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# Second generation anticoagulant rodenticide residues in barn owls 2019

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# 1 Executive Summary

The current report is the fifth in a series of annual reports that describe the monitoring of second generation anticoagulant rodenticide (SGAR) liver residues in barn owls *Tyto alba* in Britain. This work is an element of an overarching monitoring programme undertaken to track the outcomes of stewardship activities associated with the use of anticoagulant rodenticides. The barn owl is used for exposure monitoring as it is considered a sentinel for species that are generalist predators of small mammals in rural areas. The specific work reported here is the measurement of liver SGAR residues in 100 barn owls that died in 2019 in locations across Britain. The residue data are compared with those from 395 barn owls that died between 2006 and 2012 (hereafter termed baseline years), prior to changes in anticoagulant rodenticide (AR) authorisations and onset of stewardship.

As in the baseline years, the compounds detected most frequently in barn owls that died in 2019 were bromadiolone, difenacoum and brodifacoum. Overall, 87% of the owls had detectable liver residues of one or more SGAR.

*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 flocoumafen between the baseline years and 2019. There was a significantly higher proportion of barn owls with detectable liver residues of difethialone in 2019 compared to baseline years (14% vs 0.3% ).

*The ratio of birds with "low" (<100 ng/g ww) vs "high" (>100 ng/g wet wt.) concentrations for any single SGAR or for  $\sum$ SGARs.* There was no significant difference between barn owls from baseline years and from 2019 for any individual compound or for summed SGARs ( $\sum$ SGARs).

*Average concentrations of brodifacoum, difenacoum, bromadiolone and  $\sum$ SGARs in the cohort of owls with "low" residues (<100 ng/g ww) and "high" residues (>100 ng/g ww).* There was no significant difference between barn owls from baseline years and from 2019 in the concentrations of either "low" or "high" residues for all residues summed ( $\sum$ SGARs), low residues of bromadiolone and difenacoum (data tested statistically only for "low residues"), or "high" brodifacoum residues. The median concentration of "low" brodifacoum residues was higher in birds from 2019 than in baseline years.

Overall, there were few differences in liver SGAR accumulation between barn owls that died in baseline years and in 2019. The lack of significant reductions in SGAR residues in barn owls in 2019 suggests that full implementation of stewardship since 2016 has yet to result in a statistically significant reduction in exposure of barn owls to SGARs.

## 2 Introduction

The current report is the fifth in a series of annual reports describing the magnitude of second generation anticoagulant rodenticide (SGAR) liver residues in barn owls *Tyto alba* in Britain. The background to, rationale for, and aims of the study remain unchanged from those described in previous reports. They are repeated here in Sections 2.1-2.3 so that the current report can be read as a stand-alone publication.

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

Avian and mammalian predators and scavengers in rural Britain are widely 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; Sainsbury et al., 2018). Defra's Wildlife Incident Monitoring Scheme (WIIS)<sup>1</sup> and the Predatory Bird Monitoring Scheme (PBMS-<http://pbms.ceh.ac.uk/>) have shown that exposure can lead to some mortalities. 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). 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 historically were the only SGARs licensed for in and around buildings and open area use in Britain) in barn owl livers in past years is consistent with this assumption. However, these SGARs were also the most widely used compounds in Britain and residues in predators may simply reflect predominant usage (Shore, et al., 2015).

The barn owl can be considered as a sentinel for demonstrating exposure to SGARs in generalist predators of small mammals in rural areas in the UK and elsewhere; SGAR residues have been detected in this species in Canada, Denmark, France and Spain (López-Perea & Mateo, 2018). Monitoring of liver SGAR residues in barn owls in Britain has demonstrated increases in exposure largely through the 1980s and 1990s, and current widespread prevalence of residues (Walker, et al., 2014). However, there is no evidence of an associated adverse effect on barn owl populations. Previous declines in barn owl numbers are more likely to have been the indirect consequence of the earlier use of organochlorine pesticides and subsequent changes in the agricultural management of grassland (Smith and Shore, 2015). At the last comprehensive census of the population conducted during the period 1995-97, there was an estimated 4,000 breeding pairs of barn owls in the UK (Toms et al., 2001).

