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Spatial and temporal exposure patterns in non-target small mammals during brodifacoum rat control

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HIGHLIGHTS

- Brodifacoum residues: detected in all non-target small mammal taxa during rat control
- There was a negative correlation of distance to bait stations on residue occurrence.
- High residue concentrations were largely restricted to 15 m around bait stations.
- Higher concentrations but less residues occurred during baiting than after baiting.
- The highest maximal residue concentrations occurred in *Apodemus* species.

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ABSTRACT

Worldwide pest rodents on livestock farms are often regulated using anticoagulant rodenticides (ARs). Second generation ARs in particular can cause poisoning in non-target species due to their high toxicity and persistence. However, research on exposure of small mammals is rare. We systematically investigated spatial and temporal exposure patterns of non-target small mammals in a large-scale replicated study. Small mammals were trapped at different distances to bait stations on ten farms before, during and after brodifacoum (BR) bait application, and liver samples of 1178 non-target small mammals were analyzed for residues of eight ARs using liquid chromatography coupled with tandem mass spectrometry. BR residues were present in 23% out of 742 samples collected during and after baiting. We found clear spatial and temporal exposure patterns. High BR residue concentrations mainly occurred within 15 m from bait stations. Occurrence and concentrations of residues significantly decreased with increasing distance. This pattern was found in almost all investigated taxa. After baiting, significantly more individuals contained residues than during baiting but concentrations were considerably lower. Residue occurrence and concentrations differed significantly among taxa, with the highest maximal residue concentrations in *Apodemus* species, which are protected in Germany. Although *Sorex* species are known to be insectivorous we regularly found residues in this genus. Residues of active agents other than brodifacoum were rare in all samples. The confirmation of substantial primary exposure in non-target small mammals close to the baiting area indicates considerable risk of secondary poisoning of predators, a pathway that was possibly underestimated until now. Our results will help to develop risk mitigation strategies to reduce risk for non-target small mammals, as well as their predators, in relation to biocidal AR usage.

1. Introduction

Anticoagulant rodenticides (ARs) are commonly used in many parts of the world for the control of pest rodents for plant protection and hygiene purposes in both rural and urban environments (Buckle and Smith, 1994). ARs are usually coumarin or indandione derivates that inhibit blood clotting in vertebrates and result in a delayed death of poisoned individuals (Maroni et al., 2000; Thijssen and Janssen, 1994; Valchev et al., 2008). This mode of action avoids bait shyness and vitamin k_1 is available as an antidote against warfarin associated ARs (Bjornsson, 1984; Bull, 1976; Lowenthal and Taylor, 1959; Markussen et al., 2003). Exposure of non-target animals to AR, however, can occur by direct bait intake (primary exposure) or when residues of ARs are passed through the food web via prey and carrion (secondary exposure). Second generation ARs (SGARs; e.g. brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen) were introduced to the

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market in the 1970s (Lund, 1984; Thijssen et al., 1989) because of resistance of Norway rats (*Rattus norvegicus*) and house mice (*Mus musculus* spp.) to first generation ARs (FGARs; e.g. chlorophacinone, coumatetralyl and warfarin). SGARs have a higher toxicity to vertebrates and persist longer in animal tissue than FGARs (Eason et al., 2002; Fisher et al., 2003).

Predators are at great risk of secondary poisoning because the persistent ARs accumulate in the liver (Eason et al., 2002). There is worldwide evidence of secondary exposure to ARs in aerial and terrestrial predators (e.g. France: Berny et al., 1997; Raoul et al., 2003; New Zealand: Eason et al., 2002; Spurr et al., 2005; Denmark: Christensen et al., 2012; USA: Riley et al., 2007; UK: McDonald et al., 1998; Walker et al., 2010). AR-residues in population studies of mammalian predators have been reported for example in 84% of red foxes (*Vulpes vulpes*; Tosh et al., 2011a), 23% of stoats (*Mustela erminea*; McDonald et al., 1998), 30% of weasels (*Mustela nivalis*; McDonald et al., 1998) and 36% of polecats (*Mustela putorius*; Shore et al., 2003). AR-residues are highly variable in birds of prey, ranging from 10 to 100%, but most studies found 65 to 85% of bird affected (e.g. Christensen et al., 2012; Hughes et al., 2013; Murray, 2011; Newton et al., 1990; Walker et al., 2010). Large variance of AR-occurrence in these studies may reflect different application practices such as aerial applications in New Zealand (Dowding et al., 1999), field application of bromadiolone in France (Sage et al., 2008) and the restriction of BR to bait station usage in the UK (Tosh et al., 2011b).

The source of secondary exposure of predators can be target, as well as non-target individuals that consumed ARs. Barn owls (*Tyto alba*) often nest in farm buildings (De Bruin, 1994) and may be at a particularly high risk of exposure to AR poisoned animals, whether they are target, or non-target species (Newton et al., 1990; Walker et al., 2010). In Greece, the most common prey species of barn owls is the house mouse (*Mus musculus domesticus*, 26%; Bontzorlos et al., 2005), whereas in other regions of the world, including Germany, the diet of barn owls mainly consists of non-target small mammals like voles and wood mice (Görner, 1979; Langenbach, 1982; Smith et al., 1972). In these regions, bait uptake through non-target animals could play an important role for predators.

Despite ample data for secondary exposure in non-target predators that was stated above, information about non-target small mammals is lacking. Primary exposure caused by AR application for crop protection was shown in target *Arvicola* and *Microtus* species (Giraudoux et al., 2006; Hernandez et al., 2013; Sage et al., 2008). Exposure through plant protection products in the field is unlikely in Germany, because most ARs that are authorized at the EU level for plant protection are prohibited in Germany for field application. There is evidence for primary poisoning in commensal target rodents during biocidal bait usage (e.g. Cowan et al., 1995; Rowe et al., 1978). Beside commensal target rodents, many non-target small mammal species, like *Apodemus* species, bank voles (*Myodes glareolus*) and greater white toothed shrews (*Crocidura russula*) are regularly present in rural and peri-urban environments (Braun and Dieterlen, 2005). Therefore, they can be exposed to ARs (Brakes and Smith, 2005; Tosh et al., 2012; Townsend et al., 1995). Other species such as common voles (*Microtus arvalis*) and field voles (*Microtus agrestis*) mainly live in (scrub-) grassland (Braun and Dieterlen, 2005) and exposure to ARs is rare (Brakes and Smith, 2005; Cox and Smith, 1990; Elliott et al., 2014). Some of the non-target small mammal species are legally protected in Germany (e.g. *Apodemus* species as well as *Sorex* and *Crocidura* shrews; BMJV, 2005) and their exposure to ARs would be of concern.

