations for any single SGAR or for ∑SGARs*. There was no significant difference between barn owls from baseline years and from 2017 for any individual compound or for summed SGARs (∑SGARs)

- *Average concentrations of brodifacoum, difenacoum, bromadiolone and ∑SGARs in the cohort of owls with "low" residues (<100 ng/g wet wt.) and "high" residues (>100 ng/g wet wt.*). There was no significant difference between barn owls from baseline years and from 2017 in the concentrations of either "low" or "high" residues for bromadiolone, difenacoum and brodifacoum, or for all residues summed (∑SGARs). Although not statistically significant, the median and 75th percentile values of "low residues" of most compounds and ∑SGARs were lower in 2017 [and 2016] than in the baseline years

Overall, the lack of statistically significant differences in SGAR accumulation by barn owls in 2017 compared within baseline years suggests that full implementation of stewardship since 2016 has yet to be reflected by a detectable general reduction in exposure of barn owls.

2 Introduction

2.1 Exposure of non-target predators and their prey to second generation anticoagulant rodenticides (SGARs) in Britain

A wide range of avian and mammalian predators and scavengers in rural Britain is known to be exposed to Second Generation Anticoagulant Rodenticides (SGARs) (McDonald et al., 1998; Newton et al., 1999; Shore et al., 2003a; Shore et al., 2003b; Shore et al., 2006; Walker et al., 2008a; Walker et al., 2008b; Dowding et al., 2010; Hughes et al., 2013; Walker et al., 2014; Ruiz-Suárez et al., 2016). Defra's Wildlife Incident Monitoring Scheme (WIIS)[1] and the Predatory Bird Monitoring Scheme (PBMS- http://pbms.ceh.ac.uk/) have shown that some mortalities are the result. Exposure is generally thought to be secondary in most predators and scavengers but, as many species rarely feed on commensal rodents, exposure is likely due to feeding on non-target small mammal species (Rattner et al., 2014; Shore et al., 2015; Geduhn et al., 2016). In Britain, such non-target species are primarily wood mice *Apodemus sylvaticus* and bank voles *Myodes glareolus,* which will feed on bait they encounter (Brakes and Smith, 2005; Tosh et al., 2012). It has been argued that this exposure scenario may be most significant where SGARs are used around buildings and in open areas. The predominance of difenacoum and bromadiolone (compounds that are licensed for in and around building and open area use in Britain) in barn owl livers is consistent with this assumption but they are also the most widely used compounds in Britain and residues in predators may also simply reflect predominant usage (Shore, et al., 2015).

The barn owl *Tyto alba* can be considered as a sentinel for species that are generalist predators of small mammals in rural areas in the UK. Residues have been detected in this species around the globe (López-Perea & Mateo, 2018). Monitoring of liver SGAR residues in barn owls has demonstrated increases in exposure largely through the 1980s and 1990s, and an overall widespread prevalence of residues (Walker, et al., 2014). However, there is no evidence of significant adverse effects on barn owl populations of harmful levels of rodenticides in their mammalian prey and earlier declines are more likely to be the indirect consequence of the earlier use of organochlorine pesticides and subsequent changes in the agricultural management of grassland (Smith and Shore, 2015).

2.2 Changes in SGAR authorisations and implementation of stewardship

Five SGARs are currently authorised for use in the United Kingdom - difenacoum, bromadiolone, brodifacoum, flocoumafen and difethialone. Until recently, only difenacoum and bromadiolone have been authorised for use both *in and around buildings* and in *open areas* in Britain. The other three compounds were restricted to *indoor* use as a mitigation measure to reduce unintentional primary and secondary exposure and poisoning of non-target species. However,

[1] Quarterly WIIS reports are available at http://www.hse.gov.uk/pesticides/topics/reducing-environmental-impact/wildlife/wiis-quarterly-reports.htm

a review of the available ecotoxicological data for the five SGARs concluded that they were indistinguishable in terms of environmental toxicity (risks to non-target species) and should be treated in the same way in terms of authorisation in the UK (Health & Safety Executive, 2012). This led to a change in the way authorisations are assessed and all five SGARs are currently eligible for broadly similar authorisations that can include *in and around buildings* and, potentially, *open area* use. However, industry has voluntarily agreed to make no applications for authorisations for the use of brodifacoum, difethialone and flocoumafen in open areas (A. Buckle pers. comm.).