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

More recently, the UK population has been estimated to be in the range 9,000 to 12,000 breeding pairs (Prescott et al., 2019).

## 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. As previously stated, only difenacoum and bromadiolone were historically 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, 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<sup>2</sup> (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 minimise (and reduce from current levels) exposure and risk to non-target species from ARs (Buckle et al., 2017). A 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 follow-up studies were done in 2017 and 2020.
- 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 (see Prescott et al., 2019). This is to examine certain barn owl breeding parameters in the presence of the SGAR residues found in the UK barn owl population (last bullet).
- 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 (Jones et al., 2019; Buckle et al., 2020).

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<sup>2</sup> <https://webcommunities.hse.gov.uk/connect.ti/pesticides/view?objectId=10116>

- SGAR residues in the livers of barn owls from across Britain 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.

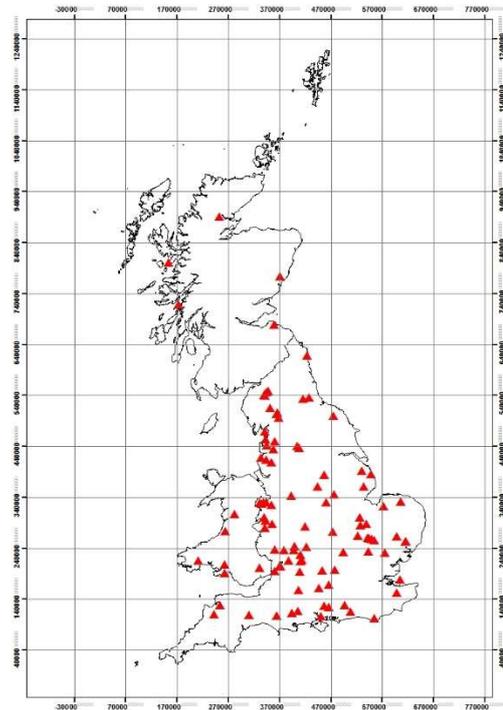
## **2.3 Aims of the current study**

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 is described by Shore et al. (2014). 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 2019. In this report, we compare SGAR residues in a sample of 100 barn owls that died in 2019 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), 2016 (during stewardship implementation), 2017 and 2018 (first and second full year after 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, 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. In all but one bird, any haemorrhaging detected at post-mortem in birds was always associated with signs of trauma and so there was no clear evidence that those individuals had died from anticoagulant rodenticide poisoning. The one remaining bird showed haemorrhaging down the left side of the thorax, as well as sub-cutaneous haemorrhaging and bruising to the musculature. The bird had been found in a field close to an owl nest box and there were no obvious causes of physical trauma. Liver subsamples were analysed for difenacoum, bromadiolone, brodifacoum, flocoumafen and difethialone.



**Figure 1. Provenance of the barn owls that died in 2019 and were analysed for liver SGAR residues**

The composition of the 100 birds collected in 2019 was 30 adults (16 males, 14 females) and 70 first-years (37 males, 33 females); first year birds were individuals hatched in the current or previous year. Overall, the percentage of adults in the 2019 sample was 30% and so within the confidence limits of the baseline dataset (mean: 29.5%, 95% confidence limits: 20.4 – 38.7%). Age has 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. For birds received by the PBMS and not analysed, tissue samples are retained in the PBMS tissue archive.

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  $\mu\text{g/g}$  wet wt. to ng/g wet wt.; for example, 0.1  $\mu\text{g/g}$  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 weight. Mean ( $\pm$  SD) recovery for deuterated bromadiolone and brodifacoum standards that were added to each of the 100 samples was  $79.6 \pm 6.8$  and  $78.4 \pm 5.8\%$ , 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: (i) so called “low” residues which are <100 ng and include non-detected values (assigned a numerical value of zero), and (ii) “high” residues which are >100 ng/g wet weight. These two datasets were analysed separately. This approach was used for liver difenacoum, bromadiolone and brodifacoum residues and for summed concentrations ( $\Sigma$ SGARs); summed residues were 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 was not possible. Change in exposure to each of these two compounds was assessed through comparison of the proportion of birds with detectable residues in baseline and in subsequent years.