Biocidal AR bait requires covered application (e.g. bait stations) in Germany. However, non-target small mammal species may be prone to primary exposure to biocidal ARs because their similar body size to target species allows them access to bait stations. There is anecdotal (Cox and Smith, 1990) and qualitative evidence (Brakes and Smith, 2005; Tosh et al., 2012) from the UK that wood mice (*Apodemus sylvaticus*) consume AR bait at bait stations placed on farms. The presence of individuals with bait-residues was restricted to a maximum distance of about 80–110 m from the baited area (Tosh et al., 2012; Townsend et al., 1995). However, there are no detailed data from systematic approaches, on spatial and temporal patterns of AR residues in non-target small mammals.

The identification of species-specific associations of residue concentration and distance from baited areas in natural settings for the application of an AR product could help to evaluate differences among locations and species regarding the risk of primary exposure. Such knowledge may also be used to estimate the risk of secondary exposure for predators at and around farms where ARs are applied and to derive spatially targeted risk mitigation approaches.

The aim of our study was to identify temporal and spatial patterns of residue distribution caused by an AR application. We used a systematic, replicated, quantitative approach to determine whether primary exposure occurs in non-target small mammals before, during and after a coordinated baiting campaign on farms in NW Germany using BR for the control of Norway rat populations. BR is regularly used in rodent control operations in the area (Buckle et al., 2012) because resistance to FGARs and to some SGARs has developed (Pelz, 2007). We were particularly interested in small mammal non-target inter-species differences in residue occurrence and concentrations, seasonal effects, and the temporal and spatial patterns of residue distribution. These aspects are highly relevant for assessing the risk for small mammals as well as predators when ARs are applied in and around buildings for biocidal or plant protection use.

2. Material and methods

2.1. Study area

In four trapping events (autumn (October/November) 2011 and 2012 as well as in late winter (February/March) 2012 and 2013) small mammals were screened for rodenticide residues during baiting campaigns with brodifacoum (BR) to control Norway rat populations. The work was conducted on farms in the Münsterland region (Fig. 1, 51.960665N, 7.626135E, North Rhine-Westphalia, Germany). The area is a mosaic of farmland (about 60%) and small forest sections (about 15%) used for timber production (GENESIS-online-database, 2013). Main crops are corn (*Zea mays*), wheat (*Triticum* spp.) and barley (*Hordeum vulgare*; GENESIS-online-database, 2013). In the region, the mean temperature is 9.2 °C and annual precipitation is 782 mm (DWD, 2014). On all farms included in this study, livestock (cattle, poultry and/or pigs) was held in stables and/or on surrounding meadows. Small mammal populations were investigated on six farms at all four trapping events (autumn 2011 and 2012 and winter 2012 and 2013; gray dots in Fig. 1), whereas populations at four farms were investigated less often (empty dots in Fig. 1) due to insufficient rat numbers based on visual surveys before bait application. At which trapping events these farms were investigated are indicated in Fig. 1. The minimum linear distance between farms where we applied BR bait was 2.9 km. Neighboring farms not included in the study were on average 310 m ± 120 m standard deviation (sd) away (minimum 160 m). Prior to the study, farmers were interviewed about AR usage behavior and rat occurrence. All farmers stated regular occurrence of rodents on their farms and used ARs to control them. Brodifacoum was used most often (5 of 10 farmers). All farmers used covered bait stations for baiting around buildings. On average farmers used ARs twice a year for three to four weeks for each baiting event. The last time farmers applied bait was several weeks to two years prior to the start of the study.

2.2. Norway rat control

Baiting was conducted according to label instructions and following the standard practice of farmers in the region as indicated in the pre-study questionnaire (see above) and lasted for three weeks. Twenty bait stations (Rattenköderbox "B", Detia Garda GmbH) were placed at each farm where rat feces and footprints were observed. Where signs

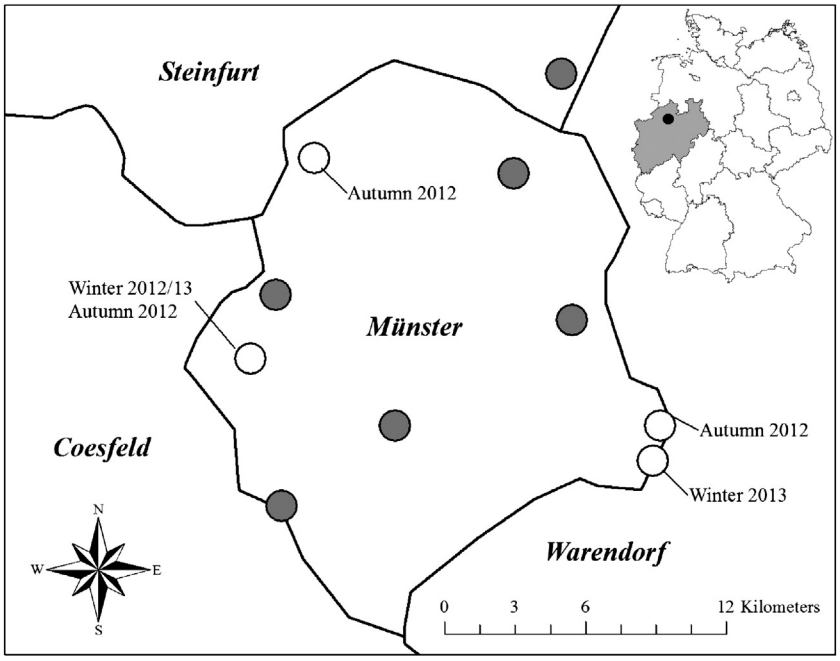

Fig. 1. Location of farms where the study was conducted in and close to Münster county in the northwest of Germany (inset shows the location of Münster in the German federal state of North Rhine-Westphalia). Most farms were investigated in all four seasonal trapping events (autumn 2011 and 2012 and winter 2012 and 2013) — gray dots. Other farms were not always sampled — empty dots and timing of trapping is indicated.

of rat occurrence were less obvious bait stations were set near specific structural elements; an approach known to be essential in effective rodent control (Endepols and Klemann, 2004; Endepols et al., 2003). Bait stations were prebaited with rolled oats for one week. At the first day of poison application, remaining rolled oats were removed and replaced by 150 g of Ratron® Brodifacoum Flocken (0.05 g/kg BR, frunol delicia® GmbH) per bait station. On average 730 g (±947 g sd) of bait per farm was removed during each baiting period. The bait formulation was granular and, beside the active ingredient and a dye, consisted mainly of rolled oats. A cable tie was used to lock bait stations. In the first week of baiting, bait stations were checked every second day. The frequency of checks subsequently depended on bait take, but was at least once a week. Removed bait was replenished at each check. Bait in all stations was completely replaced at least once within the baiting period.