The changes in authorisations for anticoagulant rodenticide (ARs) have been accompanied by the development and implementation of an industry-led stewardship scheme http://www.thinkwildlife.org/stewardship-regime/. Stewardship is intended to coordinate and deliver best practice in terms of use of ARs and thereby minimize (and reduce from current levels) exposure and risk to non-target species from ARs (Buckle et al., 2017). The stewardship scheme in the UK is being implemented by the Campaign for Responsible Rodenticide Use (CRRU- UK - http://www.thinkwildlife.org/about-crru/)

One element of stewardship is a requirement to monitor outcomes. This involves five elements:

- A periodic survey on the knowledge, attitudes and practices of all professional rodenticide users in order to observe changes over time. A baseline survey had been conducted in advance of regime implementation and a follow-up study was done in 2017.
- The breeding success at 130 selected barn owl nest sites located across five regions of the UK will be monitored to determine year on year fluctuations in nest productivity. This is to examine certain barn owl breeding parameters in the presence of the SGAR residues found in the UK barn owl population (see last bullet point).
- An annual report of WIIS data concerning vertebrate pesticides used in the UK.
- A review of the current state of knowledge of the distribution, severity and practical implications of anticoagulant resistance in UK rodents.
- SGAR residues in the livers of barn owls from across Britain will be monitored annually to determine whether there has been any change in exposure in this wildlife sentinel.

This report relates to the last of these elements, the monitoring of SGAR residues in barn owls.

The ways in which monitoring of SGAR residues in barn owls could be used to assess the impacts on non-targets of change in authorisation and associated stewardship were outlined in a report by Shore et al. (2014). That report described an analysis that examined how long it would take to detect change [of 10%, 20% and 50%] in liver SGAR concentrations from average levels of 395 barn owls that died between 2006 and 2012. The dataset of residues for 395 barn owls was considered to be a baseline against which to measure future change

Annual monitoring of liver SGAR residues in barn owls is currently conducted in support of stewardship and uses birds that died in 2016 and in later years—changes in authorisations and implementation of stewardship relate to 2016 and thereafter.

2.3 Aims of the current study

The rationale for using data on SGAR residues in barn owls that died between 2006 and 2012 as a baseline measurement against which future changes would be assessed. This time period was chosen partly because all measurements had been made using Liquid Chromatography Mass Spectrometry (LCMS), which is more sensitive than older fluorescence methods in terms of detecting residues (Dowding, et al., 2010; Shore, et al., 2015).

The current report describes liver SGAR concentrations in barn owls that died in 2017, the first full year following full implementation of stewardship that formally began midway through 2016. In this report, we compare SGAR residues in a sample of 100 barn owls that died in 2017 with those in barn owls that died between the 2006 and 2012 (baseline) years. We also include, for information purposes only, summaries of the data obtained for birds that died in 2015 (pre-stewardship) and 2016 (during stewardship implementation).

3 Methods

We analysed 100 barn owls for liver SGAR residues. The owls were collected as part of the Predatory Bird Monitoring Scheme (PBMS). Carcasses were submitted to the PBMS by members of the public throughout the year and were from across the whole of Britain, although predominantly England and Wales, as in previous years (Figure 1). All barn owls received by the PBMS were autopsied and they were found to have died from various causes, but mainly from road traffic collisions or starvation. Any haemorrhaging detected at post-mortem in birds was always associated with signs of trauma and so there was no clear evidence that any individual had died from anticoagulant rodenticide poisoning. Liver subsamples were analysed for difenacoum, bromadiolone, brodifacoum, flocoumafen and difethialone.

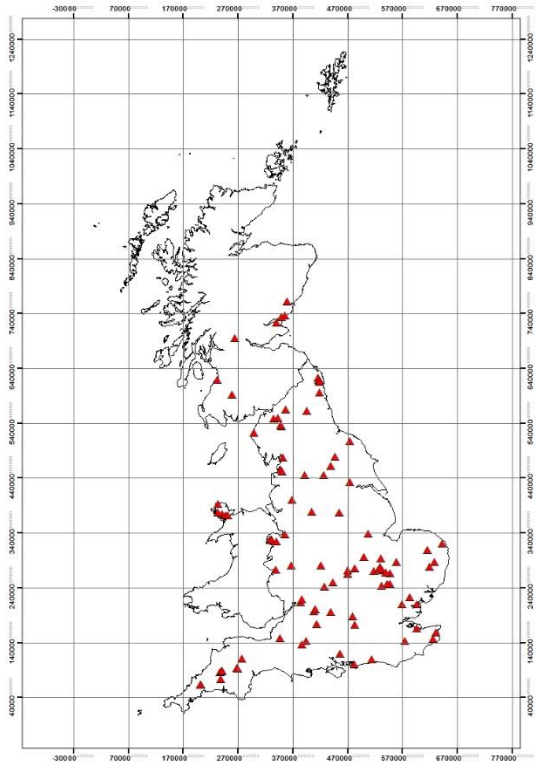

Figure 1. Provenance of the barn owls that died in [needs updating with 2017] and were analysed for liver SGAR residues