Overall, three metrics of change were assessed as per Shore et al. (2014):

- 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,  $\Sigma$ SGARs
- c) Change in “low” and “high” concentrations of brodifacoum, difenacoum, bromadiolone, and summed SGARs ( $\Sigma$ SGARs)

A summary of the proportion of birds with detectable residues of flocoumafen and difethialone in 2018 (metric (a)) is given in Section 4.1. This metric is also given for the other SGARs and for  $\Sigma$ SGARs but for information only. The above metrics for (b) and (c) are reported in sections 4.2 and 4.3, respectively. Comparisons between baseline years and 2018 for the proportions of birds that had detectable residues were by Fisher’s Exact test. Comparisons of liver SGAR concentrations between owls that died in baseline years and in 2019 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 years 2015 to 2019 for information (Figures 3-6). However, time trends were not tested statistically as the data represent only 3-4 years post-implementation of stewardship.

## 4 Results

### 4.1 General summary of liver SGAR residue data for 2019 owls

The presence or absence of liver SGAR residues in barn owls is a relatively crude binary measure of exposure. Therefore, it is not one of the agreed metrics used for assessing the outcomes of stewardship except for flocoumafen and difethialone which occur too infrequently to allow analysis using other statistical tests. However, the simple measure of “% detected” is easy to understand and is therefore presented for all compounds simply for general information.

As in the baseline and subsequent years, the compounds detected most frequently in barn owls that died in 2019 were bromadiolone, difenacoum and brodifacoum. Between 56% and 66% of 2019 owls contained detectable residues of each of these compounds (Table 1). Overall, 87% of owls in 2019 had detectable liver residues of one or more SGAR. The equivalent figure in the baseline years was 81% and it has varied between 78% (2016) and 94% (2015) subsequently (Figure 2). Some 66% of the owls in 2019 had multiple compounds in their livers.

**Table 1.** Proportion of barn owls that died in 2019 and had non-detected and detected liver bromadiolone, difenacoum, brodifacoum,  $\Sigma$ SGARs and multiple SGAR residues.

	Brom <sup>1</sup>	Difen <sup>1</sup>	Brod <sup>1</sup>	$\Sigma$ SGARs	multiple residues
<b>non-detected</b>	34	44	40	13	34
<b>detected</b>	66	56	60	87	66
<b>% detected</b>	66%	56%	60%	87%	66%

