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Accumulation of Anticoagulant Rodenticides in a Non-target Insectivore, the European hedgehog (*Erinaceus europaeus*)

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Abstract

Studies on exposure of non-targets to anticoagulant rodenticides have largely focussed on predatory birds and mammals; insectivores have rarely been studied. We investigated the exposure of 120 European hedgehogs (*Erinaceus europaeus*) from throughout Britain to first- and second-generation anticoagulant rodenticides (FGARs and SGARs) using high performance liquid chromatography coupled with fluorescence detection (HPLC) and liquid-chromatography mass spectrometry (LCMS). The proportion of hedgehogs with liver SGAR concentrations detected by HPLC was 3-13% per compound, 23% overall. LCMS identified much higher prevalence for difenacoum and bromadiolone, mainly because of greater ability to detect low level contamination. The overall proportion of hedgehogs with LCMS-detected residues was 57.5% (SGARs alone) and 66.7% (FGARs and SGARs combined); 27 (22.5%) hedgehogs contained >1 rodenticide. Exposure of insectivores and predators to anticoagulant rodenticides appears to be similar. The greater sensitivity of LCMS suggests that hitherto exposure of non-targets is likely to have been under-estimated using HPLC techniques.

Keywords: first- and second-generation anticoagulant rodenticide, insectivore, brodifacoum, bromadiolone, difenacoum, flocoumafen, coumatetralyl, warfarin, non-target

Capsule: Exposure of insectivorous hedgehogs to anticoagulant rodenticides in Britain is similar to predatory birds and mammals that specialise in eating small mammals, and hitherto exposure levels have been underestimated using HPLC techniques.

1. Introduction

Globally, rodents destroy or spoil substantial amounts of food intended for human or animal consumption (Singleton et al., 1999; Stenseth et al., 2003). Consequently, a range of methods is employed to reduce rodent density and associated damage. This is most commonly done in developed countries using anticoagulant rodenticides, vitamin K antagonists that prevent the synthesis of functional prothombrin and related blood-clotting factors. Extensive use of first-generation anticoagulant rodenticides (FGARs) during the 1950s, however, led to the evolution of genetic resistance in brown rats (*Rattus norvegicus*), with widespread cross-resistance to other compounds (Cowan et al., 1995; Thijssen, 1995). As a result, more potent second-generation anticoagulant rodenticides (SGARs) were developed which have a greater affinity to binding sites, resulting in greater accumulation, persistence and toxicity (Parmar et al., 1987; Huckle and Warburton, 1986).

Given their mode of action, both FGARs and SGARs are potentially harmful to all vertebrates, and so users are expected to adopt measures that limit direct exposure to non-target species. However, the degree to which these preventive measures are adhered to, particularly by non-professionals, is unknown. For example, in Britain some products are readily available to householders who may be less aware of the risks of non-target poisoning and/or less likely to follow manufacturer's guidelines. Non-target species may also be deliberately poisoned (Barnett et al., 2006).

Most studies investigating indirect exposure of non-target species to anticoagulant rodenticides have focussed on the consumption of poisoned rodents by predatory birds and mammals (Newton et al., 1990, 1999a; Berny et al., 1997;

93 McDonald et al., 1998; Shore et al., 1999, 2003a). However, invertebrates can be a
94 route of contamination for insectivorous vertebrates (Spurr and Drew, 1999) and,
95 although exposure of insectivorous birds has been reported (Borst and Counotte,
96 2002; Dowding et al., 2006), exposure of insectivorous mammals has not been
97 studied. Potential routes of uptake by invertebrates include: the consumption of
98 rodent faeces (Laas et al., 1985; Craddock, 2002; Eason et al., 2002); the
99 consumption of rodent carcasses; ingestion of soil-bound residues by e.g.
100 earthworms; and direct consumption of poison baits (Spurr and Drew, 1999; Dunlevy
101 et al., 2000; Craddock, 2002). Given that many ecological communities typically
102 contain larger numbers of insectivorous vertebrates relative to predators, the
103 contamination of invertebrates potentially poses the greater risk of non-target
104 poisoning in terms of species and individuals.
105 The European hedgehog (*Erinaceus europaeus*) is a medium-sized (0.8 - 1.2 kg)
106 insectivorous mammal distributed throughout Britain and across Western Europe
107 (Morris and Reeve, 2008). Hedgehogs are of particular interest in terms of exposure
108 to anticoagulant rodenticides, as they are reputed to have declined significantly in the
109 last few decades in Britain, and poisoning by industrial chemicals, including
110 rodenticides, may have been a contributory factor (Battersby and Tracking Mammals
111 Partnership, 2005). Our overall aim in this study was to investigate the scale and
112 severity of exposure of hedgehogs throughout Britain to some of the first-generation
113 (warfarin, coumatetralyl) and all of the second-generation (difenacoum,
114 bromadiolone, brodifacoum, flocoumafen) anticoagulant rodenticides that are
115 licensed for use in Britain; the indandione compounds were not determined using the
116 analytical techniques available to us in this study. The current study is the first to
117 assess anticoagulant rodenticide contamination in Britain of species at this trophic