The composition of the 100 birds collected in 2017 was 30 adults (12 males, 18 females) and 70 first-years (46 males, 24 females); first year birds were individuals hatched in the current or previous year. Overall the percentage of adults in the 2017 sample was 30% and so within the confidence limits of the baseline datatset (mean: 29.5%, 95% confidence limits: 20.4 – 38.7%). Age is known to have an effect on the magnitude of residues accumulated by barn owls (Walker, et al., 2014) and consistency between years in the proportion of adults in the sample is therefore important.

Chemical determination of residues was by Liquid Chromatography Mass Spectrometry and a summary of the analytical methods can be found in Appendix 1 of this report. AR concentrations in this report are given as ng/g wet weight (wet wt.) throughout. Data used from the report by Shore et al. (2014) were multiplied by 1000 to convert them from µg/g wet wt. to ng/g wet wt.; for example, 0.1 µg/g wet wt. is equivalent to 100 ng/g wet wt.. Limits of detection (LoD) for each compound were 1.5 ng/g wet wt. for all compounds except difethialone that had a LoD of 3.0 ng/g wet wt.. Mean (± SD) recovery for deuterated bromadiolone and brodifacoum standards that were added to each of the 100 samples was 58.0±7.6 and 71.3±9.3%, respectively.

Shore et al. (2014) outlined how new data on residues should be compared to the baseline dataset. For statistical reasons, this involves dividing the residue data into two populations — <100 ng including non-detected values which were assigned a numerical value of zero (so called

"low" residues) and >100 ng/g ww ("high" residues) — and analyzing the two separately. This approach is used for liver difenacoum, bromadiolone and brodifacoum residues and for summed concentrations (∑SGARs); summed residues are calculated as the arithmetic sum of the residues of any of the five SGARs that were measured. For flocoumafen and difethialone, there were few barn owls in the baseline dataset with liver residues of either compound and statistical comparison with concentrations in later years is not possible. Change in exposure to each of these two compounds is assessed through comparison of the proportion of birds with detectable residues in baseline and in subsequent years.

Overall, there are three metrics of change that are assessed:

a) Change in the ratio of birds with detectable residues of flocoumafen and difethialone
b) Changes in the ratio *number of owls with "high" concentrations: number of owls with "low" concentrations* for brodifacoum, difenacoum, bromadiolone, ∑SGARs
c) Change in "low" and "high" concentrations of brodifacoum, difenacoum, bromadiolone, and summed SGARs (∑SGARs)

A summary of the proportion of birds with detectable residues of flocoumafen and difethialone in 2017 (metric (a)) is given in Section 4.1. This metric is also given for the other SGARs and for ∑SGARs for information only. The above metrics for (b) and (c) are reported in sections 4.2 and 4.3, respectively. Comparisons between proportions of birds containing residues were by Fisher's Exact test and comparisons of liver SGAR concentrations between owls that died in baseline years and in 2017 were conducted by Mann-Whitney U tests. A probability level of $P<0.05$ was taken as statistically significant.

Although comparison between the baseline and current year is the metric required for stewardship reporting, change over years can also be informative and the change in metrics from baseline is shown for 2015, 2016 and 2017 for information (Figures 3-6). However, time trends were not tested statistically as the data represent only 1-2 years post-implementation of stewardship.

4 Results

4.1 General summary of liver SGAR residue data for 2017 owls

The metric of presence or absence of liver SGAR residues in barn owls is a relatively crude binary measure of exposure and, except for low prevalence compound (flocoumafen and difethialone) is not one of the agreed metrics used for assessing the outcomes of stewardship. However, this measure is relatively easy to understand and is therefore presented for all compounds simply for general information.

As in the baseline years, the compounds detected most frequently in barn owls that died in 2017 were bromadiolone, difenacoum and brodifacoum with between 48% and 82% of owls in 2017 containing detectable residues of each compound (Table 1). Overall, 90% of owls had detectable liver residues of one or more SGAR and 64% had liver residues of more than one compound. The overall prevalence of residues, as judged from the % of owls with ≥ 1 residue (Figure 2), was 81% in baseline years and has since varied between 78% (2016) and 94% (2015).