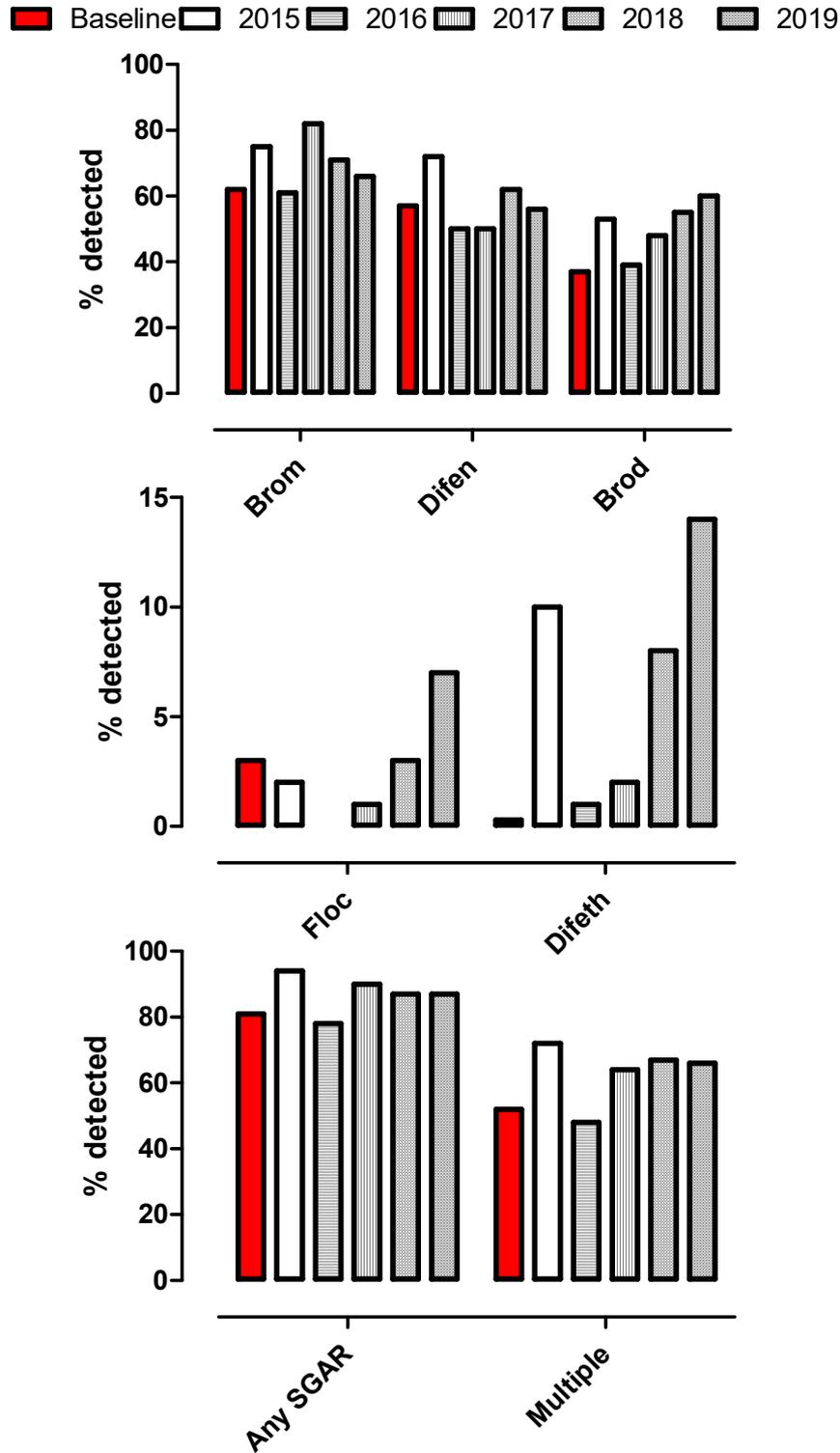
<sup>1</sup> Brom: bromadiolone, Difen: difenacoum, Brod: brodifacoum

One of the metrics for stewardship is the proportion of barn owls with detectable liver flocoumafen or difethialone residues in 2019 compared with in baseline years. There was a significantly higher proportion of birds with detectable liver residues of difethialone in 2019 than in baseline years. There was an increase that was not statistically significant (P-value <0.05) in the prevalence of flocoumafen in birds (Table 2).

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

	Flocoumafen		Difethialone	
	Baseline	2019	Baseline	2019
<b>non-detected</b>	383	93	394	86
<b>detected</b>	12	7	1	14
<b>% Detected</b>	3.0%	7.0%	0.3%	14%
<b>P-value<sup>1</sup></b>	0.080		<0.0001	

<sup>1</sup> P-value determined by Fisher's exact test.



