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Rate of exposure of a sentinel species, invasive American mink (*Neovison vison*) in Scotland, to anticoagulant rodenticides

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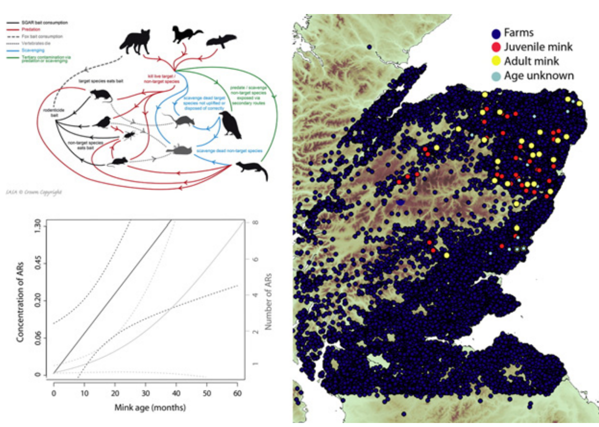
HIGHLIGHTS

- Randomly sampled, aged American mink were tested for anticoagulant rodenticides.
- 78.8% of mink had detectable residues; bromadiolone being the most commonly found.
- The probability of mink exposure to anticoagulants increased by 4.5% per month of age.
- Exposure was 1.7 times higher for mink in areas with a high density of farms.
- American mink are a potential sentinel species for exposure risks across Europe.

GRAPHICAL ABSTRACT



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ABSTRACT

Anticoagulant rodenticides (ARs) are highly toxic compounds that are exclusively used for the control of rodent pests. Despite their defined use, they are nonetheless found in a large number of non-target species indicating widespread penetration of wildlife. Attempts to quantify the scale of problem are complicated by non-random sampling of individuals tested for AR contamination. The American mink (*Neovison vison*) is a wide ranging, non-native, generalist predator that is subject to wide scale control efforts in the UK. Exposure to eight ARs was determined in 99 mink trapped in NE Scotland, most of which were of known age. A high percentage (79%) of the animals had detectable residues of at least one AR, and more than 50% of the positive animals had two or more ARs. The most frequently detected compound was bromadiolone (75% of all animals tested), followed by difenacoum (53% of all mink), coumatetralyl (22%) and brodifacoum (9%). The probability of mink exposure to ARs increased by 4.5% per month of life, and was 1.7 times higher for mink caught in areas with a high, as opposed to a low, density of farms. The number of AR compounds acquired also increased with age and with farm density. No evidence was found for sexual differences in the concentration and number of ARs. The wide niche and dietary overlap of mink with several native carnivore species, and the fact that American mink

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are culled for conservation throughout Europe, suggest that this species may act as a sentinel species, and the application of these data to other native carnivores is discussed.

1. Introduction

Anticoagulant rodenticides (ARs) are the most common type of chemicals used to control rodent pests, and work by blocking the vitamin K cycle, which is essential for the production of blood-clotting factors (Pelfrène, 2010; Stone et al., 1999). Due to the delayed action of ARs, rodents consuming AR bait may accumulate several toxic doses in their liver tissue between the first ingestion of the rodenticide and their death, and as much as 8–10 times the LD50 of some anticoagulants have been recorded in liver tissue at death (Cox and Smith, 1992; Dowding et al., 2010; Sanchez-Barbudo et al., 2012; Smith and Shore, 2015). Furthermore, the development of resistance to ARs in commensal rodents exacerbates this level of toxicity, with resistant rats containing up to 5 times more anticoagulant in their bodies than non-resistant rodents (Smith and Shore, 2015). Due to the development of resistance, there has been a shift in use away from the "first generation anticoagulant rodenticides" (FGARs) in favour of the "second generation anticoagulant rodenticides" (SGARs; Pelfrène, 2010), which are more effective, more toxic and have longer half-lives than FGARs (Eason et al., 2002; Pelfrène, 2010; Stone et al., 1999). For example, reported LD50s (mg active ingredient per kg body weight) for laboratory rodents are 6.2, 2.3 and 3 for chlorophacinone, diphacinone and warfarin (all FGARs) respectively, but are as little as 0.4, 0.55 and 0.7 for brodifacoum, difethialone and bromadiolone (all SGARs) respectively (Erickson and Urban, 2004). In mammals, hepatic half-lives range from 8 days (FGARs) to 307 days (SGARs) (López-Perea et al., 2015).

ARs are typically formulated within a cereal based bait, and any animal that consumes the bait, including non-target species such as granivorous birds (Sanchez-Barbudo et al., 2012) or insects (Godfrey, 1985; Booth et al., 2001; Spurr and Drew, 1999), may act as a source of contamination to other species that predate or scavenge them. Confirmation of the presence of residues of ARs in non-target species has been found in predatory and scavenging raptors (Hughes et al., 2013; Lambert et al., 2007; López-Perea et al., 2015; Ruiz-Suarez et al., 2014; Stone et al., 2003; Walker et al., 2008), and mammals (Dowding et al., 2010; Elmeros et al., 2011; Sanchez-Barbudo et al., 2012; Tosh et al., 2011), but reliable quantification of the extent of wildlife contamination and hence the impact of changes in AR use, are challenging.