2.3. Small mammal trapping

Each trapping event consisted of three sessions with small mammals trapped: 1) starting one week before commencement of baiting (before), 2) starting three days after commencement of baiting (during) and 3) starting on the day of bait removal (after). In the latter session bait was removed from bait stations before snap traps were set.

Snap traps along two transects (Metall-Mausefalle Fox, DeuFa) were set at each farm. Transects started at a distance of 0.5 to 5 m from a bait station and extended about 100 m away from the farm. Every 4 m a trap station of two snap traps was set and marked with a colored wooden stick. To prevent larger animals such as feral cats (*Felis silvestris catus*) from displacing traps, the first 9 trap station traps of each transect were fixed with wire to the marking stick. We established 27 trapping stations (54 traps) per transect. Each transect was subdivided in 3 groups of 9 trapping stations. Traps were set for three consecutive days during each session, or until five small mammals were collected in a group of trapping stations across both transects on a farm. Traps were shut down after three days to keep removal effects on populations minimal for further sessions. On one occasion traps were set a fourth day because of a heavy snow fall that occurred in winter 2013 and reduced trap functioning and small mammal activity.

A mixture of rolled oats and peanut butter was used for baiting snap traps. Traps were checked once a day in the morning. After the day of capture, snap trapped animals were stored at −80 °C for at least one week. Then, individuals were defrosted, weighed and sex checked and the species was determined using dental, cranial and other morphological characteristics. The liver was removed and stored at −20 °C for rodenticide residue analysis. The locations of transects and bait stations were recorded in ArcGIS software version 10.1 by Esri for further analyses.

Target rodents (Norway rats and house mice) were trapped with snap traps and we searched for carcasses. Further details will be presented elsewhere.

2.4. Analysis of anticoagulant rodenticides

The content of the eight ARs was determined with a modified method adapted from Klein and Alder (2003) by liquid chromatography coupled with tandem mass spectrometry in electrospray ionization mode after solid supported liquid extraction of liver samples.

For validation of results we used several standard substances and surrogates. Certified standard substances of chlorophacinone, warfarin, coumatetralyl, difenacoum, bromadiolone, brodifacoum, flocoumafen, the surrogates diphacinone-d_4 and coumachlor, the internal standards chlorophacinone-d_4 and warfarin-d_5 and a acetonitrile-solution of difethialone (10 ng/µl) were purchased from Dr. Ehrenstorfer GmbH. The surrogates acenocoumarol and phenprocoumon were obtained from Novartis and Arevipharma, respectively. The working standards were dissolved in acetonitrile (Ultra gradient HPLC, J. T. Baker). The calibration standards were prepared with concentrations ranging from 0.1 to 100 ng/ml in a mixture (1:1) of methanol (Pestanal, Sigma-Aldrich) and deionized water (Arium 611UV, Sartorius).

The whole defrosted liver tissue sample (0.2 to 2 g) was weighed in a polypropylene tube and spiked with a mixture of the four surrogates (200 ng in 100 µl). The homogenization of samples was carried out in two steps to reduce foaming by an Ultra Turrax (T25, IKA) with 20 ml methanol for 3 min and after the addition of 10 ml of water (2:1/v/v) a second time for 1 min. After centrifugation for 5 min at 5000 rpm (Heraeus Megafuge 1.0) 5 ml of sodium chloride-solution (20%) was added to a 15 ml aliquot of the supernatant, shaken by vortex mixer

(VF 2, IKA) and transferred on a diatomaceous earth column (Chem Elut, 20 ml, 12198008, Agilent). After 15 min the solid supported liquid extraction was done with 100 ml dichloromethane (Pestanal, Sigma-Aldrich). An aliquot of 2 ml from this solution was evaporated (Rotavapor RV 6, Büchi). The residue was spiked with a 100 μl mixture of chlorophacinone-d_4 (25 ng) and warfarin-d_5 (10 ng) used for the 1,3-indandione and the 4-hydroxycoumarine substances as internal standards, respectively. The solvent was evaporated under N_2-flow. The extract was redissolved in 1 ml methanol:water (1:1 v:v) and subsequently filtrated through a syringe filter (PTFE, 0,2 μm, Ø 13 mm, Roth) in a autosampler vial.

ARs analyses were realized by the liquid chromatograph UltiMate 3000 RS (Dionex) coupled with the mass spectrometer QTRAP 5500 (AB SCIEX) used in electrospray ionization mode. The chromatographic column (Luna PFP (2) 50 × 2 mm × 3 μm and Kinetex PFP 50 × 2.1 mm × 5 μm, equipped with KrudKatcher Ultra HPLC In-Line Filter, 0.5 μm Depth Filter at 70 °C, Phenomenex) was loaded with 5 μl of the sample solution. The mobile phase consisted of a gradient elution of two solvents (A: methanol + 0.5% acetic acid + 5 mM ammonium acetate; B: water + 0.5% acetic acid + 5 mM ammonium acetate). It started with 10% A, reaching 90% A at 3 min, continued for 1 min and then switched to 10% A for column equilibration for about 2 min. The flow rate was 800 μl/min. The quantification of ARs was performed in electrospray ionization negative mode with a source temperature of 450 °C and an ion spray potential of −4.5 kV. Identification and quantification took place with precursor – product ion – transition by Multiple Reaction Monitoring (chlorophacinone: 373.1 → 201.0, warfarin: 307.0 → 161.0, coumatetralyl: 291.0 → 140.9, difenacoum: 443.1 → 135.0, bromadiolone: 526.9 → 249.9, brodifacoum: 251.0 → 78.9, flocoumafen: 541.0 → 382.0, difethialone: 538.9 → 80.8, acenocoumarol: 352 → 145.0, diphacinone-d_4: 343.1 → 167.0, phenprocoumon: 278.9 → 250.0, coumachlor: 340.9 → 160.8, chlorophacinone-d_4: 377.1 → 200.9 and warfarin-d_5: 312.1 → 161.0 m/z). Confirmation was done by spectra comparison between sample and references based on Enhanced Product Ion-spectra (response > 1000 cps), additionally.

The calibration curves were linear over the range of the eight concentration levels used: 0.1, 0.5, 1.0, 5.0, 10, 25, 50 and 100 ng/ml (r^2 > 0.99). The signals to noise ratios of the lowest concentration level were always >6:1. All samples were measured twice. The concentrations of ARs were calculated with peak areas by Analyst 1.6.1 and the data presented refers to liver fresh weight.