Table 1. Proportion of barn owls that died in 2017 and had non-detected and detected liver bromadiolone, difenacoum, brodifacoum, ∑SGARs and multiple SGAR residue

	Bromadiolone	Difenacoum	Brodifacoum	∑SGARs	multiple residues
non-detected	18	50	52	10	36
detected	82	50	48	90	64
% detected	82%	50%	48%	90%	64%

One of the comparator metrics for stewardship is to compare the proportion of 2017 barn owls containing flocoumafen and difethialone with that for owls in baseline years. There was no statistically significant difference between owls from baseline years and from 2017 in the frequency of detection of either compound (Table 2).

Table 2. Proportion of barn owls that had non-detected and detected liver concentrations of flocoumafen and difethialone

	Flocoumafen		Difethialone	
	Baseline	2017	Baseline	2017
non-detected	383	99	394	98
detected	12	1	1	2
% Detected	3.0%	1.0%	0.3%	2.0%
P-value[1]	*0.482*		*0.105*	

[1] *P-value determined by Fisher's exact test., P<0.05 considered statistically significant.*

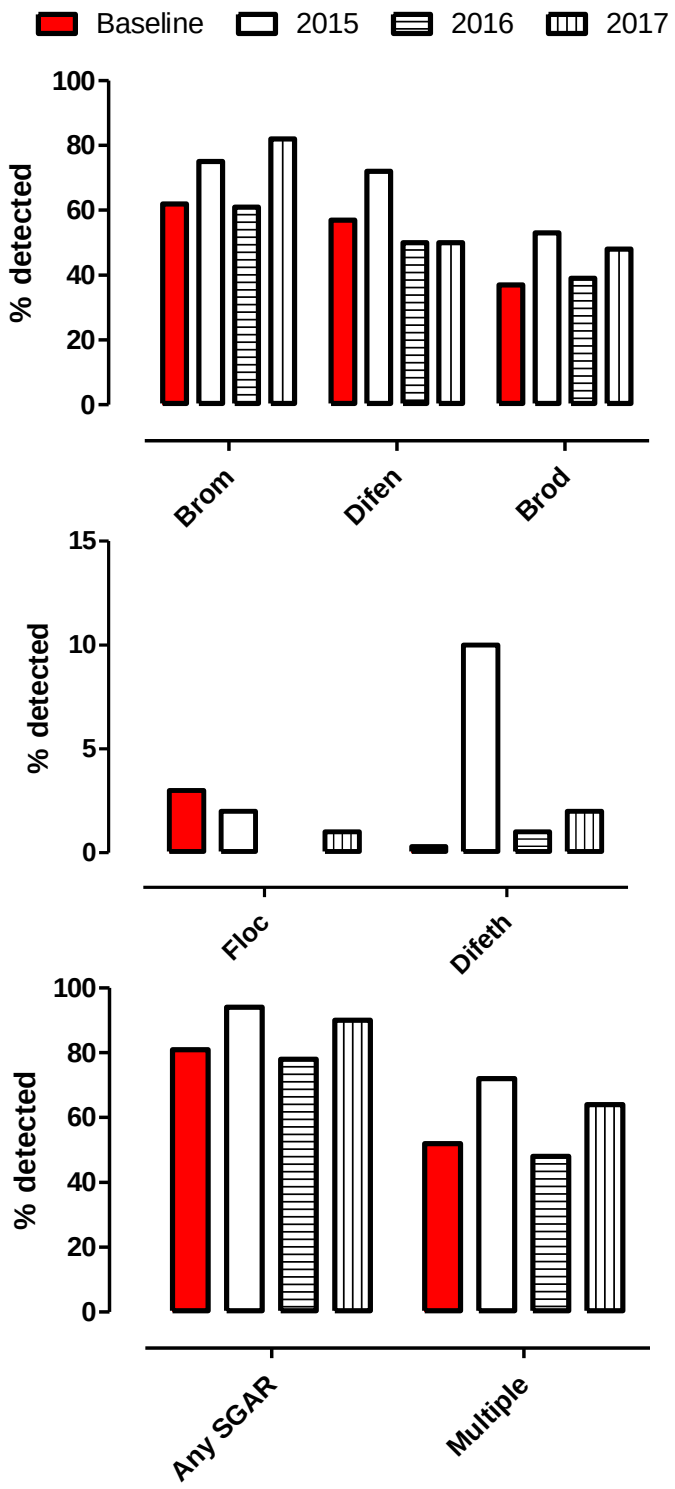

Figure 2. Percentage of barn owls with detected residues of SGARs in their liver. No birds found in 2016 had detectable residues of flocoumafen in their liver. Brom: bromadiolone; Difen: difenacoum; Brod: brodifacoum; Floc: flocoumafen, Difeth: difethialone.