Exposure to ARs is commonly monitored opportunistically via samples of dead animals. In Scotland, opportunistic encounters of specific species, most commonly raptors, are submitted into the Wildlife Incident Investigation Scheme (WIIS). Opportunistic sampling may however have limitations in quantifying exposure in wildlife if individuals submitted are unrepresentative of the whole population of the focal species. The carcases submitted may be predisposed towards AR exposure. For instance, starving animals may be more inclined to forage close to areas with high levels of human activity where their encounter rate with poisoned rodents, partly intoxicated or dead, is likely to be much higher than in remote areas, and the chance of someone finding and submitting a carcase is also relatively high. In the US, bird carcase detection has been shown to be approximately twice the rate in urban than in rural areas, and three times more likely to be reported in urban than in rural areas (Ward et al., 2006). Even animals submitted as a result of a road traffic accident (again with a high human encounter rate) might be a biased sample since partially intoxicated individuals could be more vulnerable to collisions with vehicles (Newton et al., 1999). Certainly prey species exposed to ARs exhibit abnormal behaviors which increase their risk of mortality (Smith and Shore, 2015). In North America, fishers with higher residues of ARs suffered higher rates of mortality, suggesting that exposure to ARs may predispose the animal to dying from other causes (Thompson et al., 2014). Furthermore, potentially confounding factors, such as the age and sex of sampled individuals or the geographical provenance and land use, may give a biased perception of the extent of contamination. Thus, where individuals analysed for AR contamination are collected opportunistically, it may be unwise to extrapolate the rate of contamination from the sample to the wider population and estimate the true risk ARs pose. An ideal sampling regime would provide estimates of the contamination risk to species exposed to ARs per unit time that could be reliably compared between e.g. ecosystems, land use types and regulatory regimes.

In northern Scotland, a large-scale participatory project to control invasive non-native American mink (*Neovison vison*) has been underway since 2006, yielding 970 individuals by 2013, collected over an area of 20,000 km^2 (Bryce et al., 2011; Melero et al., 2015). This provided us with an unbiased sample source of a priori healthy carnivore individuals on which exposure to ARs could be analysed. Given the generalist diet of mink (see review in Melero et al., 2014), the species can be considered a sentinel species of exposure to chemicals for other sympatric carnivores species. In the UK, this includes many native carnivores, some fully protected such as otters (*Lutra lutra*; SSI, 2007; Strachan, 2007), badgers (*Meles meles*; PBA, 1992; Rainey et al., 2009), pine martens (*Martes martes*; SSI, 2007; WCA, 1981; Croose et al., 2014), and Scottish wildcats (*Felis silvestris*; SSI, 2007; Kilshaw et al., 2015), as well as non-protected carnivores, such as red foxes (*Vulpes vulpes*), stoats (*Mustela erminea*), weasels (*Mustela nivalis*) and polecats (*Mustela putorius*) (see Harris et al., 2008 for distribution of non-protected carnivores).

In this study we aimed to quantify levels of AR exposure in Scottish wild carnivores using mink as a sentinel species. In addition, we examined potential factors involved in the rate of exposure to ARs, by examining how exposure per unit time varied by land use and the sex of the individuals, and by considering how relationships between mink age and exposure were affected by these covariates.

ARs are commonly used to prevent rodent damage to stored agricultural crops and feed (Hughes et al., 2012, 2014), where they are permitted for use indoors, and for some compounds, for use outdoors away from buildings, for example, to control rats around stacks of hay and straw (Farmers Academy, 2015). Therefore, in terms of land use, the accessibility of mink to farms was expected to be the main environmental covariate affecting mink exposure to ARs via predation of available, contaminated prey. Thus, AR exposure was compared to the connectivity to farms, a metric reflecting the potential influence of these sources of AR weighted by their sizes (number of fields per farm) and their distance to each mink at their capture location. Given that mink are strongly sexually dimorphic, with the smaller females preying upon smaller items than males (Dunstone and Birks, 1987), we also considered whether male and female mink had different levels of exposure. Further, because culled mink were accurately aged, and because of the long half-lives of the SGAR metabolites in particular, we estimated the per time unit rate of accumulation of SGAR via ingestion of contaminated prey (the slope of exposure and age relationships), which we suggest has the potential to serve as a robust metric suitable for multi-site comparisons of the risk ARs pose to predators in the natural environment.

2. Materials and methods

2.1. Sample collection

Liver samples were obtained from necropsies of a total of 99 mink selected amongst 979 that were captured between 2007 and 2013 in rural areas of northern Scotland as part of an invasive non-native species control project (Bryce et al., 2011; graphical abstract). All mink were captured in cage traps typically placed on floating platforms and sacrificed in accordance with the Wildlife & Countryside Act, 1981 and only secondarily used for answering applied ecology research questions (e.g. Melero et al., 2015; Oliver et al., 2016). Guidance to people trapping mink was to use unbaited traps, and rely on the curiosity of the mink to investigate their environment for capture. However, some volunteer trappers used dead rabbit or canned sardines. Given the broad diet of mink (Melero et al., 2008b), neither approach was considered to induce capture bias with respect to likely sources of AR contamination. Whole livers, the primary organ in which anticoagulant rodenticides accumulate (Dowding et al., 2010; Fournier-Chambrillon et al., 2004), were excised and stored at −20 °C until sample preparation and analysis. Place of capture, year of capture and sex were recorded on site or during necropsy. Based on the appearance of dental pulp of canine teeth at X-ray, mink were aged as younger or older than 10 months (Helldin, 1997). Mink less than 10 months old were assumed to have been born the previous May, which is the month of peak births (Dunstone, 1993). Those judged to be older than 10 months were further aged using tooth cementum analyses performed by Matson Laboratory LLC (MT, USA).

2.2. Analytes of interest

The ARs examined in this study include those that have been most commonly used in rodent control activities in the UK: warfarin; coumatetralyl; diphacinone; chlorophacinone (all FGARs), and bromadiolone; difenacoum; flocoumafen, brodifacoum (SGARs). The mink were not screened for difethialone, since ARs containing this active were only available in the UK from July 2011, and approximately 8 mink from the sample were captured after this time.

2.3. Preparation of matrix-matched calibration curves

Standards for ARs were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All standards were certified reference materials (purity ranging from 98% to 99.5%). Stock solutions of individual pesticides were prepared from certified reference material into methanol (≈400 μg/ml) and aliquots taken to compose standard mixtures (5 μg/ml) of warfarin, coumatetralyl, diphacinone, chlorophacinone, bromadiolone, difenacoum, flocoumafen and brodifacoum. From this, an intermediate solution at 0.4 μg/ml was prepared by diluting 2 ml of mix stock to a final volume of 25 ml with methanol. This intermediate solution was used to prepare solvent standards at different concentrations: 0.05 μg/ml, 0.02 μg/ml, 0.004 μg/ml, 0.002 μg/ml; all in methanol.