118 level. Furthermore, we analysed tissue residues using both high performance liquid
119 chromatography coupled with fluorescence detection (hereafter HPLC) and liquid-
120 chromatography mass spectrometry (LCMS). To date, characterisation of exposure
121 of non-target species has mostly used HPLC (for example, McDonald et al., 1998;
122 Shore et al., 2003a, 2006a; Walker et al., 2008) but LCMS is potentially a more
123 sensitive technique and, perhaps more importantly, enables compounds with similar
124 chemical structure to be differentiated with greater confidence since identification is
125 based upon mass rather than elution times. Our specific objectives were: to compare
126 and contrast the (i) frequency of occurrence and (ii) average residue magnitude of
127 FGARs and SGARs in hedgehogs by analysing liver concentrations using both HPLC
128 and LCMS techniques; (iii) to determine whether there were differences in levels of
129 contamination between males and females and between geographical regions; and
130 (iv) on the basis of these results, compare the extent of sub-lethal exposure of
131 hedgehogs in Britain with that of predatory birds and mammals, and assess whether
132 hedgehogs are at risk of acute toxicity from their exposure.
133
134 **2. Materials and Methods**
135
136 During 2004-2006, 20 adult hedgehog carcasses were collected from wildlife
137 rehabilitation hospitals from each of six (Scotland, Wales, Midlands and West, South-
138 Western, South-Eastern, and Eastern) of the seven regions of Britain as defined by
139 the Department for Environment, Food and Rural affairs when assessing rodenticide
140 usage (Dawson et al., 2003); we were unable to obtain samples from the remaining
141 region (Northern England). All 120 hedgehogs used in the study had either died
142 following admission or were euthanased due to their injuries or illness.

Each carcass was weighed, sexed and stored at -20^{o}C until dissection, when it was inspected for lesions, injuries or other abnormalities. These observations, along with information collected at admission, were used to determine the cause of death or reason for euthanasia. The whole liver, the primary organ for accumulation of rodenticides (Huckle and Warburton, 1986), was removed, weighed to two decimal places and stored in aluminium foil at -20^{o}C until further analysis.

2.1. Residue analyses

Anticoagulant rodenticide residues were quantified using both HPLC and LCMS. The four main SGARs licensed for use in the UK (brodifacoum, bromadiolone, difenacoum and flocoumafen) were quantified using both techniques. The two most commonly applied FGARs in the UK, coumatetralyl and warfarin (Dawson and Garthwaite, 2004), were also analysed using LCMS only. All reagents were from Rathburn Chemical Co. Ltd, Walkerburn, Scotland and of a grade suitable for HPLC and LCMS analysis.

Extraction procedures for second-generation compounds followed Hunter (1985) and Jones (1996). Samples were analysed in randomised batches of 15. Each liver was defrosted at room temperature and a subsample of approximately 1g (mean wet weight±SE=0.98±0.01g) ground to a homogenous paste using acid-washed furnace-cleaned sand and anhydrous sodium sulphate. A 30ml aliquot of extraction solvent (50:50 acetone/chloroform) was mixed thoroughly with the ground tissue, stood for 1 hour, then decanted and collected in a 100ml measuring cylinder through a funnel containing glass wool and anhydrous sodium sulphate. The ground tissue was subsequently washed with 30ml aliquots of extraction solvent and washings were

added to the original extraction aliquot until a total volume of 100ml was collected. The mixture was mixed by inversion and left to stand at room temperature for a minimum of 12 hours. Subsequently the extract was divided into 50ml for analysis by HPLC and 30ml was archived at 4ºC in the dark for later analysis by LCMS. Both samples were reduced to zero volume by evaporation of solvent in a fume cupboard and the remaining 20ml was poured to waste.

The reduced extract was re-dissolved in 1ml of extract solvent and 4ml acetronitrile and cleaned using an SPE Isolute C_{18} (EC) 1g column (Internation Sorbent Technology, Mid-Glamorgan, UK) connected to an SPE 500-mg NH_2 column solvated with methanol. Columns were conditioned with 5ml methanol followed by 5ml acetronitrile. The re-dissolved extract was loaded onto the C_{18} column and washed with three 5ml aliquots of acetronitrile at <4ml/minute. The C_{18} column was then removed and 4ml ammoniacal methanol was washed through the NH_2 column (flow <4ml/min). The resulting eluant was combined with 5ml methanol, reduced to near dryness (to remove ammonia) and re-dissolved in 0.5ml methanol. Samples were finally transferred to a chromatography vial via a 4mm syringe filter (Whatman International Ltd, Kent, UK).