4.2 Number of owls with liver AR residues above and below 100 ng/g wet wt.

This analysis was conducted for brodifacoum, difenacoum, bromadiolone and ∑SGARs only.

There was no significant difference between barn owls from baseline years and from 2017 in the ratio of birds with "low" (<100 ng/g wet wt.) vs "high" (>100 ng/g wet wt.) concentrations for any single SGAR or for ∑SGARs (Table 3 & Figure 3). The percentage of owls with "high" residues in all four monitoring periods are summarised in Figure 3.

Table 3. Number of barn owls that had "low" (non-detected and <100 ng/g wet wt.) and "high" (>100 ng/g wet wt.) concentrations of SGARs in their liver

	Bromadiolone		Difenacoum		Brodifacoum		∑SGAR	
Conc.	Baseline	2017	Baseline	2017	Baseline	2017	Baseline	2017
<100 ng/g "low"	376	95	375	98	381	94	329	85
>100 ng/g "high"	19	5	20	2	14	6	66	15
% high	4.8%	5.0%	5.1%	2.0%	3.5%	6.0%	16.7%	15%
P-value[1]	*0.258*		*0.276*		*0.261*		*0.764*	

[1] P-value determined by Fisher's exact test., P<0.05 are considered statistically significant

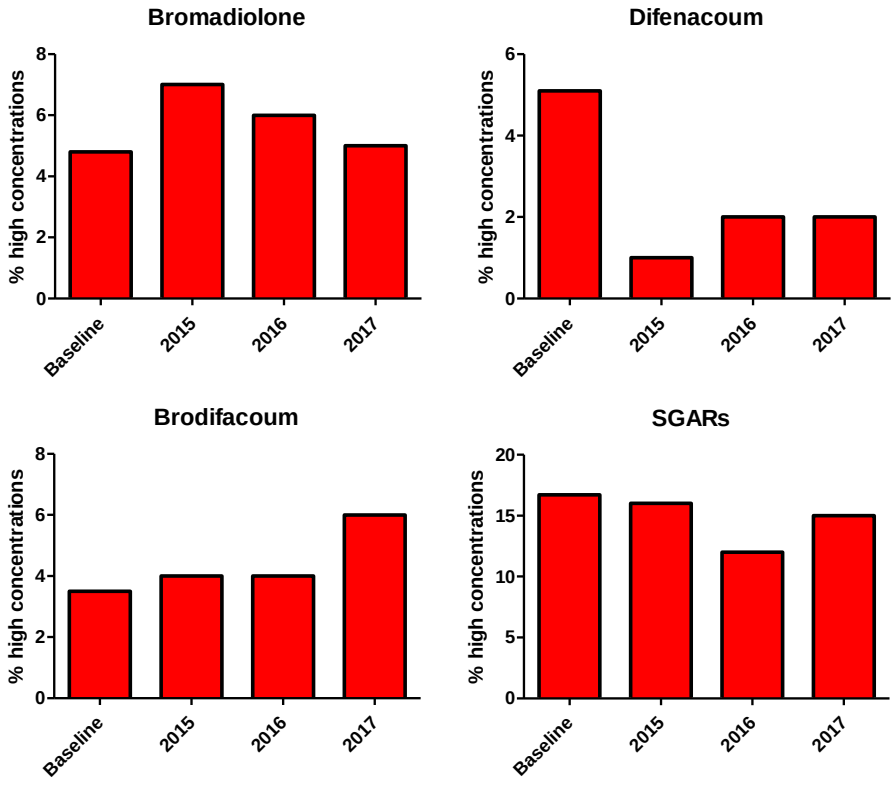

Figure 3. Proportion of barn owls with "high" (>100 ng/g wet wt.) liver SGAR concentrations.

4.3 Concentrations of brodifacoum, difenacoum, bromadiolone and ∑SGARs in the cohort of owls with residues <100 ng/g wet weight ("low" residues) and >100 ng/g wet weight ("high" residues)

For all individual compounds (Table 4) and the sum SGARs (Table 5) the median "low" and "high" concentrations measured in owls from 2017 were lower than or the same as those from the baseline years, although none of the differences between years were statistically significant.

Although comparison between the baseline and current year is conducted, change over years can also be informative and is shown in figures 4 and 5. Generally the 75th percentile and median concentrations for "low" concentrations were lower in 2016 and 2017 than in baseline years (Figure 4).

Table 4. Median, 25th percentile (Q1), and 75th percentile (Q3) concentrations (ng/g wet wt.) of bromadiolone, difenacoum and brodifacoum in barn owl livers. Non-detected values were assigned a score of zero. Sample numbers (N) given in Table 3.