To prepare rodenticide matrix-matched standards, 2.5 ml of each solvent standard concentration plus 0.25 ml of a concentrate of chicken liver (4 g/ml in methanol) were introduced into a 5 ml volumetric flask with methanol containing 5 mM Di-butylammonium Acetate (DBAA) to obtain the standards at 0.025 μg/ml, 0.01 μg/ml, 0.002 μg/ml and 0.001 μg/ml with a final matrix concentration of 0.2 g/ml. Another mixture of rodenticides was prepared as above, to be used as a confirmation mixture. Both the matrix-matched and the solvent standards were prepared every 7 days to ensure the correct quantification of samples. Linear calibration curves were constructed using QuanLynx software (Waters Corporation, MA, USA), which correlates peak areas and concentration.

An experiment was conducted to check the validity of the chicken liver for preparing matrix-matched calibration curves. All the procedures described above were repeated using residue-free mink liver ($n = 18$) instead of chicken liver, and the quantification was compared to each other. As shown in Fig. 1, the results in both matrices were well correlated (Pearson correlation test; $R^2 = 0.984$, $p < 0.0001$), and thus, the employment of chicken matrix-matched standard curves for the quantification of ARs in mink liver was validated.

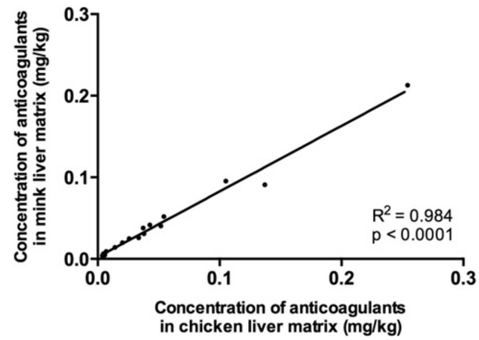

Fig. 1. Correlation of anticoagulant rodenticides in chicken liver and mink liver matrices.

2.4. Sample preparation and clean-up

Liver tissue was finely chopped and a portion (≤4 g) was weighed into a beaker (100 ml); 40 ± 1 mg of solid ascorbic acid was added and mixed thoroughly using a glass rod. Anhydrous sodium sulphate (50 g ± 10 g, adjusted for the weight of the liver sample extracted) was added to absorb moisture. The mixture was left to dry for 20–30 min until friable then transferred into an extraction bottle (250 ml) and 100 ± 10 ml of extraction solvent chloroform/acetone (1:1 v/v, 0.075% ascorbic acid) was added. The bottle was securely capped and placed on a shaker for at least an hour at 145 strokes per minute. The crude extract was filtered off through a Whatman No1 filter paper (18.5 cm) with washings into a round bottom flask (150 ml) and evaporated just to dryness by rotary evaporation (bath temperature not exceeding 40 °C). The dry residue was redissolved in approximately 2 ml of cyclohexane/ethyl acetate (1:1 v/v) and the resulting extract was transferred quantitatively to a volumetric flask (4 ml) and made up to volume with the same solvent mixture.

Automated gel permeation chromatographic (GPC) clean-up was undertaken to enhance recovery, and used a Gilson 233-XL/402 system and Bio-bead SX-3 column (340 × 25 mm). The Bio-bead column was prepared as previously described (Hunter and Sharp, 1988) except that the solvent mixture employed was cyclohexane/ethyl acetate (1:1 v/v). The GPC flow rate used was 5 ml/min.

Liver tissue extracts were filtered through glass fibre syringe filters (25 mm, 1.2 μm) and 2 ml applied to the GPC column (approx. 2 g of extract). The first 70 ml of eluate was discarded, and the next 100 ml collected. The cleaned-up extract was evaporated just to dryness (bath temperature not exceeding 40 °C) and re-dissolved, with the aid of ultrasonication in 5 mM methanolic DBAA solution (10 ml) for analysis by Liquid Chromatography Mass Spectrometry (LC–MS/MS). When sample weight was <4 g, the final volume of 5 mM methanolic DBAA was calculated to maintain the ratio of 0.2 g/ml.

2.5. Chemical analysis

Chromatographic analyses were performed using an Acquity UPLC system coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Waters Corporation, MA, USA). The chromatographic separation was performed using a 50 × 2.1 mm, 1.7 μm analytic column (Waters Acquity UPLC BEH C18) at 35 °C. Mobile phases were (A) water/methanol 95/5 v/v, 5 mM ammonium acetate, and (B) methanol, 5 mM ammonium acetate. The flow was set at 480 μl/min. The volume injection was 5 μl. The total run time was 7 min and the gradient was programmed as follows: min 0, 70% A; min 0.52, 70% A; min 0.66, 40% A; min 1.05, 40% A; min 3.31, 15% A; min 4.90, 15% A; min 5.00, 0% A; min 6.00, 0% A, min 6.05, 70% A; and min 7.00, 70% A.

Retention times of each compound were initially determined in the full scan mode (mass range: m/z 45–600). The time-selected multiple reaction monitoring (MRM) method was constructed by infusion of

Table 1
LC–MS/MS method settings of the anticoagulant rodenticides.

Rodenticide	MRM-screen (m/z → m/z)	Cone voltage (V)	Collision energy (eV)	MRM-confirmation (m/z → m/z)
Warfarin	307 → 161	60	20	307 → 250
Coumatetralyl	291 → 141	40	30	291 → 247
Diphacinone	339 → 167	50	25	339 → 144
Chlorophacinone	373 → 201	40	25	375 → 203
Bromadiolone	525 → 250	80	40	527 → 250
Difenacoum	443 → 293	70	30	443 → 135
Flocoumafen	541 → 161	70	35	541 → 289
Brodifacoum	521 → 135	70	35	521 → 187
	523 → 135	70	35	523 → 187

m/z → m/z: Precursor ion → product ion transitions.

methanolic solutions of pure standards directly into the source. Optimum cone voltage and collision energy values were determined for each analyte. The molecular ion species was identified i.e. [M–H] and selected as the (negative) precursor ion. The precursor ion → product ion transitions listed in Table 1 were used for screening, confirmation and construction of associated calibration curves.