2.2. High performance liquid chromatography

High performance liquid chromatography (HP Series 1100, Agilent Technologies, Bracknell, Berkshire, UK) was performed using a ODS Hypersil 200mm x 4.6mm 5µm column (Thermo electron corporation, Runcorn, Cheshire, UK) at 30^{o}C. A 15µl aliquot of cleaned-up extract was injected onto the column using 76:24 methanol:water (v/v) supplemented with 0.25% (v/v) acetic acid and 40mM

ammonium acetate, as the mobile phase pumped at 1.0ml/min isocratically. SGARs were detected by fluorescence spectronomy (HP 1100 series fluorescence detector) using three excitation wavelengths (313nm, 320nm and 350nm) simultaneously to allow for correction of co-eluting peaks that interfered with the fluorescence of the rodenticides. The emission for each excitation wavelength was measured at 380nm. The excitation wavelength of 313nm gave the greatest emission signal at 380nm and was thus used for quantification. The ratio the emission response elicited by the 320nm wavelength to that elicited by 313nm and the ratio elicited by 350nm to that elicited by 313nm were both used to aid identification. A chromatographic peak was identified as a specific SGAR if the ratios of the signals for each excitation wavelength matched the ratios in the standards and if the absolute retention time of the peak fell within the retention time window of the calibration standards.

2.3. Liquid chromatography mass spectrometry

The archived extraction samples were cleaned using methods previously outlined and analysed by liquid-chromatography tandem mass spectrometry conducted on a Zorbax Eclipse C18 3µm column (150 x 2mm). The analysis was conducted using an isocratic mobile phase consisting of acetronitrile:water containing 0.1% formic acid in the ratio 75:25 and at a flow rate of 200µl/min. The column was maintained at 35^{o}C; injection volume was set at 15µl. A Surveyor HPLC system (Thermo Corporation, Hemel Hempstead, Hertfordshire, UK) was used to separate the sample and deliver it to an LCQ Duo, API ion trap mass spectrometer (Thermo Corporation, Hemel Hempstead, Hertfordshire, UK).

Analyses were performed using electrospray ionisation in the negative mode. The capillary temperature was set at 270^{o}C with an ionisation voltage of -36.0V. The sheath and auxiliary gasses used were helium and nitrogen maintained at 80psi and 20psi respectively. Sensitivity was increased using single ion monitoring, scanning for the molecular ion of each of the rodenticides. Selectivity and conformational analysis was undertaken using tandem mass spectrometry.

2.4. Quality assurance

Quantification of residues was carried out by comparison with rodenticide standards (Chemservice, Greyhound Chromatography, Merseyside, UK) for all the FGARS and SGARS that were quantified. For HPLC analysis, the linear calibration range was 50-500ng/ml and the limit of detection (LoD) for peaks identified as SGARs was determined from the linear regression of the multilevel calibration using the equation $Y=Y_0+3S_{y/x}$, where Y is the LoD response, Y_0 is the intercept and $S_{y/x}$ is the standard error of the regression line. The HPLC LoDs for bromadiolone, difenacoum, flocoumafen and brodifacoum based on the standards were 0.03, 0.01, 0.01 and 0.02µg respectively, which were analogous to previous analyses of polecat (*Mustela putorius*) livers (Shore et al., 2003a). The LoDs for LCMS were obtained using a similar method and were 0.002µg for all compounds.

For LCMS analysis, three concentrations (100, 50 and 10ng/ml) of the standards for all the FGARs and SGARS were run alongside procedural blanks after every eight samples to determine day-to-day quantitation. Calibration curves were obtained using a range of concentrations (500, 400, 200, 100, 50, 20, 10, 5, 1 and 0.1ng/ml) of

these standards; the average areas of ten determinations of each standard concentration were used to produce these curves.

For both HPLC and LCMS analysis, procedural blanks (reagents only) were analysed alongside samples to detect possible contamination during sample preparation. Chicken liver samples were each spiked with known concentrations of each SGAR and were prepared, stored and analysed in the same way as unknown samples to determine sample matrix recovery and percent recovery data. For HPLC the mean (±SE%) recovery, determined from analyses of eight spiked samples, were 108±11.5%, 81.6±5.0%, 95.2±9.8% and 93.3±9.0% for difenacoum, bromadiolone, flocoumafen and brodifacoum respectively. Corresponding figures for LCMS recovery were 59.2±9.9%, 27.3±12.0%, 59.2±9.9% and 65.9±7.3%, determined from analyses of four samples spiked for each SGAR. The apparently lower recovery associated with LCMS than HPLC may have been an artefact reflecting poor stability of spiked samples when archived. The bromadiolone and difenacoum concentrations in the actual samples of hedgehog livers were not significantly lower when quantified by LCMS than when measured by HPLC (see Results). Concentration data in tissue samples were not recovery-corrected.

2.5. Statistical analysis

The numbers of samples with detectable and non-detectable rodenticide residues as determined by HPLC and LCMS were compared using Fisher's exact tests. Liver concentrations were not normally distributed and average residue concentrations are given as medians. Median liver concentrations in animals with detectable residues were compared using Mann-Whitney U tests. Wilcoxon matched

pairs tests were used to compare residue concentrations detected by the two techniques within the same individual. Binary logistic regression was used to examine the effect of region, batch number and sex on the presence/absence of contamination; batch was included as a factor to confirm that batching samples for analysis did not introduce any analytical biases. All analyses were conducting using SPSS, Release 15.0 (Field, 2005).