		Bromadiolone			Difenacoum[2]			Brodifacoum		
Conc.		Median	Q1	Q3	Median	Q1	Q3	Median	Q1	Q3
< 100 ng/g wet wt. (low)	Baseline	5.0	0.0	17.8	3.1	0.0	12.3	0.0	0.0	5.9
	2017	3.5	2.0	10.9	0.0	0.0	6.7	0.0	0.0	3.2
	MW value[1]	17769			16217			17378		
	P-value	*0.938*			*0.059*			*0.608*		
> 100 ng/g wet wt. (high)	Baseline	179	114	224	136	115	160	347	133	923
	2017	140	117	214	121	-	-	172	134	250
	MW value[1]	43.00			-			28.00		
	P-value	*0.776*			-			*0.266*		

[1] Mann-Whitney U value

[2] Only two barn owls had detected "high" residues of difenacoum and so it was not possible to compare between concentrations for the baseline years and 2017.

Table 5. Median, 25th percentile (Q1), and 75th percentile (Q3) concentrations (ng/g ww) of ∑SGARs in barn owl livers. Non-detected values were assigned a score of zero. Sample numbers (N) given in Table 3.

		Sum SGAR		
Conc.		Median	Q1	Q3
"Low"	Baseline	15.4	2.8	38.5
	2017	9.1	2.4	29.9
	MW value[1]	13094		
	P-value	*0.364*		
"High"	Baseline	171	123	272
	2017	143	128	229
	MW value[1]	446		
	P-value	*0.555*		