Analyses were performed using electrospray ionization in the negative mode. An interchannel delay of 0.005 s, an interscan time of 0.02 s, dwell time 0.02 s and span corresponding to 0.2 Da were used. Argon of 99.9% purity (BOC Manchester, UK) was used as a collision gas (2.89×10^{-3} mbar cell pressure). A nitrogen generator (Peak Scientific, Renfrew, UK) and a compressor system (Atlas Copco, Cumbernauld, UK) were used to supply nitrogen as the de-solvation, cone and nebulizer gas. These were set at universally applied values of approximately 500 l/h (de-solvation gas flow rate) and 50 l/h (cone gas flow rate). The ion source was operated at 120 °C, the de-solvation temperature held at 500 °C and the capillary voltage was maintained at 0.3 kV. The LC–MS/MS instrument was controlled and the data processed using MassLynx 4.1 and QuanLynx Application Manager software (Waters Corporation (Micromass), Manchester, UK).

The limit of determination (LOD) was set at 0.005 mg/kg for all the ARs. When necessary, the samples were diluted in order to fit within the limits of the calibration curve. Acceptable recoveries fell within the range 60%–140% with the mean being between 70% and 90% at low and high levels. However as recoveries for chlorophacinone and diphacinone were between 20% and 80%, the method is therefore considered qualitative/semi-quantitative for these two compounds. Measured concentrations were not corrected for recovery rates.

2.6. Quality control of AR measurements (QA/QC)

All of the measurements were performed in duplicate, and mean values were used for the calculations. In each batch of samples, two 4-point calibration curves were injected: one at the beginning and the other at the end of the batch. A low-level calibrator (0.002 μg/ml) was included every four samples. Each batch also contained a routine liver matrix sample spiked at high-level (0.1 mg/kg) processed at the same time as samples, including GPC clean-up. In addition, a liver matrix sample spiked at low-level (0.02 mg/kg) was included with every fourth batch analysed. Two blanks were also included in each batch of samples i.e. a reagent blank, containing 100% methanol, and a matrix blank (procedural blank). The results were considered to be acceptable when the quantification of the analytes in the QC was within 40% of the deviation of the theoretical value.

2.7. Statistical analyses

Two variables were defined to quantify the rate of contamination in mink: the concentration and the cumulative number of rodenticides in mink liver, each likely to reflect the ingestion of contaminated prey. Concentration was defined as the total concentration of all rodenticides ($\sum$AR) measured as mg/kg and then rank-ordered to control for overdispersion. The cumulative number of rodenticides was the sum of the different AR compounds found per mink ($\sum N_{AR}$).

To test the hypotheses of sexual dimorphism in feeding habits and the bio-accumulation of ARs with age, we tested the effects of sex and age (in months) on the concentration and the cumulative number of rodenticides. To test the effect of the availability of potentially contaminated prey from farms, we used the connectivity index S^F following Hanski and Thomas (1994), defined as:

$$S_i^F = \sum_{j=1}^{n} \exp\left(-\frac{d_{ij}}{d'}\right)A_j$$

where S^F is the connectivity of each mink i to the surrounding matrix (sum) of all farm holdings j, d_{ij} is the distance (km) between each mink i to each farm holding j, and A_j is the size of the farm, defined as the number of fields per farm holding j. Connectivity increases with the number of fields within a farm but decreases exponentially with the distance to the farms weighted by the parameter d' (also known as $\alpha = 1 / d'$), which reflects the mobility of mink. Values of d' indicate the size of the farmed area that influences AR contamination of mink, reflecting the scale of mink foraging. We estimated the value of d' based on a profile likelihood approach whereby models for the concentration and the cumulative number of rodenticides, analysed independently as response variables (see below), were iteratively fitted to the data using values of S^F estimated using a range of values for d' (1–120 km) chosen to best reflect mink movements (Oliver et al., 2016). The most likely value of d' was obtained by the model with the lowest model deviance value. Values of d' were then back-transformed to actual distances (i.e. applying $-\ln(1 / d')$).

All statistical analyses were performed using generalised linear models (GLMs). The ranked concentration of ARs was fitted to a Gaussian distribution. The cumulative number of ARs was fitted to a Poisson distribution with a log link. For all models the null hypotheses were that there were no differences between the estimates of the covariates and the baseline factorial category (i.e. female = 0 as the intercept). For each analysis, a global model was first defined and model selection was conducted by sequentially dropping non-standardised covariates based on AIC (Akaike, 1973; Burnham and Anderson, 1998). Model averaging and estimates weighting across the most likely models (ΔAIC < 1) were used to incorporate model uncertainty in the parameter estimates using the R package MuMIn (Bartoń, 2014). Analyses were carried out in R 3.2.0 using package lme4 (Bates et al., 2014).

3. Results

Of the 99 animals sampled in this study, 54 were captured in the period 2007–2008; 15 in the period 2009–2010; and 30 in the period 2011–2012. Forty eight percent ($n = 45$) were male and 52% ($n = 54$) were female. Most mink were juveniles (less than 10 months of age, $n = 57$; median age = 6 months old, average = 10.83, range = 2 to 59). Mink were tested for exposure to a total of 8 AR (4 FGARs and 4 SGARs), with 79% ($n = 78$) of the animals exhibiting detectable residues of at least one of these compounds in their livers; 56% with two or more compounds; 21% with 3 or more, and 5% with 4 compounds (average 1.56 compounds, range 0–4 for the whole sample). The most common SGARs found were bromadiolone and difenacoum, one or both being present in all mink with residues ($n = 77$) with the exception of one animal, which contained only brodifacoum. The single-feed, more toxic SGARs (brodifacoum, flocoumafen) were found in 10% of mink ($n = 9$). Coumatetralyl was the only FGAR detected ($n = 22$), but was only found in liver samples that also contained at least one SGAR. The average concentration of AR across all animals sampled ($n = 99$) was 0.23 mg/kg (median = 0.11, p25th–p75th = 0.009 and 0.357 mg/kg, respectively), with almost 50% of positive cases

($n = 37$) exhibiting levels of $\sum$ AR above a reported toxicity threshold of 0.20 mg/kg (Table 2).