**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  $\Sigma$ SGARs only.

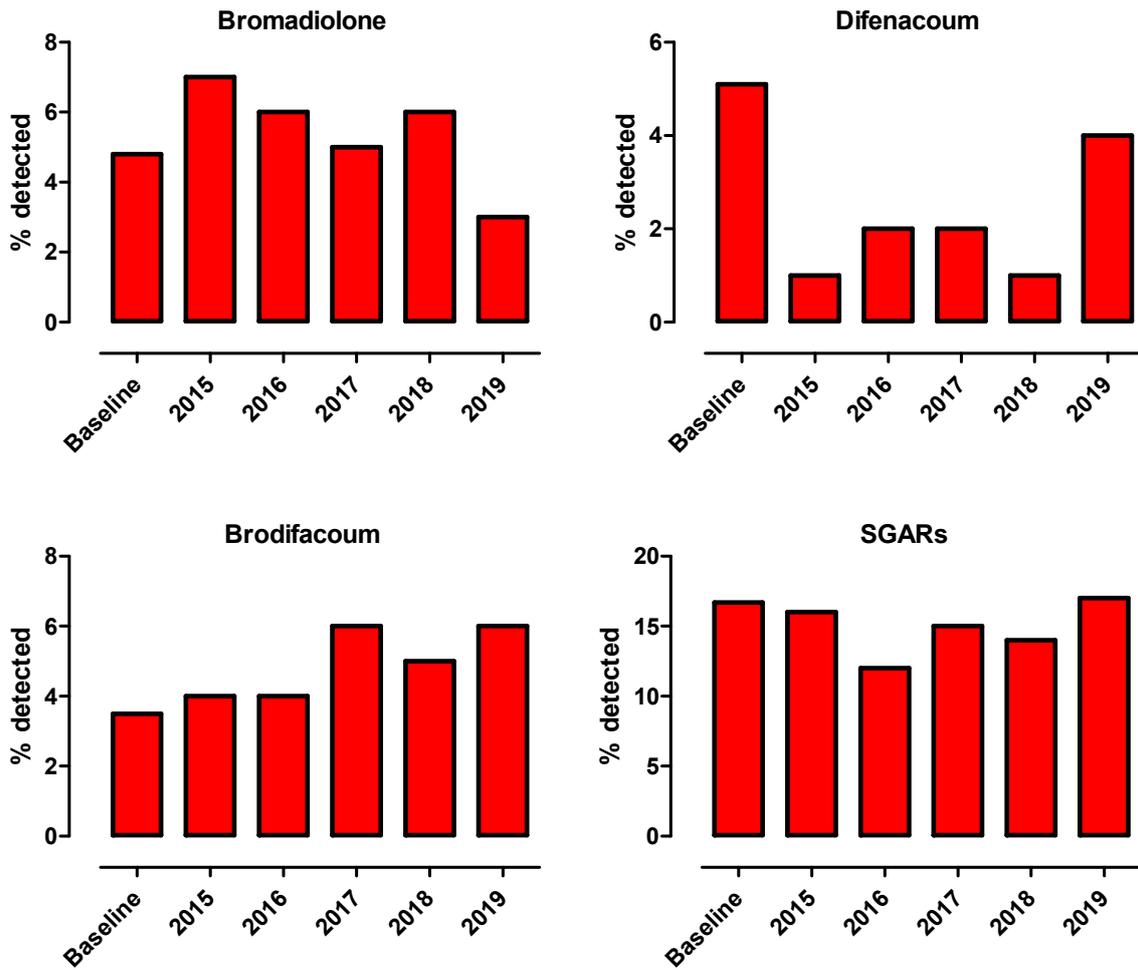
There was no significant difference between barn owls from baseline years and 2019 in the ratio of birds with “low” (<100 ng/g wet wt.) vs “high” (>100 ng/g wet wt.) concentrations for any single SGAR or for  $\Sigma$ SGARs (Table 3 and Figure 3).

The percentages of owls with “high” residues in all six monitoring periods are shown in Figure 3. The values were generally equal to or below 6% for each individual SGAR and below 20% for  $\Sigma$ SGARs.

**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

Conc.	Bromadiolone		Difenacoum		Brodifacoum		$\Sigma$ SGAR	
	Baseline	2019	Baseline	2019	Baseline	2019	Baseline	2019
<100 ng/g “low”	376	97	375	96	381	94	329	83
>100 ng/g “high”	19	3	20	4	14	6	66	17
% high	4.8%	3.0%	5.1%	4.0%	3.5%	6.0%	16.7%	17%
<b>P-value<sup>1</sup></b>	0.591		0.799		0.261		1.000	

<sup>1</sup> 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 $\Sigma$ SGARs in the cohort of owls with residues <100 ng/g wet weight (“low” residues) and >100 ng/g wet weight (“high” residues)

For  $\Sigma$ SGARs, there was no significant difference between barn owls from baseline years and 2019 in the magnitude of either “low” or “high” residues (Tables 4 and 5). This was also true for “low” residues of bromadiolone and difenacoum, while there were too few birds with “high” residues in order to test whether the magnitude of residues were significantly different between baseline years and 2019 for these two active ingredients. For brodifacoum, the “low” residues were significantly higher in birds from 2019 compared with the baseline years, but there was no significant difference between 2019 and baseline years for the magnitude of “high” residues.