The rate of recovery of the analytical procedure was performed with four turkey (*Meleagris gallopavo*) liver samples purchased at a food store before analyses of field samples were conducted. Two grams of fresh turkey liver was spiked with analytes and surrogates (0.200 μg/g) and analyzed as described above. Mean recovery values and in brackets the relative standard deviations (liver samples spiked with all ARs) were for chlorophacinone 83% (14%), warfarin 118% (4%), coumatetralyl 100% (6%), difenacoum 78% (7%), bromadiolone 77% (4%), brodifacoum 58% (6%), flocoumafen 65% (4%) and difethialone 41% (7%) and for the surrogate acenocoumarol 112% (5%), diphacinone-d_4 106% (9%), phenprocoumon 101% (1%) and coumachlor 91% (2%). No interferences were observed in two blank turkey liver samples, which ran in the same batch as the spiked turkey liver samples. All study samples were spiked with the surrogate mixture for ongoing validation of the analytical performance. The measured concentrations were not corrected for recovery rates.

Bait recovered at the end of the baiting period from two different lots (autumn 2011 and winter 2013) was analyzed identically to liver samples, but resulting BR-concentrations were corrected for recovery rate.

2.5. Data editing and statistical analysis

Trap success per farm was calculated as the number of individuals trapped per taxon per 100 trap nights, adjusted by subtraction of sprung traps. The latter included all traps, which were triggered accidentally by rain, snow, animals etc. Transects per farm were pooled. Friedman tests were used to test for differences in trap success among trapping sessions and Wilcoxon tests to compare trap success among small mammal taxa based on the number of farms at all trapping events.

The linear distance in meters between the point of capture and the closest bait station was measured with the tool "near" in ArcGIS®. Bait stations that were set in attics or within brick buildings were excluded from this analysis because there was no direct access for non-target species. Animals approaching bait stations there would have had to take detours of at least 5 m and therefore using direct line distance was not appropriate. For the 4% of trap locations affected by that scenario we considered the closest bait station in the open for analysis. These bait stations were on average 4 m further away from the trap location than the excluded bait station.

For the presence and absence data of BR-residues we used a binomial generalized linear mixed effects model (GLMM) fitted by maximum likelihood. As our residue data per individual was clustered within farms and trapping events, we added both as random factors. Metric distance and nominal season and session factors required mixed modeling. The model was selected by backward adjustment using the Akaike information criterion. Data were divided into individuals trapped before and individuals trapped during and after baiting, and were analyzed separately. Multi comparisons for taxa within the model were done with Tukey contrast. Percentages of individuals containing BR per farm were used for graphical presentation. Distance classes were used in figures, although they were not included in the linear modeling.

Negative binomial regression (nbGLM) was used to identify factors correlating with BR-residue concentration of BR-positive individuals during and after baiting as concentrations were not normally distributed. No random factor was integrated in this model, due to small numbers of BR-positive small mammals. The model was selected by backward adjustment using the Akaike information criterion. Multi comparisons for taxa within the model were done with Tukey contrast. Concentration axes in scatter plots were log transformed, but original concentrations were used in the modeling.

BR liver residue concentrations higher than 1 μg/g were assumed to be lethal for the species considered (EPA, 1998; Erickson and Urban, 2004; Mosterd and Thijssen, 1991; O'Connor and Booth, 2001; Redfern et al., 1976 and Špakauskas et al., 2005; details in discussion). Therefore, this threshold was used for classification of BR-concentrations for residues found before baiting and in the figures comparing different BR concentrations during and after baiting.

A Spearman-rank test was used to test for a correlation between BR and warfarin residue occurrence.

All statistical analyses were conducted with R-project 3.0.1 (RCoreTeam 2013) and the level of significance was $p < 0.05$.

Our measure of variance is the standard deviation of the mean.

3. Results

3.1. Trap success

In total, 1244 non-target small mammals were collected from 30 trapping events (autumn 2011: 6 farms, 356 individuals (ind.); winter 2012: 7 farms, 289 ind.; autumn 2012: 9 farms, 482 ind.; winter 2013: 8 farms, 117 ind.). *Apodemus* and *Microtus* species, *M. glareolus* and *Crocidura* and *Sorex* shrews were trapped regularly on farms and were used for further analysis. Fourteen individuals of *Micromys minutus* were trapped, but because of this low sample size, they were not included in comparison of trap success. Trap success varied among trapped species (Fig. 2) before, during and after baiting (before: $\chi^2 = 54.5$, df = 4, $p < 0.001$; during: $\chi^2 = 35.1$, df = 4, $p < 0.001$; after: $\chi^2 = 36.6$, df = 4, $p < 0.001$). *Apodemus* species were trapped more often than all other species before, during and after baiting ($p < 0.05$ in all pair-wise tests, Fig. 2). *M. glareolus* and *Microtus* voles did not differ significantly in