The concentration of ARs increased by 4.5% per month of life (Fig. 2a), and the rate of accumulation was 1.7 times higher in locations with proportionally more farms (third quartile of connectivity) relative to those in areas with fewer farms (first quartile of connectivity), due to the connectivity–age interaction (Table 3). Male and female mink had similar contamination rates irrespective of age (additive effect of ARs and age sex interaction were non-significant; Tables 3 and 4).

The influence of farm connectivity was best explained 2 km away (0–3 km 95% CI) from the source of contamination (hence, within each mink's territory; best $d' = 8$; 0.5–18, 95% CI; Fig. 3). The model predicted that by the time mink reached 20 months of age, most contained at least 0.2 mg/kg ARs in their livers (Fig. 2a).

The cumulative number of ARs present in mink also increased with age and connectivity to farm (best $d' = 4$; 0.5–7 km 95% CI; Fig. 2b, Table 3b). The model predicted that most mink were contaminated with at least two compounds at 24 months of age (Fig. 2b). The accumulation of ARs was slower for males; although the difference was not significant. There was no evidence of any asymptote in the number of ARs encountered in a mink lifespan over the range of mink age available (Fig. 2b, Table 3b), although clearly there are only a limited number of ARs to which mink may be exposed, and the confidence intervals for this statistic are large, suggesting that some older animals may only be exposed to a single AR.

4. Discussion

Unlike most other studies that either do not provide age data, or use broad age categories, the accurate ageing of mink in this study allowed us to model AR exposure with age, along with sex, and proximity to farms at known densities. Overall, 79% of mink ($n = 99$) culled in northern Scotland exhibited detectable residues of AR compounds in their livers; with over half exposed to two or more compounds, and a fifth to three or more compounds. Mink were increasingly likely to have acquired ARs as they aged, with virtually all mink of two years of age contaminated to 0.2 mg/kg, which corresponds to a previously reported potential toxicity threshold (Grolleau et al., 1989; Newton et al., 1999). Mink living in the more densely farmed area accumulated AR at the rate of 4.5% per month, which was significantly higher than 2.5% in the least intensely farmed parts of the study area. The monthly rate of acquisition of ARs by mink was significantly related to connectivity to farm holdings, our chosen measure of the intensity of farming activities in the locality of sampled mink. Our estimates of connectivity best predicting the concentration were relatively precise (0–3 km 95% CI), indicating that the most influential farms as source of ARs were at

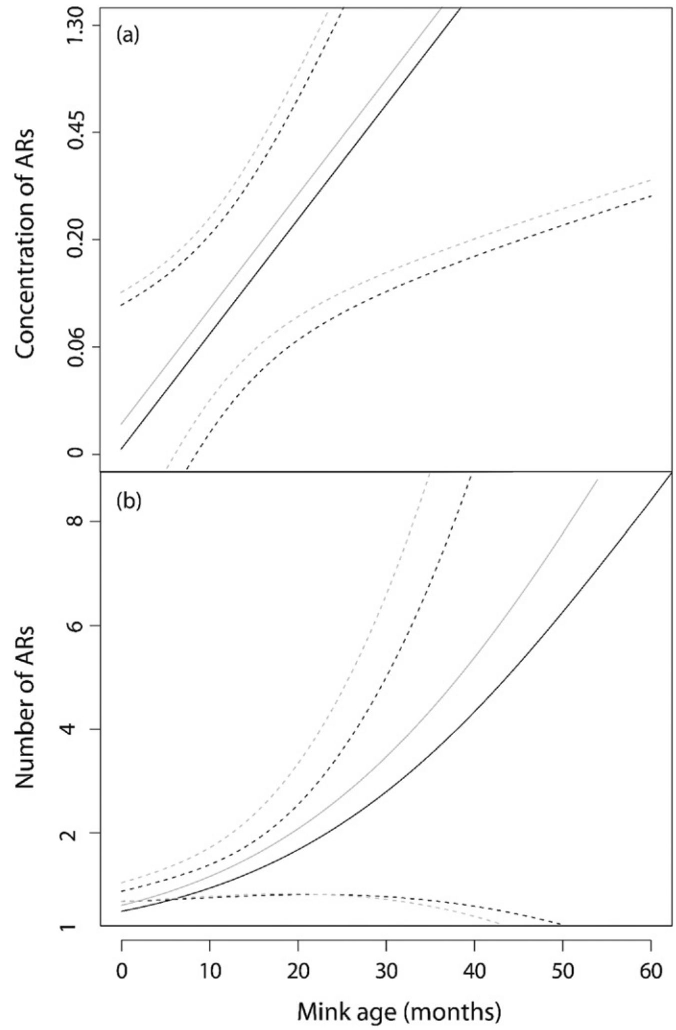

Fig. 2. Model predictions for (a) the concentration of ARs, and (b) the cumulative number of ARs in mink in relation to mink age (in months) for males (black) and females (grey), keeping connectivity at its median values. Continuous lines relate to the estimated fit of the best model weighted for models within the best values of d' (8 and 4; dashed lines denote the 95% CIs).

≤2 km, therefore within mink home range and foraging distances (Zuberogoitia et al., 2006; Melero et al., 2008a). The interaction between age and farm connectivity strongly suggests that farming practices

Table 2
Liver concentration (mg/kg) of different anticoagulant active ingredients in all mink and mink with detectable residues.