[1]Mann-Whitney U value

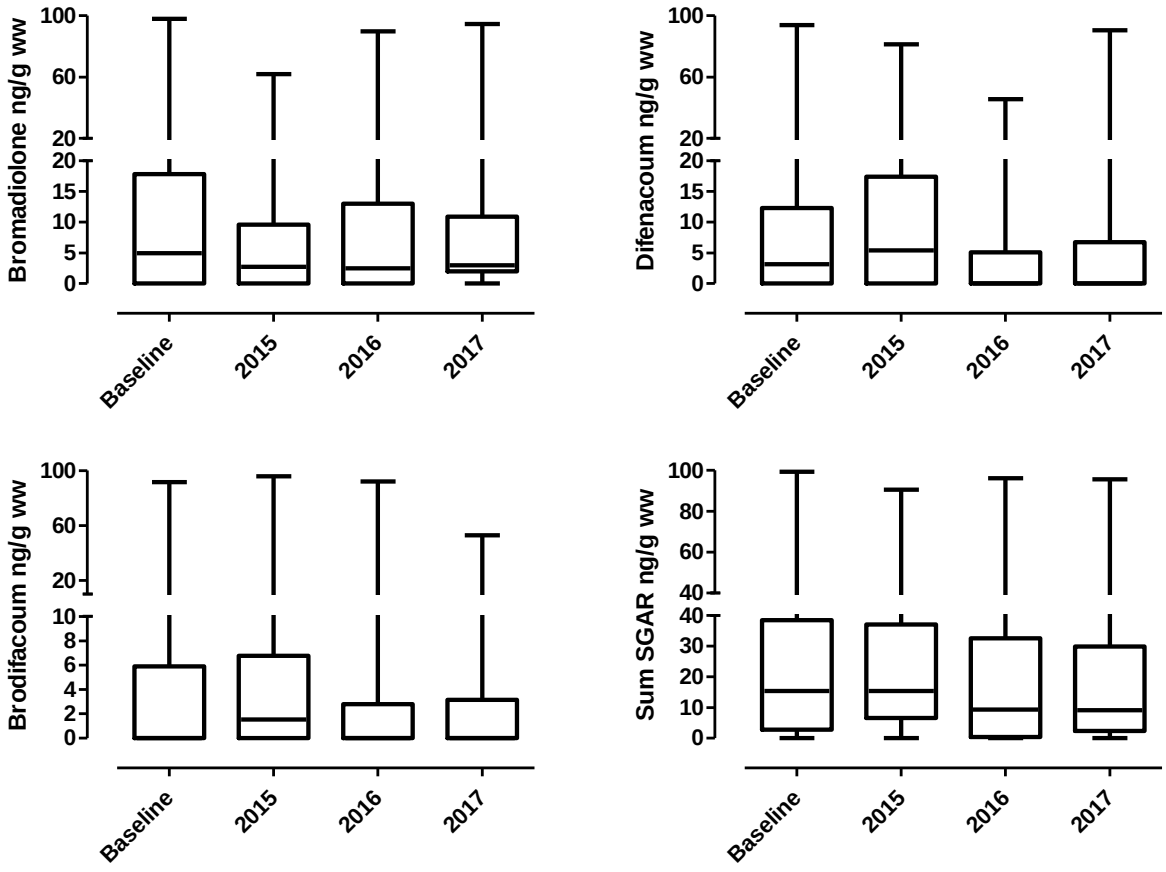

Figure 4. Box and whiskers plot of brodifacoum, difenacoum, bromadiolone and ∑SGARs liver concentrations in the cohort of owls with residues <100 ng/g wet weight ("low" residues) found dead in the 2006-2012, 2015, 2016, and 2017. Horizontal line, box and whiskers represent median, 25-75th quartile range and minimum maximum range, respectively.

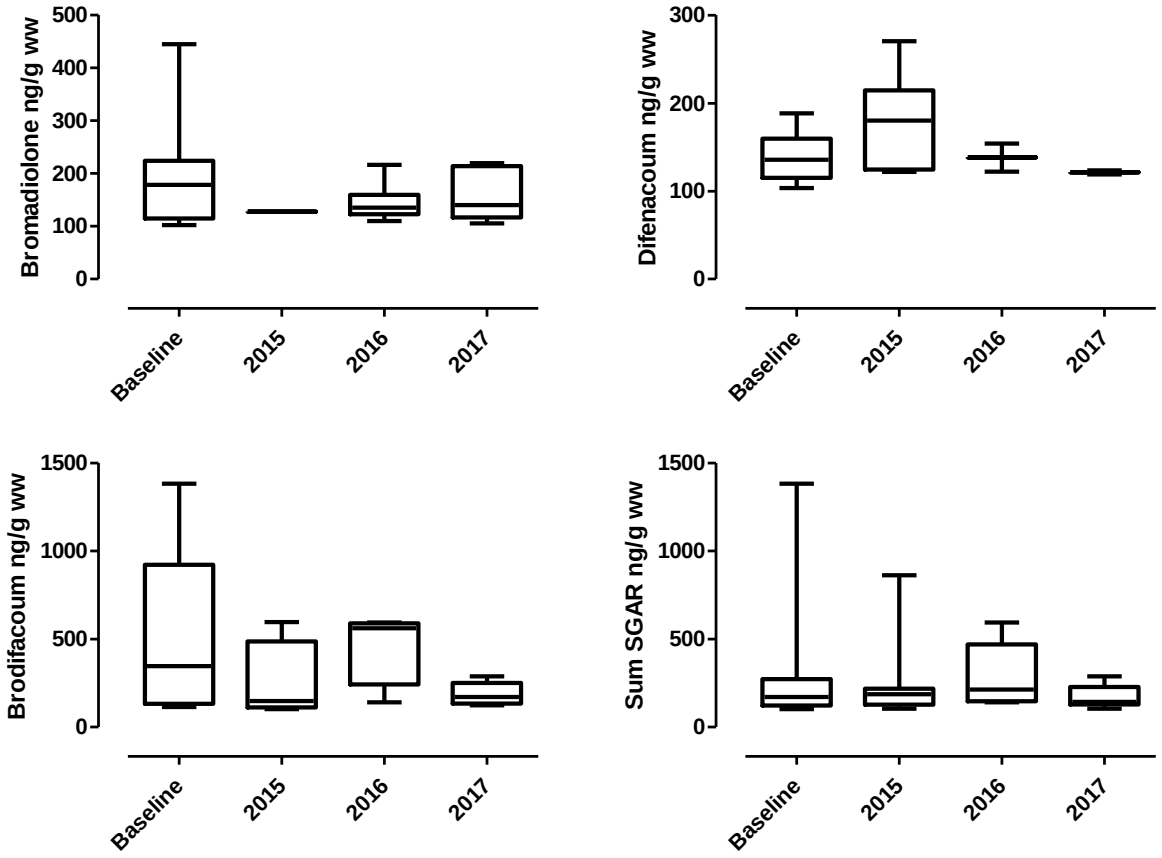

Figure 5. Box and whiskers plot of brodifacoum, difenacoum, bromadiolone and ∑SGARs liver concentrations in the cohort of owls with residues >100 ng/g wet weight ("high" residues) found dead in the 2006-2012, 2015, 2016, and 2017. Horizontal line, box and whiskers represent median, 25-75th quartile range and minimum maximum range, respectively.