3. Results

Reasons cited by wildlife hospitals for admission of the hedgehogs used in this study were: injury (*n*=55); unknown (*n*=46); natural causes (*n*=18); and suspected poisoning (*n*=1), although this diagnosis was not confirmed clinically or chemically. No obvious signs of haemorrhage other than that associated with trauma were found during *post-mortem* examinations (*n*=120).

Using HPLC, detectable liver concentrations of brodifacoum, bromadiolone, difenacoum and flocoumafen were found in four, 13, 16 and zero animals respectively (Table 1); in total, SGARs were detected in 27 individuals (23% of the animals analysed: Table 2). In contrast, SGARs were detected in 69 (57.5%) hedgehogs when the analysis was conducted by LCMS (Table 2). FGARs (only determined by LCMS) were detected in 27 (22.5%) animals (Table 2). Overall, residues of at least one FGAR or SGAR were detected in two thirds of hedgehogs when samples were analysed by LCMS. Fifty-three (44%) individuals had liver residues of one compound; 21 (18%), five (4%) and one (1%) animal contained residues of two, three and four compounds respectively.

290 The greater frequency of detection of SGARs by LCMS than HPLC was largely
291 because more instances of difenacoum and bromadiolone contamination were
292 detected by LCMS (Table 2); the difference in frequency of detection between the
293 analytical methods was significant for difenacoum (Fisher's Exact test, $P<0.001$) and
294 approached significance for bromadiolone (two-tailed Fisher's Exact test, $P=0.10$).
295 Much of this higher frequency of detection was due to the greater sensitivity of the
296 LCMS. Liver difenacoum and bromadiolone concentrations below 0.025µg/g wet
297 weight (ww) and 0.05µg/g ww, respectively, were not detected by HPLC, whereas
298 these concentrations comprised 25-50% of the LCMS detections for these
299 compounds (Fig. 1). Overall, detection of these low level difenacoum and
300 bromadiolone residues by LCMS accounted for an extra 30 hedgehogs (25% of the
301 sample) being identified as containing rodenticide.
302 The average magnitude of residues (Table 3), not just the frequency of
303 occurrence, also varied with analytical technique. When only hedgehogs with HPLC
304 and/or LCMS detectable residues were included in the statistical analysis, the
305 median liver bromadiolone concentration was lower when determined by LCMS than
306 by HPLC (Mann Whitney U test: $U=61.0$, $n_1=23$, $n_2=13$, $P<0.01$; Fig. 2). This reflected
307 the presence of low-level bromadiolone concentrations (typically < 0.1 µg/g ww; Fig.
308 1) that were detected by LCMS but not by HPLC (and so were not included in the
309 HPLC dataset of animals with detected residues). When the statistical analysis was
310 further restricted to a matched pair comparison of just animals with bromadiolone
311 residues detected by *both* analytical methods, there was no significant difference
312 between LCMS and HPLC measurements (Wilcoxon matched pairs test: $n=10$, $Z=-$
313 0.663, $P>0.05$). This again suggested that differences between HPLC- and LCMS-
314 determined measurements were solely due to detection of low-level concentrations

by LCMS. However, this was not true for difenacoum. Median liver concentrations of difenacoum in animals with detectable residues did not differ with the method of determination (U=427.5, n_1=16, n_2=57, P>0.05), despite the presence of a relatively large number of low-level difenacoum residues in the LCMS sample (Fig. 1). This may reflect differential responses (involving enhancement or quenching of response) of the two techniques, as matched-pair analysis indicated that residues were higher in animals when measured by LCMS (n=9, Z=-2.429, P<0.05).

Analyses of potential differences in residue magnitude with sex and region were based on LCMS data. Geographical region was not significantly associated with the presence/absence of (i) FGARs (coumatetralyl and warfarin), (ii) bromadiolone and difenacoum combined (the most commonly found SGARs), (iii) all four SGARs, or (iv) all FGARs and SGARs combined (Table 4). Sex did, however, approach significance in two of the four models (bromadiolone and difenacoum combined, P=0.052; all SGARs, P=0.072; Table 4), with a greater frequency of occurrence of contamination in males than females.

4. Discussion

The major proportion of hedgehog diet consists of invertebrates, particularly molluscs, beetles and earthworms (Wroot, 1984). Invertebrates have different blood-clotting mechanisms to vertebrates and so are less susceptible to anticoagulant rodenticides than birds and mammals (Shirer, 1992; Pain et al., 2000; Craddock, 2002; Johnston et al., 2005). However, ground-dwelling invertebrates can access and feed on rodenticides, including those placed in bait stations (Spurr and Drew, 1999; Dunlevy et al., 2000; Craddock, 2002), and retain ingested compound in their

bodies for four weeks or longer (Booth et al., 2001; Craddock, 2002). Additional exposure of invertebrates to rodenticides may also arise through ingesting contaminated soil (where baits have not been protected or have been displaced or removed from bait stations), rodent food caches and rodent carcasses. Thus, predation of contaminated invertebrates is likely to be a major pathway by which hedgehogs are exposed to anticoagulant rodenticides. However, hedgehogs will consume small mammal carcasses if they are available (Yalden, 1976) and may also access spilt, cached or unprotected baits directly, and these may be alternative secondary and primary exposure routes.