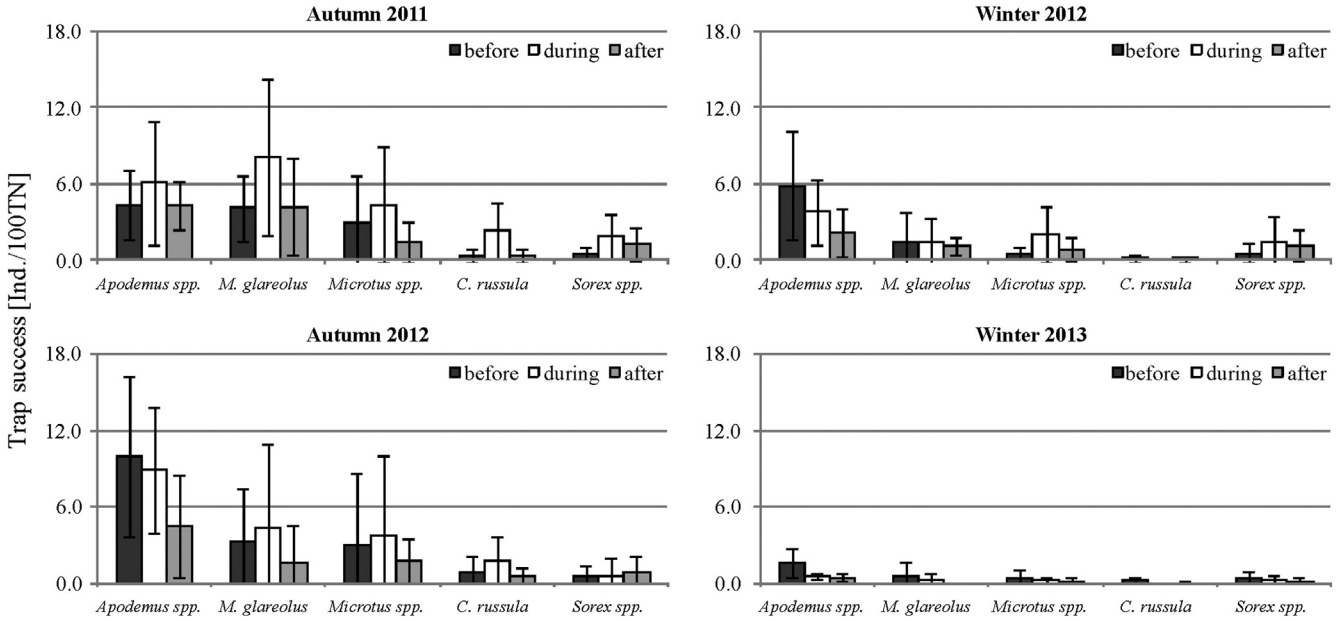

Fig. 2. Trap successes of non-target small mammals before, during and after brodifacoum bait application on farms in the Münsterland, North Rhine-Westphalia in Germany. Six farms were investigated in autumn 2011, 7 in winter 2012, 9 in autumn 2012 and 8 in winter 2013. Trap success (trapped individuals per 100 trap nights [Ind./TN]) is means ± sd.

trap success ($p > 0.415$). Trap success was higher for *M. glareolus* than for shrews except for the 'after' session, when trap success was similar for *M. glareolus* and *Sorex* shrews ($p = 0.988$) (Fig. 2).

3.2. Residues before baiting

Before baiting 10.1% of 436 analyzed small mammals contained brodifacoum (BR) residues. Both winter trappings were conducted 13 to 15 weeks after the preceding autumn trapping. The second autumn trapping (2012) took place 28 weeks after the preceding winter trapping. Focusing on farms that were investigated in an autumn and a consecutive winter trapping event (Fig. 3 N = 13 (autumn 2011 to winter 2012: 6 farms and autumn 2012 to winter 2013: 7 farms; 383 analyzed individuals)), the number of samples containing BR residues before baiting started (41 individuals) was more than five times higher in winter (20.6%) than in the previous autumn (3.8%) ($z = 4.24$, $p < 0.001$). Occurrence of samples containing BR decreased with increasing distance of trap location to bait stations ($z = -2.16$, $p = 0.031$). Concentrations of >1 μg/g liver were present in two *Apodemus* individuals in winter 2012 from different farms. These animals were trapped 21 m and 79 m, respectively, from a bait station. One additional individual with a BR liver concentration of >1 μg/g was trapped on a farm where we did not apply BR in the previous autumn.

3.3. Spatial and temporal patterns in non-target small mammals during and after baiting

Residues of BR were found in 168 of 742 analyzed non-target small mammal liver samples (22.6%) taken during and after the baiting period. In autumn 2011 there were BR-residues in 20.3% of small mammals (±9.2%, 6 farms, N = 241), in winter 2012 in 21.8% (±16.1%, 7 farms, N = 192), in autumn 2012 in 20.7% (±18.0%, 9 farms, N = 265) and in winter 2013 in 31.0% (±21%, 8 farms, N = 44).

3.3.1. Residue occurrence

The probability of capturing a small mammal containing BR decreased significantly with increasing distance from bait stations ($z = -8.52$, $p < 0.001$). The furthest an individual was trapped from a bait station that contained BR was 87 m. During baiting, the percentage of BR-positive individuals per farm decreased from 53.6% (±38.2%) closest to bait stations to 5.7% (±13.6%) furthest away from bait stations (>60 m). After baiting a similar pattern was found; 59.2% (±41.9%) to 9.3% (±15.8%), respectively (Fig. 4). There was a statistically non-significant tendency for more BR-residues in winter than in autumn ($z = 1.81$, $p = 0.070$) and a significant difference in BR-residue occurrence between sessions ($z = 3.66$, $p < 0.001$, Fig. 4). Residues in individuals occurred about 1.5 times more often directly after baiting (29.6% ±11.1%) than during baiting (20.4% ±10.1%, Fig. 4).

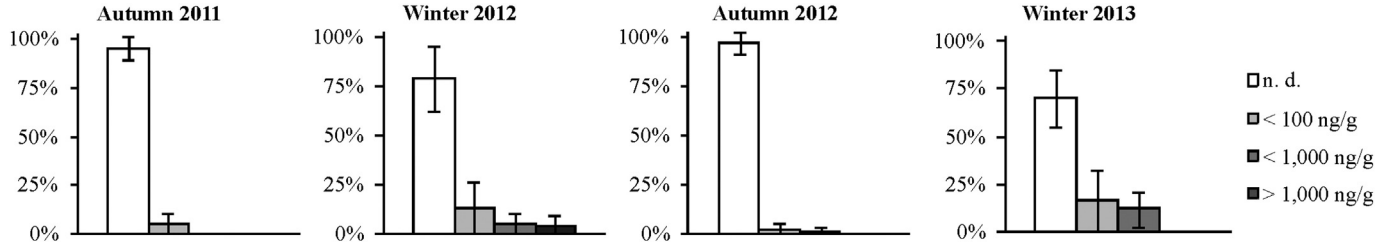

Fig. 3. Brodifacoum (BR) occurrence before baiting. Distribution of BR-residues before baiting in non-target small mammals from farms investigated in autumn and the following winter. Percentages of individuals per farm are means ± sd grouped in classes of n. d. (not detectable), <0.1 μg/g, 0.1 to 1 μg/g and >1 μg/g BR in liver tissue.

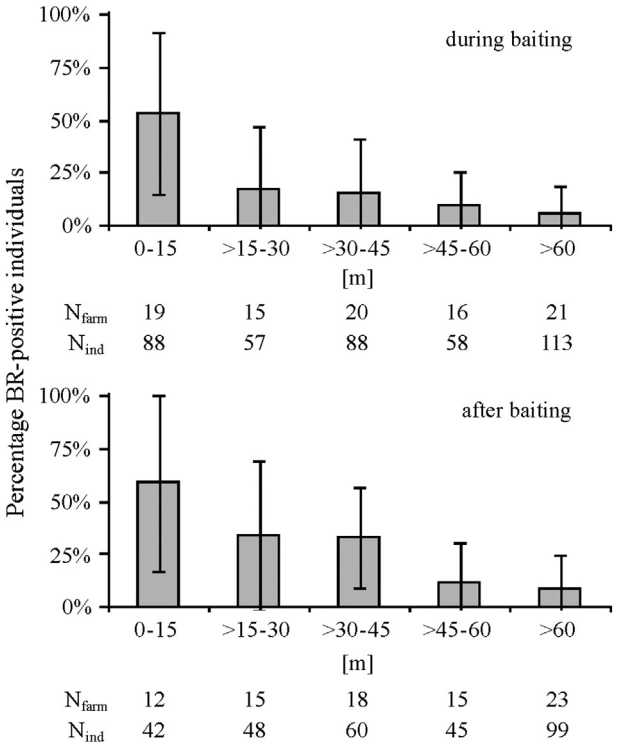

Fig. 4. Temporal pattern in residue occurrence. Percentages of non-target small mammals containing brodifacoum (BR) residues during (above) and after (below) Norway rat control with BR at different distances to bait stations. Percentages of BR-positive individuals per trapping event on a farm (N_{farm}) are means ± sd (farms where less than two individuals per distance class were captured were excluded). Number of individuals per distance class is stated as N_{ind}.