5 Discussion

Overall, there were no statistically significant differences in liver SGAR accumulation between barn owls that died in baseline years and those that died in 2017. As in baseline years, the prevalence of residues in barn owls in 2017 remained widespread and most residues (85% for ∑SGARs) were <100 ng/g wet wt. While no statistically significant changes were observed in residue magnitude in barn owls with "low" concentrations, median and 75th percentile values were lower in both 2016 and 2017 than in the baseline years. The inter-year variation in barn owls that had "high" concentrations is less consistent than that observed in barn owls with "low" concentrations. This may be a consequence of the lower sample numbers observed in the "high" residue cohort. The prevalence of flocoumafen and difethialone residues remained low with the proportion of birds with detectable residues in 2016 and 2017 birds being similar to baseline years.

Overall, the lack of significant differences in SGAR accumulation by barn owls in 2017 compared within baseline years suggests that full implementation of stewardship since 2016 has yet to be reflected by a statistically significant reduction in exposure in barn owls, as judged by the metrics used in the current report, although it may be encouraging that "low" residues were generally lower in owls in 2016 and 2017. It may be expected for cultural and ecological reasons that there will be time-lags between implementation of stewardship, change in use patterns, and detection of change in SGAR accumulation in barn owls. Indeed, the likely time-lag for such detection based solely on the variability of residues between barn owls, was highlighted in the report by Shore et al. (2014).

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8 Appendix 1 – Analytical method for determination of SGARs in liver tissues

A sub sample (0.25g) of each liver was thawed, weighed accurately, ground and dried with anhydrous sodium sulfate. Each sample was spiked with labelled standards (d^5- Bromodialone, and d4- Brodifacoum, QMx). Chloroform: acetone (1:1 v/v) was added to each sample and the samples were thoroughly mixed using a vortex.

Samples were extracted on a mechanical shaker (Stuart SF1, Bibby Scientific) for 1h, then centrifuged at 5000 rpm for 5 minutes and the supernatant was transferred to a clean tube. This process was repeated with clean solvent, but the second time, samples were on the mechanical shaker for only 30 minutes. The combined extract was evaporated to dryness using nitrogen, re-dissolved in chloroform : acetone (1:1; v/v) and filtered (O.2 mm PTFE filter). The filtered sample was evaporated to dryness and re – dissolved in acetone: DCM (1:23; v/v).

The sample was re-filtered (0.2mm PFEE filter) and then cleaned using automated size exclusion chromatography (Agilent 1200 HPLC system). The clean extract was evaporated and the residue was re-suspended in chloroform: acetone: acetonitrile (1:1:8; v/v). The extract was further cleaned using solid phase extraction cartridges (ISOLUTE® SI 500mg, 6ml). The cartridges were washed with methanol and activated with acetonitrile. The samples were eluted with acetonitrile and this solvent was then exchanged for the mobile phase.

Analysis was performed using a 'Ultimate 3000' HPLC coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific, Hemel Hemsptead; UK) interfaced with an ion max source in Atmospheric Pressure Chemical Ionisation mode (APCI) with negative polarity and operated with Xcalibur software ™ (V.2.0.7.). Analyte separation (10 µL inj. volume) was performed on a Hypersil Gold column (Thermo, 1.9 µm particle size, 50 mm x 2.1mm I.D.) using a H2O : MeOH mobile phase gradient.

The analytes were eluted from the column using a programme which mixed different ratios of mobile phase A: 0.77g/L Ammonium acetate in water and Mobile phase B: 0.77g/L Ammonium acetate in Methanol at a rate of 0.3 ml min-1. Gradient elution started from 70% A and 30% B, increased to 60% B in 2 min and held until 6 min; it was then ramped to 70% B at 8.5 min and finally to 100% B at 12 min, held for 1 min and then returned to starting conditions.

MS/MS was performed in single reaction mode (SRM) using APCI in the negative mode, and characteristic ion fragments were monitored for each compound. Argon was used as the collision gas. Chromatographic peaks were integrated using Xcalibur™ which was also used to generate linear calibration curves with R2>0.99.

For quality control and assurance, in each batch a blank and in house QC were used. The performance of the method was assessed in terms of the limit of detection (LOD), recovery of the internal standards for the analytes and linearity. The rodenticides standards (Dr

Ehrenstorfer) were matrix matched. Recovery for the total procedure was calculated using the labelled standards.

Limits of detection (LoD) for each compound were 1.5 ng/g wet wt. for all compounds except difethialone that had a LoD of 3.0 ng/g wet wt.. Each liver sample was spiked with deuterated bromadiolone and brodifacoum and mean (± SD) recovery for deuterated bromadiolone and brodifacoum that was added to each of the 100 samples was 58.0±7.6 and 71.3±9.3%, respectively.