Whatever the route of exposure, it is clear from our results that contamination of hedgehogs with anticoagulant rodenticides is commonplace. These compounds may therefore similarly pose a risk to other species at the same trophic level, such as insectivorous birds (Rammell et al., 1984; Empson and Miskelly, 1999; Robertson and Colbourne, 2001). The frequencies with which we detected SGAR residues by HPLC were towards the mid (brodifacoum, bromadiolone) or low (difenacoum) end of the spectrum documented for predatory birds and mammals in Britain (Table 1), but were comparable in some instances to prevalence rates in species considered to be specialist predators of small mammals, such as the polecat (Shore et al., 2003a), barn owl (*Tyto alba*) (Newton et al., 1999b) and tawny owl (*Strix aluco*) (Walker et al., 2008). Likewise, the magnitudes of residues were also broadly similar to those measured in predatory birds and mammals in Britain (Table 3). Thus, hedgehogs in Britain appear to be at similar risk of exposure and effects from anticoagulant rodenticides as non-target predatory birds and mammals.

Our data also suggest that exposure of hedgehogs is geographically widespread. The absence of any significant difference between the proportion of individuals with

residues and region indicates that the scale of exposure of hedgehogs does not vary markedly across Britain, consistent with studies of polecats (Shore et al., 2003a), even though the apparent use of rodenticides in arable regions varies geographically (Dawson et al., 2003). In part, however, the likelihood of detecting correlated patterns between prevalence rates in animals and regional patterns of use will be affected by exactly which specific compounds are used. This is because compounds, and particularly FGARs and SGARs, differ in their biological half-life and toxicity (Eason et al., 2002). Furthermore, geographical variation in arable use of rodenticides is unlikely to be of relevance to those animals that were from urban areas. There are no published data for rodenticide use in urban areas in Britain and so it is not possible to assess how urban use may relate to exposure of hedgehogs. Finally, our finding that male hedgehogs tended to be more likely to accumulate rodenticides than females may also have a spatial, albeit small scale, explanation. Males have a greater ranging behaviour than females (Reeve, 1994) and this is likely to increase the likelihood of individuals finding baits and contaminated forage.

The overall similarity between hedgehogs and specialist avian and mammalian predators of small mammals was unexpected. This may simply indicate that secondary exposure is more common than previously anticipated for food chains in which small mammals are not a major component. However, this similarity may mask other factors, such as differences in the likely exposure of non-target species in urban and rural areas. We had no information on the exact location in which our hedgehogs were found. Our reliance on analysing the carcasses of animals admitted to wildlife hospitals may have biased the sample towards urban areas because their relatively high human population density may mean that sick/injured hedgehogs are more likely to be found. In contrast, most UK studies on secondary exposure in

predatory birds and mammals have analysed animals that are predominantly from rural areas. It is not clear whether an urban-biased sample would tend to increase or decrease the likelihood of detecting exposure. Rodenticides are widely used on farms in rural Britain but are also commonly used throughout urban and suburban landscapes by both professional practitioners and the general public. The density of baits and contaminated prey relative to population numbers of non-target species in rural and urban areas is completely unknown. Furthermore, it is possible that hedgehogs may be particularly susceptible to exposure in urban areas where untrained domestic users may be prone to unintentional misuse. Animals may also be more likely to suffer traumatic injuries in human-dominated habitats through collisions with motor vehicles or injuries arising from misadventure (Reeve and Huijser, 1999). If such injuries occur independently of levels of rodenticide uptake, such a sample would give a reliable indication of levels of sub-lethal contamination in those areas, but if rodenticide uptake increases the likelihood of injury (Fournier-Chambrillon et al., 2004), then urban samples in particular may over-estimate exposure rates. Comparison of exposure rates of hedgehogs or other species from known urban and rural locations is merited.

The analysis of our sample of hedgehog tissues using LCMS as well as HPLC has shown that exposure, particularly low-level exposure, is markedly underestimated by HPLC. The proportion of hedgehogs exposed to SGARs increased by two- to three-fold when the analysis was conducted by LCMS. We postulate that current estimates of the exposure of predatory birds and mammals to SGARs have been similarly under-estimated where they have been determined using HPLC measurements.