Compound	%	All mink sampled $n = 99$		Mink with detectable AR residues $n = 37$	
		Mean ± SD	Median (range)	Mean ± SD	Median (range)
FGAR					
Warfarin	0.0	NA	NA	NA	NA
Chlorophacinone	0.0	NA	NA	NA	NA
Coumatetralyl	22.2	0.015 ± 0.050	<LOD (<LOD – 0.300)	0.067 ± 0.089	0.026 (0.004–0.300)
Diphacinone	0.0	NA	NA	NA	NA
Multi-feed SGAR					
Bromadiolone	74.7	0.186 ± 0.251	0.063 (<LOD – 1.296)	0.249 ± 0.263	0.141 (0.006–1.296)
Difenacoum	52.5	0.022 ± 0.049	0.004 (<LOD – 0.315)	0.042 ± 0.062	0.016 (0.003–0.315)
Single feed SGAR					
Brodifacoum	9.1	0.003 ± 0.018	<LOD (<LOD – 0.171)	0.038 ± 0.054	0.015 (0.004–0.171)
Flocoumafen	2.0	0.0002 ± 0.002	<LOD (<LOD – 0.017)	0.012 ± 0.008	0.012 (0.006–0.017)
ΣARs	77.8	0.227 ± 0.276	0.110 (<LOD – 1.296)	0.288 ± 0.281	0.194 (0.004–1.296)

AR: anticoagulant rodenticide; FGAR: first generation anticoagulant rodenticide; SGAR: second generation AR; ΣARs: sum of all ARs; <LOD: below limits of determination; NA: not applicable.

Table 3
Parameter estimates of the effect of covariates with their associated standard errors for variables included in the best model weighted for the models including the best values of d' for (a) the concentration of ARs, and (b) the cumulative number of ARs.

Parameter	Estimate	SE	Z	p-Value
(a) Concentration of ARs				
Age	4.5	1.82	2.51	0.01
S^F	1.58	0.0001	2.27	0.02
Sex	−4.65	5.61	−0.82	0.40
Age ∗ S^F	−0.0001	5e−5	−2.41	0.01
(b) Cumulative number of ARs				
Age	0.06	0.03	2.35	0.02
S^F	0.0002	5e−5	2.83	<0.001
Sex	−0.01	0.02	−0.94	0.35
Age ∗ S^F	−8e−6	4e−6	−2.38	0.02

S^F: connectivity of mink to the surrounding farm holding matrix.

represent a major source of contamination of ARs in this species. AR residues in foxes have also been positively associated with farming practices in Germany; specifically with livestock (pig) densities (Geduhn et al., 2015). Although the current analysis did not test the relationship between total ARs, and occurrence or distribution of the different farming sectors, in 2014, 57% of Scotland's pigs were reared in parts of northern Scotland where many of the sampled mink were trapped (see graphical abstract; http://www.gov.scot/Topics/Statistics/Browse/Agriculture-Fisheries/agritopics/Pigs). Our analyses do not rule out the influence of other contributors to the rate of contamination, since even mink caught in areas with low farm density were contaminated. The contribution of rodent control by gamekeepers, where rats are a significant pest of game rearing activities should be assessed in future studies (Mcdonald and Harris, 2000; Sánchez-García et al., 2015). Also, urban sources of ARs will arise from sewer baiting of rats by local authorities, and use in domestic and industrial circumstances to control ingress of commensal rodents (Battersby et al., 2002).

Although livers were tested for eight different ARs, only five compounds were found, with bromadiolone being the most frequently found (75% of all animals tested). Difenacoum was the second most frequently detected AR (53%) in the mink, followed by coumatetralyl (22%), brodifacoum (9%) and flocoumafen (2%). The pattern of AR exposure, with comparatively low exposure to the FGARs and especially the single-feed SGARS, reflects the known usage patterns of ARs on arable, grassland and fodder crop farms (that tend to support grazing livestock) over broadly the same time period that mink were collected (Hughes et al., 2012; Hughes et al., 2014). In this study, mink were contaminated on average with two distinct AR compounds by two years of age, and that acquisition of different AR compounds was best explained by farm connectivity across 0.5–7 km (95% CI), more than twice the maximum distance over which generalised AR contamination took place. Since commercial products are not manufactured with combinations of different ARs (López-Perea et al., 2015), this distance might be explained by farmers favouring a particular product with a specific AR compound, and mink travelling further distances, covering more individual farms, are more likely to acquire multiple AR compounds.

In this study samples were taken from apparently healthy mink that were trapped and culled, rather than a potentially biased sample of opportunistically collected carnivores, that might have included individuals found dead, moribund or road-killed. The expected direction of any bias arising in opportunistic samples might be to overestimate true contamination rates. Despite this, and worryingly, the observed accumulated concentration of AR contamination in seemingly healthy animals in this study was higher than those reported in other mustelids in the UK before 2000, and higher than the concentration reported in some other European countries. For example, 36.0% ($n = 50$) of European polecats (*M. putorius*) which had been killed on roads in Wales and England, 30.0% ($n = 10$) of weasels (*M. nivalis*) and 22.5% ($n = 45$) of stoats (*M. erminea*) that had been killed by gamekeepers on shooting estates in England sampled in 1996/97, were reported to have detectable amounts of ARs in their livers (McDonald et al., 1998; Shore et al., 2003). Unfortunately, non-standardised sampling between studies precludes establishing whether this reflects a high degree of penetration of these chemicals in the trophic chain in Scotland compared with the rest of the UK, or changing patterns of AR use over time. The latter option is supported by a recent study of foxes (shot, and opportunistically sampled from road kills) collected from across the UK (n: Northern Ireland = 155; England & Wales = 29; Scotland = 44), which suggested similar levels of contamination in Scotland as the rest of the UK (Tosh et al., 2011). However, only 15% of trapped American mink ($n = 47$) and 10% of Eurasian otters opportunistically collected ($n = 11$; 20 respectively) were exposed to ARs in France (Fournier-Chambrillon et al., 2004; Lemarchand et al., 2010); while 39% of Scottish otters ($n = 23$) were found exposed to ARs between 2004 and 2015 (EA Sharp, SASA, pers. comm.; unpublished data). Whether the high contamination rate observed in Scottish mink reflect real differences or merely sampling effect caused by e.g. differences in the age of individuals, and hence the length of exposure, of the typically small number of individuals sampled is not known. Furthermore, differences in assay methodology and sensitivity (limits of detection, recovery of compound, and whether or not corrections for recovery are applied) mean that at present, comparisons between contemporary and older studies should be treated with some caution (Shore et al., 2015). Using assay sensitivities broadly comparable with those of the current study, it is reported that 97% ($n = 61$) of stoats and 93% ($n = 69$) of weasels collected opportunistically in Denmark, and 78% ($n = 58$) of American fishers (*Martes pennanti*) in California that had been trapped, radiotagged and carcases collected if later found dead, contained ARs residues (Elmeros et al., 2011; Gabriel et al., 2012). These degrees of exposure are comparable

Table 4
Model selection based on AIC and ΔAIC for (a) the concentration of ARs and (b) the cumulative number of rodenticides ARs in mink. Best models are marked in bold.