BR-residue occurrence significantly differed among species (Table 1). It was lowest in *Microtus* voles (Table 1). *C. russula* showed higher BR occurrence than *Apodemus* mice, but *Apodemus* mice, *M. glareolus* and *Sorex* shrews did not differ significantly in BR-exposure rates (Table 1). Occurrence of BR-residues was highest at distances within 15 m to bait stations in all small mammal taxa, except shrews, which showed the highest BR-occurrence at 15 to 30 m distance (Fig. 5).

Trap success in *Apodemus* mice, *M. glareolus* and *Microtus* voles allowed identification of spatial distribution of BR-occurrence within these taxa but data were combined from the "during" and "after" sessions except for *Apodemus* mice, which were trapped almost twice as often as the other species. In all three taxa there were relatively more individuals trapped that carried BR-residues within 15 m of bait stations and a slight decrease of BR-residues in animals trapped 15 to >60 m away (Fig. 5). Hardly any BR-residues were found in *Microtus* voles at distances >15 m. Two or more *Apodemus* mice were trapped <15 m from bait stations in 13 trapping events on farms during baiting, but only on two farms after baiting. *C. russula* stayed close to the farms, and only on two occasions more than one individual was trapped at a distance of >15 m.

In a preliminary analysis BR residues in target species occurred in 52 of 53 Norway rats and in 13 of 15 house mice. Detailed results will be presented elsewhere.

3.3.2. Residue concentrations

The highest BR-residue concentrations were found within 15 m from a bait station and liver concentrations significantly decreased with increasing distance ($z = -6.88$, $p < 0.001$, Fig. 6). Concentrations >1 μg/g (in total 31 individuals; 4.2% of all samples during and after baiting and 18.5% of animals containing BR-residues) were rare at distances >15 m, whereas concentrations <0.1 μg/g (54.2% of BR-positive animals) occurred at distances up to 87 m. Concentrations from 0.1 to 1 μg/g (27.4% of BR-positive individuals) were present up to about 50 m from bait stations (Fig. 6). BR-residue concentrations were considerably lower after (median = 0.050 μg/g) than during baiting (median = 0.178 μg/g; $t = -2.14$, $p = 0.032$). More individuals with concentrations >1 μg/g occurred during baiting (22 out of 84 BR-positive individuals) than after baiting (9 out of 84 BR-positive individuals; Fig. 6). The season was removed from the model, as there was no significant difference between residue concentrations of individuals trapped in winter and in autumn.

BR-residue concentrations significantly differed among species (Table 1, Fig. 6), with higher mean concentrations in *Apodemus* than in *C. russula* and *Sorex* shrews, and higher residues in *M. glareolus* than in *C. russula*. Residues >1 μg/g were present in all taxa, except *Sorex* shrews. Maximum concentration was highest in *Apodemus* mice and lowest in *Sorex* shrews (Table 1). 16 out of 65 *Apodemus* mice had BR-residues >1 μg/g and *Myodes* voles in 5 of 43 BR-positive cases. In *Microtus* voles only one individual carried residues after baiting, whereas there were residues in 8 *Microtus* voles during baiting, four of them at concentrations >1 μg/g and within 11 m from bait stations.

Preliminary results of BR-positive individuals of target species indicated a median BR-residue concentration of 6.461 μg/g and a maximum of 25.124 μg/g in Norway rats and a median concentration of 25.282 μg/g and a maximum of 35.982 μg/g in house mice.

3.4. Occurrence of non-brodifacoum rodenticides

Additionally to BR, samples were screened for seven other ARs. Occurrence ratios of these substances were generally low (Table 2), except chlorophacinone in autumn 2011 (17%), warfarin in autumn 2011 (3%) and winter 2012 (4%), and bromadiolone in winter 2013 (3.6%). Warfarin could not be detected in samples before baiting (in autumn 2011 and winter 2012). However, in autumn 2011 and winter 2012 during and after baiting, warfarin occurred in 83.3% of samples containing BR > 1 μg/g (N = 12), in 24.1% of samples with BR concentrations between 0.1 and 1 μg/g (N = 29) and in 1.9% of BR-positive samples with BR concentrations below 0.1 μg/g (N = 54). Warfarin concentration of warfarin-positive individuals from autumn 2011 and winter 2012 correlated with BR concentration ($N = 4$, $r_s = 0.70$, $p < 0.001$). Difenacoum was detected in 7 samples in autumn 2011, 6 of them containing BR and 5 of them with concentrations >2 μg/g.

Based on these results two bait lots were analyzed for residues of ARs other than brodifacoum and considerable amounts of warfarin (7.3 μg/g) were found in one lot and difenacoum (2.1 and 0.5 μg/g respectively) in both lots. Concentrations of other substances were 0 or <1 μg/g.

4. Discussion

Brodifacoum (BR), an AR, that was used most often by farmers of our study region, was widespread across non-target small mammal species trapped during and directly after in- and outdoor Norway rat control operations on farms. There were clear spatial and temporal patterns with high residues mainly occurring within the immediate surroundings of farms, and liver concentrations considerably decreased with increasing distance. These results confirm the findings of Townsend et al. (1995) who found higher bait occurrence in *Apodemus* species within than outside a baiting area and up to 80 m from rodent control. Our study demonstrates that this spatial pattern occurred consistently in all investigated taxa, except *Sorex* species. Tosh et al. (2012) showed that residues in house mice occur within 30 m of bait stations and in wood mice at a maximum distance of 110 m from a farm where baiting was conducted. There were AR residues in some *Apodemus* mice although no ARs were used on farms. We also found residues in small mammals up to 87 m away from bait stations and residues of ARs other than BR in some individuals. Compared to the BR exposure of small mammals within 15 m of bait stations, AR residues less often

Table 1
Residue pattern in non-target small mammals. (A) Occurrence (percentage [%] of individuals containing brodifacoum (BR)) and concentrations of BR-residues in liver tissue of non-target small mammal taxa during and after baiting Norway rats with BR-bait at farms in Münsterland, Germany. "Sig." marks significant differences between species and "sd" stands for standard deviation. (B) Statistical details of taxa analysis (except *M. minutus*, because sample size was too low) from Tukey pair-wise comparisons within a binomial general linear mixed model (occurrence) or negative binomial regression (concentration).