Although comparison between the baseline and current year is the metric required for stewardship monitoring, change over years can also be informative and is shown in Figures 4 and 5. There were no striking temporal trends — the 75th percentile and median concentrations for “low” concentrations tended to be lowest in year 2016 and 2017 (Figure 4). The descriptive statistics for “high” concentrations have generally been similar among years (Figure 5).

**Table 4.** Median, 25<sup>th</sup> percentile (Q1), and 75<sup>th</sup> 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.

Conc.		Bromadiolone <sup>2</sup>			Difenacoum <sup>2</sup>			Brodifacoum		
		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
	2019	3.1	0.0	9.4	2.0	0.0	5.6	2.7	0.0	6.6
	MW value <sup>1</sup>	16543			16246			14769		
	P-value	0.146			0.122			0.003		
> 100 ng/g wet wt. (high)	Baseline	179	114	224	136	115	160	347	133	923
	2019	121	-	-	148	-	-	198	122	253
	MW value <sup>1</sup>	-			-			42		
	P-value	-			-			0.967		

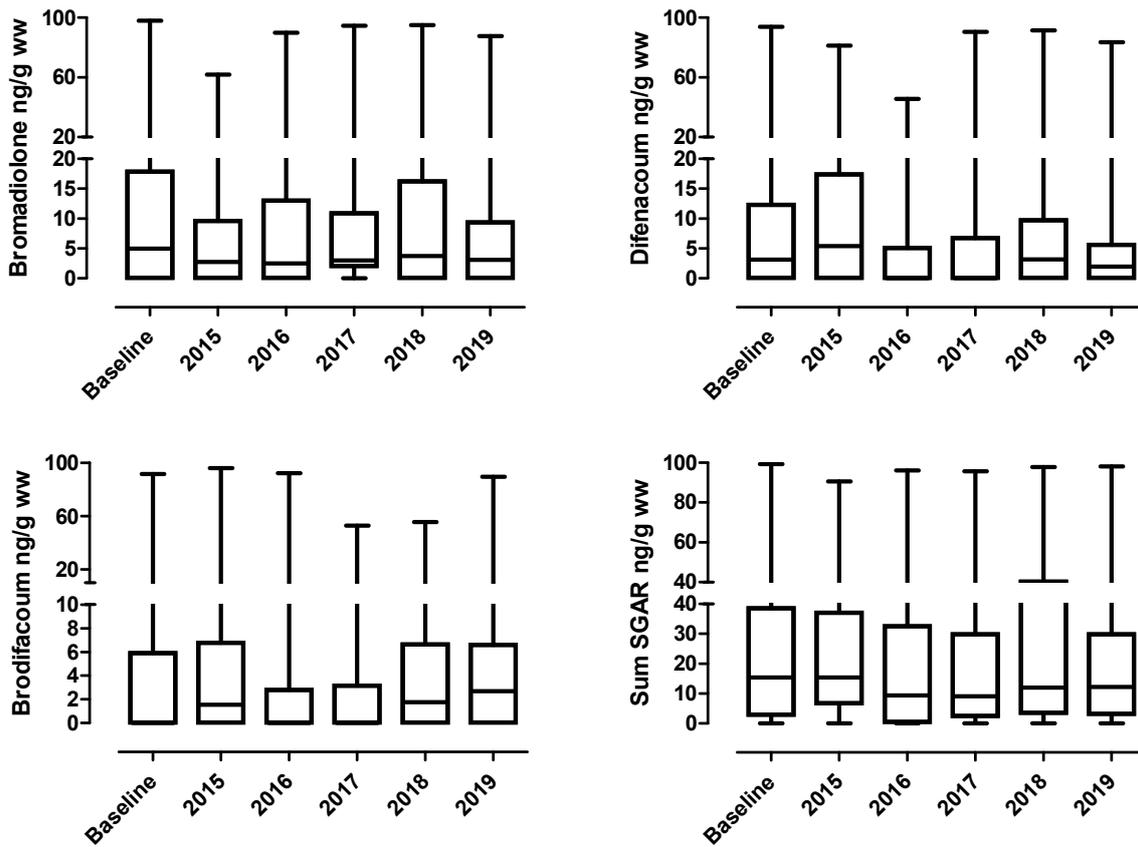
<sup>1</sup> Mann-Whitney U value

<sup>2</sup> Only three and four barn owls had detected “high” residues of bromadiolone and difenacoum, respectively, and so it was not possible to compare between concentrations for the baseline years and 2019.