Rank	Covariates	df	AIC	ΔAIC
(a) Concentration of ARs				
1	**Age ∗ S^F + sex**	**4, 86**	**859.00**	**0**
2	Age ∗ S^F ∗ sex	7, 83	861.27	2.73
3	Age + S^F + sex	3, 87	863.00	4
(b) Cumulative number of ARs				
1	**Age ∗ S^F + sex**	**90**	**282.23**	**0**
2	Age + S^F + sex	90	286.68	4.45
3	Age ∗ S^F ∗ sex	90	286.69	4.46

S^F: connectivity of mink to the surrounding farm holding matrix.

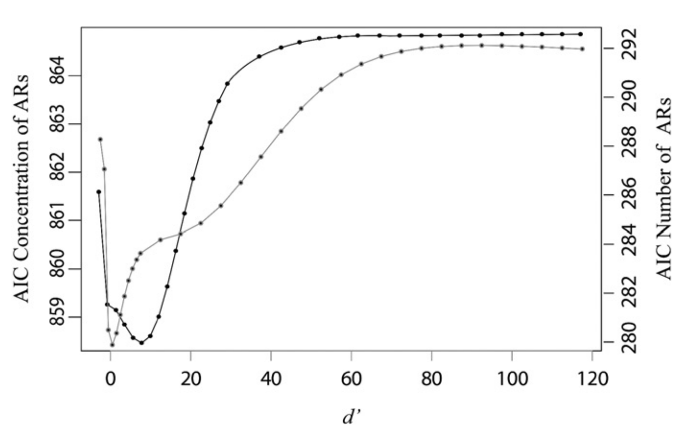

Fig. 3. AIC profile obtained by fitting the model of the concentration of ARs (black), and the cumulative number of ARs (grey) in relation to the connectivity (S^F) data estimated across values of the parameter d' between 0 (i.e. capture location) and 120 km. The lowest AIC values indicate the best models, leading to $d' = 8$ and $d' = 4$ for the concentration and the number of ARs respectively.

with that found in stoats (100%, $n = 11$ and 85%, $n = 115$) after intensive rodent eradication operations using broadcast baiting methods in New Zealand (Alterio et al., 1997; Eason et al., 2002 respectively).

Almost 50% of the positive cases ($n = 37$) detected residues above a previously reported potential toxicity threshold (0.20 mg/kg), which has been associated with mortalities in mustelids and other mammals (Grolleau et al., 1989; Berny et al., 1997; Newton et al., 1999). Evidence of toxicity is generally based on macroscopic haemorrhaging, which cannot be accounted for as physical trauma injuries, but is associated with relatively high concentrations of hepatic ARs. Bromadiolone poisoned stoats were associated with liver concentrations of 0.23 mg/kg (Grolleau et al., 1989), whereas Fournier-Chambrillon et al. (2004) found that hepatic bromadiolone residues of 0.7 mg/kg were responsible for AR poisoning in two polecats and a mink. In another study, mortality in a single polecat was associated with a difenacoum liver residue of 1.4 mg/kg (Shore et al., 1996). In coyotes, mortality has been associated with hepatic brodifacoum residues of between 0.25 and 1.0 mg/kg (Poessei et al., 2015), while very similar total AR concentrations were not associated with lethality at all in hedgehogs (Dowding et al., 2010). Mortality in bats was associated with levels of between 0.19 and 0.68 mg/kg (Dennis and Gartrell, 2015), although various studies arising from New Zealand consider toxicity thresholds to have been reached at various concentrations ranging from 0.3, 0.5 and 0.7 mg/kg (see Spurr et al., 2005). In general, Sanchez-Barbudo et al. (2012) found that haemorrhaging was typically associated with higher levels of AR, although the response could be variable. This variability has been observed both within and between species (Shore et al., 2015), and the relationship between mortality and $\sum$AR is complex (Eason et al., 2002; Rattner et al., 2014). For ARs to exert a lethal effect on mammals and birds, it is necessary for all specific binding sites, predominantly located in the liver and pancreas, to be saturated with AR, and in addition, for AR to be present in excess of this (Mosterd and Thijssen, 1991; Thijssen, 1995). The concentrations of the specific binding sites in the livers have been reported to vary between species of mammals and birds, although they are of the same order of magnitude. Thus the liver residue levels in species that have died as a result of ARs would also be expected to vary between species, but within the same order of magnitude (Erickson and Urban, 2004; C. Prescott, pers. comm.). While signs of anticoagulant toxicity can sometimes be obvious due to haemorrhaging at the macroscopic level, it can also be very difficult to verify that an animal has actually died as a result of anticoagulant ingestion, particularly if haemorrhaging occurs at the microscopic level (Shore et al., 2015). Nonetheless, survival despite contamination is unequivocal, and in these animals, liver residue levels provide a minimum measure of their binding site concentrations (C. Prescott, pers. comm.). Furthermore, it has been reported that individual animals, once they have recovered from sub-lethal exposure, may develop compensatory tolerance to ARs (Eason et al., 2002), and that American mink may be less susceptible to these chemicals (Kaukeinen, 1982). In this study, the high liver concentrations found in a large proportion of apparently healthy mink, while indicative of exposure, are not indicative of lethality, at least in this species, although they might be indicative of a more deleterious impact on other carnivores. Another approach to assessing lethality where carcases with confirmed anticoagulant-induced haemorrhaging are available, is to construct a probabilistic model relating the risk of lethal poisoning by SGARs with hepatic concentrations (Thomas et al., 2011).