A

	Occurrence of BR-residues			BR-concentrations of BR-positive individuals [µg/g wet weight]						
	N	[%]	Sig.	N	Mean	sd	Sig.	Median	0.25; 0.75 QT	Max.
Apodemus spp.	307	21	A	65	2.713	5.895	A	0.059	0.019; 0.794	31.125
C. russula	38	66	B	25	0.573	1.052	B	0.053	0.021; 0.366	4.287
Myodes glareolus	168	26	AB	43	1.908	5.104	AC	0.036	0.019; 0.302	20.180
M. minutus	10	10		1	2.020			2.020		2.020
Microtus spp.	130	7	C	9	2.356	3.310	AB	0.240	0.093; 2.863	7.990
Sorex spp.	89	28	AB	25	0.214	0.190	BC	0.164	0.074; 0.300	0.766

B

Tukey pair-wise comparisons		Occurrence of BR-residues		BR-concentrations of BR-positive individuals	
		z	p	z	p
Apodemus spp.	*C. russula*	−3.51	0.005	4.51	<0.001
	Myodes glareolus	−1.35	0.730	1.66	0.519
	Microtus spp.	4.24	<0.001	1.08	0.873
	Sorex spp.	−0.86	0.948	2.81	0.047
C. russula	*Myodes glareolus*	2.49	0.109	−3.17	0.015
	Microtus spp.	5.94	<0.001	−2.13	0.239
	Sorex spp.	2.48	0.113	−1.04	0.888
Myodes glareolus	*Microtus* spp.	4.89	<0.001	0.15	0.999
	Sorex spp.	0.24	0.999	1.63	0.543
Microtus spp.	*Sorex* spp.	−4.45	<0.001	1.09	0.870

occurred and concentrations were lower suggesting little exposure further away from farms that had been baited.

The identification of spatial patterns of species-specific presence of BR residues in non-target small mammals from our study can be used to estimate the risk of secondary exposure in non-target predators more precisely. The risk of secondary poisoning seems highest for predators that hunt in the direct surroundings of baited farm areas, as the highest concentrations and proportion of small mammals carrying BR-residues were found within 15 m of bait stations. In contrast, the risk for secondary exposure of predators through non-target small mammals seems low

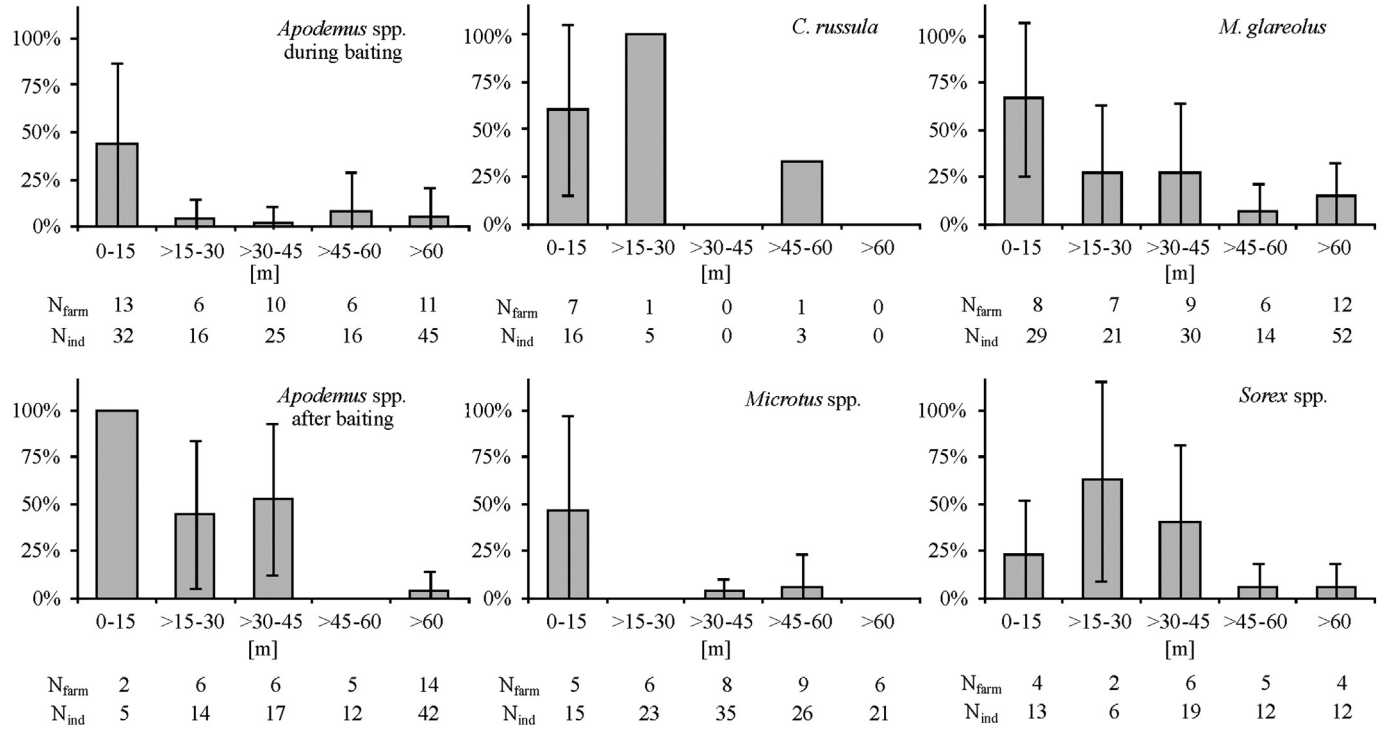

Fig. 5. Small mammal taxa differences in residue occurrence. Left: Brodifacoum (BR) occurrence in *Apodemus* species trapped during and after Norway rat control with BR at different distances to bait stations. Center and right: BR occurrence in other non-target species from individuals pooled from trapping during and after Norway rat control. Percentages of BR-positive individuals per trapping event on a farm (N_{farm}) are means ± sd (farms where less than two individuals per distance class were captured were excluded). Number of individuals per distance class is stated as N_{ind}.