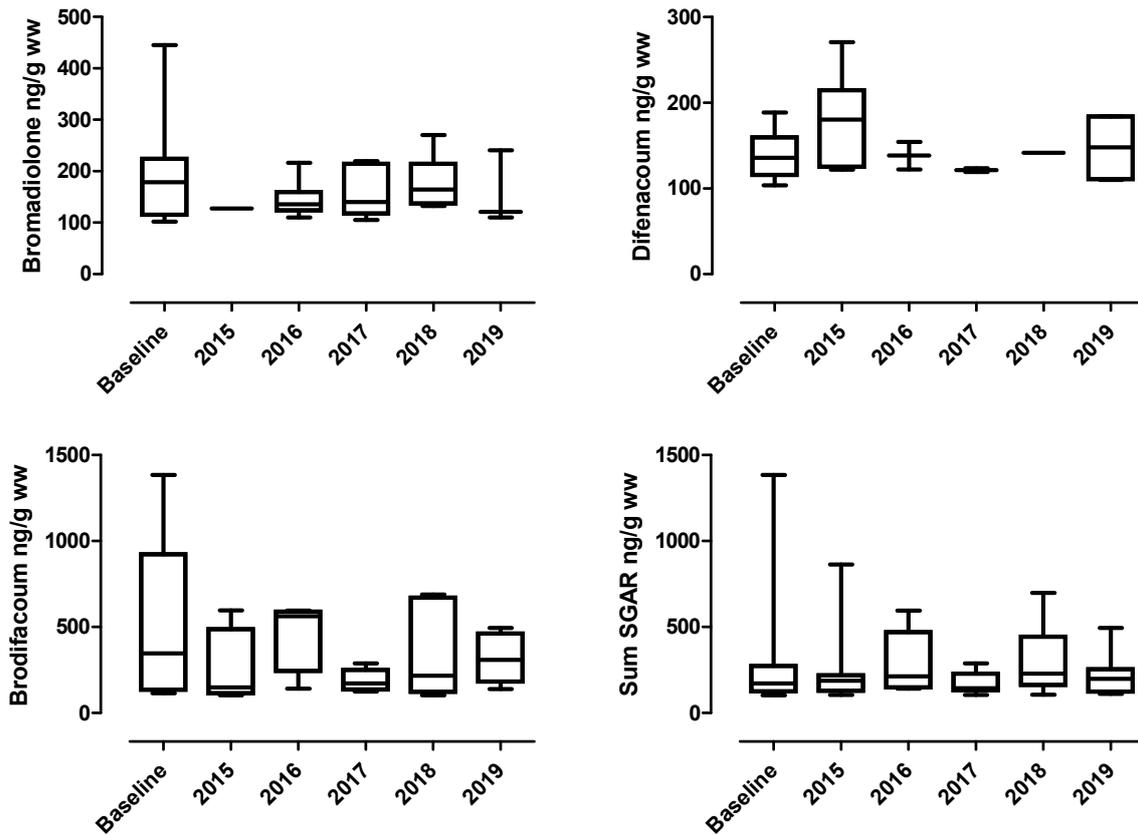
**Table 5.** Median, 25<sup>th</sup> percentile (Q1), and 75<sup>th</sup> percentile (Q3) concentrations (ng/g ww) of  $\Sigma$ SGARs in barn owl livers. Non-detected values were assigned a score of zero. Sample numbers (N) given in Table 3.