A key contribution of this study is the first estimates of the rate of contamination with increasing age, calculated from the slopes of the contamination age relationships. The rates of exposures are high, in the order of 4.5% of the population per month of life in mink. This exposure rate results in virtually all mink being exposed above a reported potential toxicity threshold of 0.2 mg/kg by 2 years of age, and assumes cumulative exposure due to the long half-lives of the SGAR compounds in particular. For example, the hepatic half-life of bromadiolone, the most frequently detected active ingredient found in this study, has been estimated in rats at between 170 and 318 days (see Erickson and Urban, 2004 for review). While few mink live to 2 years in culled populations, other carnivores routinely do (e.g. otters and martens) such that if extrapolated, our result suggest widespread penetration of potentially toxic levels of AR and therefore, potential population impacts. Carnivores can be aged relatively easily using canine X-ray and section, and it would be highly desirable for future studies to report age-corrected estimates of AR exposure, where possible using exposure rate per unit time for comparing prevalence of AR non-target contamination between regions and different regulatory regimes.

The precise routes of exposure in this study remain unconfirmed, although dietary analysis of mink has shown that they will take target rodents such as rats (*Rattus norvegicus*), as well as non-target rodents such as field voles (*Microtus agrestis*), wood mice (*Apodemus sylvaticus*), water voles (*Arvicola terrestris*) and shrews (*Sorex* spp.) (Akande, 1972; Cuthbert, 1979; Melero et al., 2014). Recent studies from Germany have found high AR residues in non-target species which were trapped at various distances from AR bait boxes. The highest maximum residues were found in field mice (*Apodemus*), followed by voles (*Microtus*, *Myodes*), then shrews (*Sorex*, *Crocidura*). However, 21% of *Apodemus* species contained AR residues; 7% and 26% respectively in *Microtus* and *Myodes* species; and 28% and 66% respectively in *Sorex* and *Crocidura* species (n total = 732). The majority of rodents with AR residues were trapped within 15 m of the bait boxes (Geduhn et al., 2014, 2016). These data strongly support previous reports of secondary exposure risks via non-targets in the UK (Brakes and Smith, 2005).

Of particular concern is the incidence of the most potent, single-feed SGARs, brodifacoum (9% of all mink) and flocoumafen (2%) (Table 2), which at the time of mink trapping, were only approved for indoor use (EC, 2004; EC, 2007). Given these restrictions, routes of exposure suggest regular movement of rodents in and out of buildings which is plausible only for house mice, wood mice and rats, or unapproved use outside of buildings.

Similarly high rates of exposure and high concentrations of ARs found in this study, have been reported in foxes and some raptors from Scotland (Tosh et al., 2011; Hartley et al., 2013; Hughes et al., 2013). These levels of exposure may suggest possible risks to other non-target species, although the current data may be more indicative of risks to mustelids of high conservation status, especially given the degree of dietary overlap between mink and native mustelids (Gorman, 2008). Where there is both niche and dietary overlap, it is possible that native mustelids may be exposed to toxic levels of ARs. Analyses performed on 23 Eurasian otters from Scotland over a similar time period found that 39% were exposed to ARs, and that just under 9% exhibited $\sum$AR above 0.20 mg/kg (E. Sharp, pers. comm.; unpublished data). While the niche and diet of otter and mink overlap (Clode and MacDonald, 1995; Bonesi et al., 2004; Melero et al., 2008b), and otters can be found in a wide variety of habitats across Scotland (SNH, 2015), otters specialize mainly on aquatic prey, while mink can exploit both aquatic and terrestrial species (see Melero et al., 2014). It is also possible that mink outcompete sympatric carnivores for access to poisoned rodents, and may inadvertently be lowering their exposure risks. Although there are no published data from Scotland on AR residues in European pine marten (*M. martes*), concerns have been raised regarding AR impacts in protected American fishers (*M. pennanti*) (Gabriel et al., 2012).

5. Conclusions

This study has demonstrated a relatively high level of AR exposure in mink. The long half-lives of the SGARs in particular (WHO, 2007; Vandenbroucke et al., 2008), means that across the lifetime of most mink, AR residues increase in both concentration and number of active compounds. This relationship is highly affected by the presence of farms in terms of number and the size of the farms found in the area around mink trapping locations.

Mustelids are particularly susceptible to AR contamination probably as a result of their varied prey base, which includes target and non-target rodents (Shore et al., 2003; Gabriel et al., 2012; Melero et al., 2014). These data support the use of mustelids, and in particular the American mink, as a sentinel of environmental AR contamination in rural areas. The rigour of comparisons of the degree of penetration of wild carnivore populations by AR could be increased if done in a standardised manner by using feral mink that are widely culled for conservation. The invasive alien species framework for the identification of invasive alien species of EU concern (EC, 2013) lists the American mink as one of 16 invasive mammals, of which several may be candidate species for comparative AR mitigation studies. For instance across mainland Europe, the raccoon (*Procyon lotor*) is widely found living in the wild, although not in the UK. Nonetheless, the American mink has been found in 28 European countries (Bonesi and Palazon, 2007), and in some of these countries, they are intensively controlled (Maran et al., 2012). Our results suggest that correcting prevalence for age, hence the time of exposure to AR, would greatly increase the power of comparisons.

Under Directive 98/8/EC concerning the placing on the market of biocidal products, several anticoagulants were described as "high toxic, non-selective and can pose a high risk of primary and secondary poisoning to non-target animals and children". For these reasons, European Member States are required to implement and assess the success of risk mitigation measures (EC, 2009). The measurement of AR residues in American mink has the potential to provide an intra-continental reference database, against which risk mitigation measures may be judged at the international level.

Competing financial interest declaration

There are no actual or potential conflicts of interest to declare for any author.

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