Although exposure of hedgehogs to anticoagulants may be widespread, there is no evidence from our study that this commonly causes lethal poisoning. The *post mortem* examination of the animals in our study did not identify any instances of haemorrhage that appeared consistent with rodenticide poisoning. Although there is no precise liver concentration in hedgehogs or other species that is diagnostic of lethal poisoning, SGAR residues in excess of 0.2µg/g ww are considered to be of concern in barn owls (Newton et al., 1999a) and residues of >1µg/g ww are generally considered to be very high. Irrespective of the measurement technique in our study, the percentage of hedgehogs with summed SGAR residues above 0.2µg/g ww and 1µg/g ww was <11% and <5% respectively. The detection of liver residues exceeding 1µg/g ww suggests that lethal poisoning by rodenticides is likely to occur in some hedgehogs, but the lack of haemorrhaging and relatively low magnitude of most residues suggests that, for animals in our study, contamination with rodenticides was generally not a contributory factor in their admission to wildlife hospitals. Overall, however, poisoning of non-target wild animals by anticoagulant rodenticides is difficult to monitor and studies such as ours may underestimate poisoning events because animals with fatal doses may become lethargic some hours before death and die in cryptic locations (Newton et al., 1999a). Furthermore, there is a general lack of knowledge about whether sub-lethal exposure, as appears to be common in hedgehogs, may be associated with any sub-lethal impacts or an increased susceptibility to toxicity following repeated exposures.

5. Conclusion

This study has shown that the European hedgehog, an insectivorous species, has similar rates of exposure (judged from the proportion of animals with HPLC-

detected liver concentrations and the size of those residues) to those of specialist predators of small mammals. Given that hedgehogs only rarely eat rodents, these results indicate that anticoagulant rodenticides are finding their way into ecosystems via transfer pathways other than through consumption of contaminated rodents. Furthermore, our data indicate that analysis of samples using LCMS can increase the estimate of exposure by two- to three-fold, largely through the detection of low-level residues, and that the use of HPLC may have markedly under-estimated the true scale of exposure of other non-target species to anticoagulant rodenticides.

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Figure legends

Fig. 1. Frequency distribution of bromadiolone and difenacoum liver concentrations in hedgehogs detected by HPLC and LCMS.

Fig. 2. Median and interquartile ranges of liver concentrations of first- and second-generation anticoagulant rodenticides in hedgehogs with detectable residues as quantified using HPLC and LCMS. Sample sizes are given in Table 2.

Table 1

Percentage occurrence of the residues of the first-generation anticoagulant rodenticide coumatetralyl (coum) and the second-generation anticoagulant rodenticides brodifacoum (brod), bromadiolone (brom), difenacoum (difen) and flocoumafen (floc) in the livers of predatory birds and mammals in British wildlife as identified using high performance liquid chromatography. ND indicates residue not detected; - indicates chemical was not investigated

Species	***n***	**Coum**	**Brod**	**Brom**	**Difen**	**Floc**	**Total[a]**	**Ref[b]**
Hedgehog (*Erinaceus europaeus*)	120	-	3.3	10.8	13.3	ND	22.5	1
Polecat (*Mustela putorius*)	100	-	3.0	12.0	22.0	ND	36.0	2
Stoat (*Mustela erminea*)	40	15.0	2.5	6.7	-	-	22.5	3
Weasel (*Mustela nivalis*)	10	30.0	-	10.0	-	-	30.0	3
Red fox (*Vulpes vulpes*)	92	7.6	5.4	26.1	16.3	-	45.7	4
Barn owl (*Tyto alba*)	717	-	3.9	11.0	16.7	1.1	26.1	5
Barn owl (*Tyto alba*)	52	-	5.8	28.8	30.8	ND	42.3	6
Buzzard (*Buteo buteo*)	40	-	2.5	5.0	32.5	2.5	37.5	6
Tawny owl (*Strix aluco*)	172	-	4.7	11.6	5.8	ND	19.2	7
Red kite (*Milvus milvus*)	20	-	-	-	-	-	70.0	8
Kestrel (*Falco tinnunculus*)	36	-	-	-	-	-	67.0	8

[a] Total percentage of individuals positive for one or more chemicals. [b] Reference: 1 - present study; 2 - Shore et al. (2003a); 3- McDonald et al. (1998); 4 - Shore et al. (2003b); 5 - Newton et al. (1999b); 6 - Shore et al. (2006a); 7 - Walker et al. (2008), 8 - Shore et al. (2000); 9 - Shore et al. (2006b).

608 Table 2

609 Number and percentage (out of sample of 120) of hedgehogs with first- (FGAR) and
610 second-generation anticoagulant rodenticides (SGAR) detected using high
611 performance liquid chromatography (HPLC) and liquid-chromatography mass
612 spectrometry (LCMS)

613

	Hedgehogs with residues detected by			
	HPLC		LCMS	
	%	*n*	%	*n*
Coumatetralyl (FGAR)			14.2	17
Warfarin (FGAR)			8.3	10
Brodifacoum (SGAR)	3.3	4	5.0	6
Bromadiolone (SGAR)	10.8	13	19.2	23
Difenacoum (SGAR)	13.3	16	47.5	57
Flocoumafen (SGAR)	0	0	0.8	1
Total SGARs only	22.5	27	57.5	69
Total FGARs and SGARs	-		66.7	80

614 Coumatetralyl and warfarin only determined using LCMS

615

616

Table 3

Mean ± SE (*n*) concentration (µg/g ww) of second-generation anticoagulant rodenticide residues in British wildlife identified using high performance liquid chromatography. Figures are the concentrations only for those animals where residue was detected