<b>Conc.</b>		<b>Sum SGAR</b>		
		Median	Q1	Q3
<b>“Low”</b>	Baseline	15.4	2.8	38.5
	2019	12.2	3.1	29.9
	MW value <sup>1</sup>	12959		
	<i>P-value</i>	0.472		
<b>“High”</b>	Baseline	171	123	272
	2019	198	122	253
	MW value <sup>1</sup>	529		
	<i>P-value</i>	0.722		

<sup>1</sup>Mann-Whitney U value



**Figure 4.** Box and whiskers plot of brodifacoum, difenacoum, bromadiolone and  $\Sigma$ SGARs liver concentrations in the cohort of owls with residues <100 ng/g wet weight (“low” residues) found dead in the 2006-2012, and single years 2016 to 2019. Horizontal line, box and whiskers represent median, 25-75<sup>th</sup> quartile range and minimum maximum range, respectively.



**Figure 5.** Box and whiskers plot of brodifacoum, difenacoum, bromadiolone and  $\Sigma$ SGARs liver concentrations in the cohort of owls with residues >100 ng/g wet weight (“high” residues) found dead in the 2006-2012 (Baseline), and year 2015 to 2019. Horizontal line, box and whiskers represent median, 25-75<sup>th</sup> quartile range and minimum maximum range, respectively.

## 5 Discussion

Overall, there were few differences in liver SGAR metrics between barn owls that died in baseline years and those that died in 2019.

The one bird that showed haemorrhaging at necropsy not associated with signs of trauma had a brodifacoum liver concentration of 494 ng/g wet weight. Given the necropsy observations and the relatively high SGAR concentration in its liver, it is possible to conclude that SGAR exposure contributed to the death of this bird. However this is the only incidence of this kind since monitoring for the rodenticide stewardship began in 2016; 400 birds in total.

As in baseline years, residues were prevalent in barn owls in 2019 but most residues (83% for  $\Sigma$ SGARs) were <100 ng/g wet wt. There were only two statistically significant differences between baseline years and 2019. These was an increase in the prevalence of both difethialone and in the concentrations of brodifacoum in the cohort of owls with residues <100ng/g wet weight (“low” residues”).

The rise in difethialone presence reflects that this SGAR was new to the market in baseline years. Overall, detection rates remain relatively low even in 2019.

The increase in the concentrations of brodifacoum in the “low” residues cohort partly reflected an increase in the proportion of owls with detectable “low” residues compared with baseline (Fisher’s exact test,  $P < 0.0001$ ). This is consistent with there having been a small rise in exposure to this compound when comparing 2019 with baseline years. However, it should be noted that median and the 75th percentile of brodifacoum “low” residues cohort owls have varied during the study period (Figure 4) with no indication of a progressive change over time.

The lack of significant reductions in SGAR residues in barn owls in 2019 suggests that full implementation of stewardship since 2016 has yet to result in a statistically significant reduction in exposure of barn owls to SGARs.

## 6 Acknowledgements

This project was funded by the Campaign for Responsible Rodenticide Use (CRRU) UK.

We thank the Predatory Bird Monitoring Scheme (PBMS) for provision of barn owl livers for analysis. The PBMS collection of owl carcasses was supported by the Natural Environment Research Council award number NE/R016429/1 as part of the UK-SCAPE programme delivering National Capability.

The PBMS is a citizen science project and relies on members of the public to submit bird carcasses to the scheme. Their efforts are key to the success of the PBMS and projects, such as the current one, which are dependent on the samples collected, and we thank all members of the public who have sent in bird carcasses.

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## 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  $d^4$ - 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 (0.2 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<sup>®</sup> 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 Hempstead; UK) interfaced with an ion max source in Atmospheric Pressure Chemical Ionisation mode (APCI) with negative polarity and operated with Xcalibur software<sup>™</sup> (V.2.0.7.). Analyte separation (10  $\mu$ L inj. volume) was performed on a Hypersil Gold column (Thermo, 1.9  $\mu$ m particle size, 50 mm x 2.1mm I.D.) using a H<sub>2</sub>O : 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<sup>-1</sup>. 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<sup>™</sup> which was also used to generate linear calibration curves with  $R^2 > 0.99$ .

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 ( $\pm$  SD) recovery for deuterated bromadiolone and brodifacoum that was added to each of the 100 samples was  $79.6\pm 6.8$  and  $78.4\pm 5.8\%$ , respectively.



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