Species[a]	*n*	Brodifacoum	Bromadiolone	Difenacoum	Ref[b]
Hedgehog	120	0.05±<0.01 (4)	0.59±0.24 (13)	0.10±0.03 (16)	1
Polecat	50	0.06±0.01 (3)	0.12±0.03 (12)	0.30±0.07 (22)	2
Stoat	9	0.12	0.20±0.10 (3)	-	3
Weasel	3	-	0.25 (1)	-	3
Barn owl	88	0.02±<0.01 (9)	0.09±0.02 (23)	0.03±0.01 (35)	4
Kestrel	40	0.08±0.03 (6)	0.18±0.04 (16)	0.08±0.02 (29)	4
Red kite	8	0.35±0.22 (5)	0.11±0.01 (3)	0.20 (1)	5
Tawny owl	172	0.25±0.14 (8)	0.21±0.05 (20)	0.06±0.02 (10)	6

[a] For Latin names, see Table 1; [b] reference: 1 present study; 2 - Shore et al. (2003a); 3 - McDonald et al. (1998); 4 - Shore et al. (2006b); 5 - Carter and Burn (2000), 6 - RF Shore (unpubl. data).

Table 4

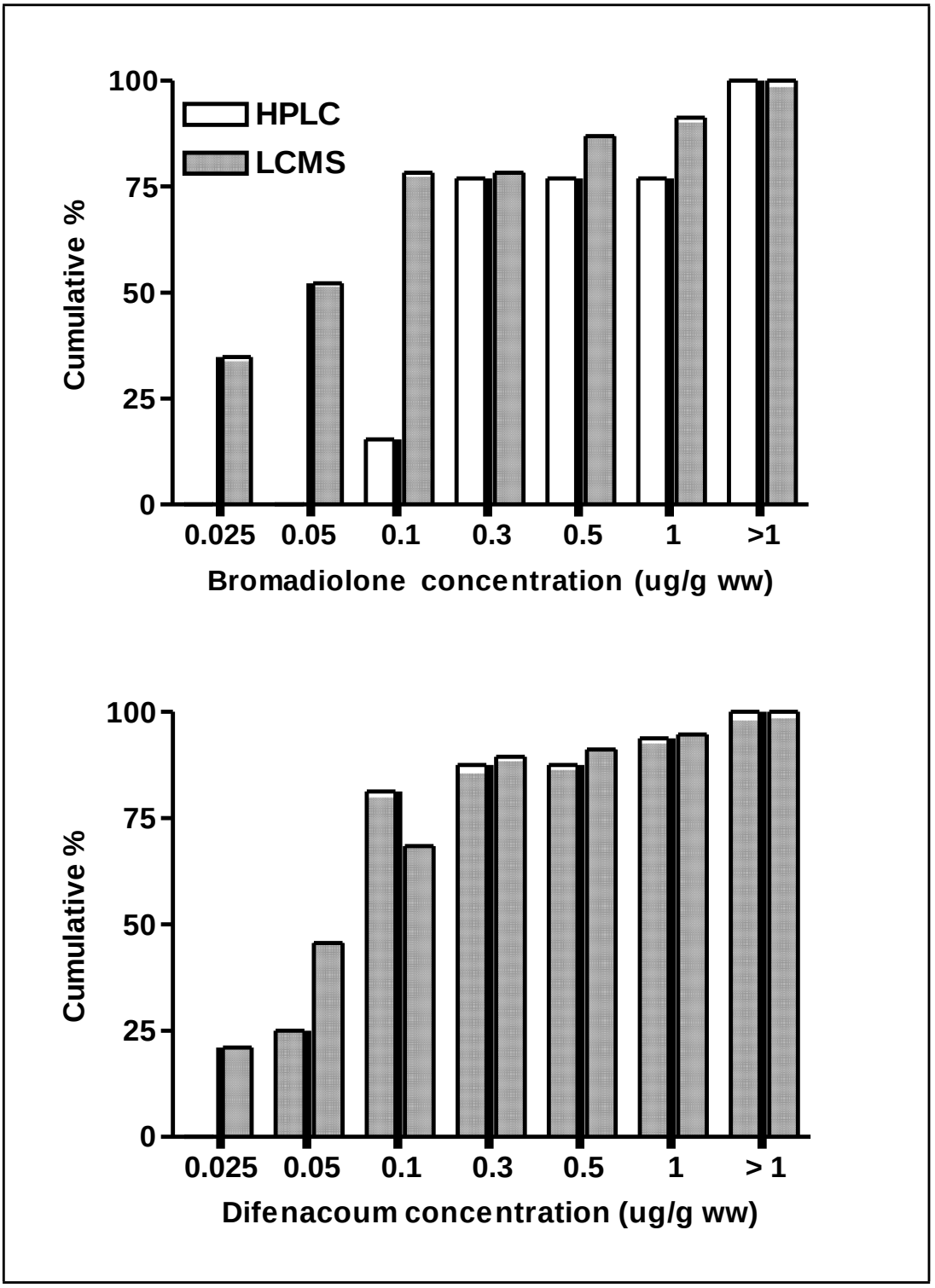
Binary logistic regression models examining the relationship between region, batch and sex and the presence/absence of (a) first-generation anticoagulant rodenticides (coumatetralyl and warfarin), (b) the second-generation anticoagulant rodenticides bromadiolone and difenacoum, (c) all second-generation anticoagulant rodenticides (brodifacoum, bromadiolone, difenacoum and flocoumafen) and (d) all first- and second-generation anticoagulant rodenticides in hedgehogs from across Britain (*n*=120)

Model	**Variable**	***B***	**S.E.**	**Wald**	**d.f.**	***P***
a	Batch			3.426	8	0.905262621204005235451272477297080593

Male:female ratio for hedgehogs from different regions were: South-Eastern 12:8; South-Western 15:5; Eastern 11:9; Midlands and West 8:12; Wales 12:8; Scotland 7:13

639 Fig. 1

640



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642 Fig. 2

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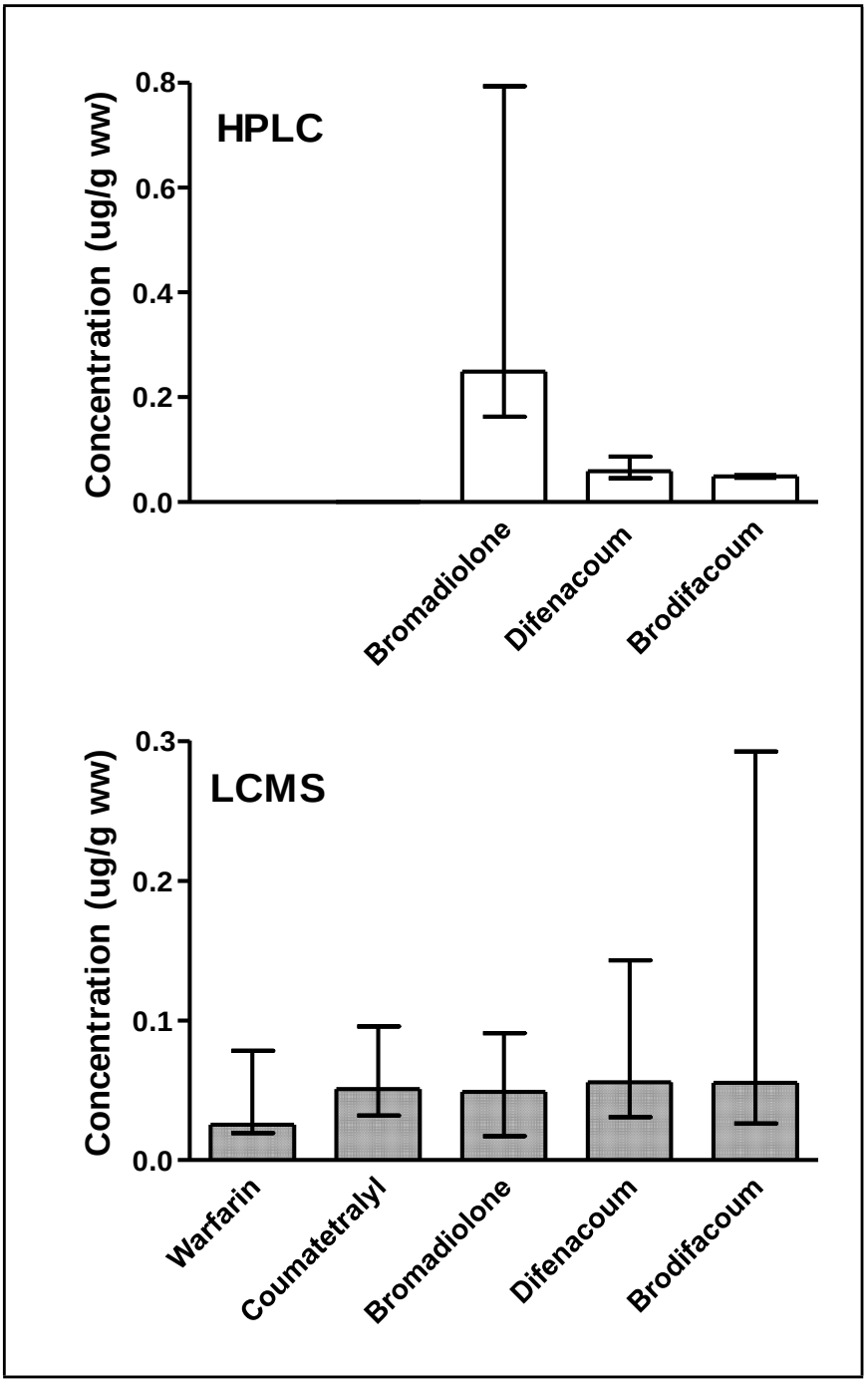

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