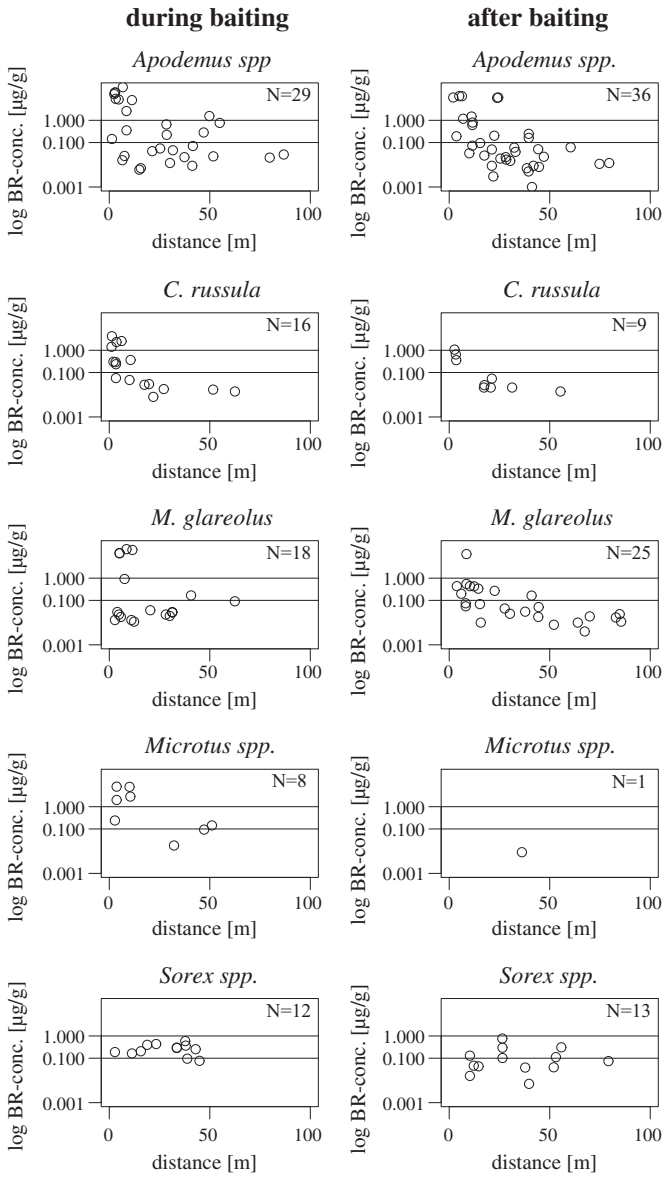

Fig. 6. Distance concentration relation. Brodifacoum (BR) residue concentrations of BR-positive non-target small mammals during baiting (left) and after baiting (right) with BR for Norway rat control at farms in Münsterland, Germany. Concentrations (µg/g liver wet weight) are plotted on a log-scale against distance to the baiting area for each small mammal taxon.

when prey is collected further away. Barn owls are known to nest in farm buildings (De Bruin, 1994) and they were present on several farms in our study. Furthermore, they hunt in agricultural landscapes (Bontzorlos et al., 2005), sometimes even within buildings (De Bruin, 1994). There are several studies that showed barn owls to be exposed to ARs (e.g. Newton et al., 1990; Walker et al., 2010) and other small mammal predatory birds like red kites (*Milvus milvus*) and kestrels (*Falco tinnunculus*; Walker et al., 2010). Red foxes regularly visit farms (Braun and Dieterlen, 2005) and polecats prey on rodents at farmyards (Birks, 1998) and have been shown to be exposed (Shore et al., 2003; Tosh et al., 2011a). Furthermore, Proulx and MacKenzie (2012) found considerably more badgers (*Taxidea taxus*) and red foxes in areas with low levels of AR poisoning compared to areas with high AR poisoning.

It is difficult to interpret AR liver concentrations because little is known about the relationship between liver residue concentrations, toxicity and biological effects of ARs in small mammals. The LD_{50} of BR is 0.26 to 0.56 µg/g body weight in laboratory rats and about 0.4 to 0.52 µg/g in laboratory mice (Erickson and Urban, 2004; O'Connor and Booth, 2001; Redfern et al., 1976; Špakauskas et al., 2005). One day after a single dose of 0.2 µg/g BR laboratory Norway rats showed mean liver concentrations of 0.792 (Mosterd and Thijssen, 1991) and 1.107 µg/g respectively (EPA, 1998). Assuming linear relation between the amount of ingested BR and liver concentration, ingestion of a LD_{50} in Norway rats would result in liver concentrations of 1.030 to 3.100 µg/g. For laboratory mice, liver concentrations of 1.584 to 2.214 µg/g would be expected. Therefore, we indexed individuals containing residues >1 µg/g (1.724 µg/g when corrected by recovery rate) as potentially moribund. Such concentrations occurred almost without exception at distances <15 m to the next bait station. This also demonstrated that high BR-residues were strongly associated with the immediate surroundings of the baiting area. It seems that individuals containing high concentrations had home ranges that included a bait station.

However, sublethal effects could occur at lower ingested amounts of ARs. Prothrombin complex activity (PCA) was reduced to about 35% in Wistar rats dosed with 0.2 µg/g BR after 24 h (Mosterd and Thijssen, 1991) but Leck and Park (1981) found a reduction to 18.5% of normal PCA in Wistar rats after a dose of 0.1 µg/g. Prescott et al. (2007) found an ED_{50} of 0.217 and 0.222 µg/g for male and female non-resistant Norway rats. Comparisons to other studies concerning true AR concentrations are difficult due to bait marker usage in most studies (e.g. Brakes and Smith, 2005; Cox and Smith, 1990; Townsend et al., 1995). Only Tosh et al. (2012) and Elliott et al. (2014) investigated residue concentrations within non-target small mammal species at biocidal AR-usage. In Tosh et al. (2012), concentrations of different ARs were generally lower (0.003 to 0.615 µg/g corrected by recovery rate) than in the present study, but the studies are difficult to compare because of different application methods, sampling distances (up to 260 m in Tosh et al., 2012) and lower recovery rates for all ARs in Tosh et al. (2012).

For the first time a clear temporal pattern in exposure rates in small mammals can be shown. BR-residues were more prevalent after (29.6%) than during (20.4%) baiting, whereas residue concentrations were higher during than after baiting. This indicates the presence of sublethally contaminated individuals for 2–3 weeks after commencement of baiting. Twice as many individuals contained residues >1 µg/g BR

Table 2
Occurrence of non-brodifacoum rodenticides. Occurrence [%] and median concentrations [µg/g] of anticoagulant rodenticides in liver samples of non-target small mammals before, during and after brodifacoum baiting for Norway rat control on farms in North Rhine-Westphalia, Germany.

	Autumn 2011 (N = 355)		Winter 2012 (N = 289)		Autumn 2012 (N = 417)		Winter 2013 (N = 117)	
	% ± sd	µg/g	% ± sd	µg/g	% ± sd	µg/g	% ± sd	µg/g
Bromadiolone			0.3 ± 0.9	0.008	1.9 ± 1.8	0.030	3.6 ± 5.1	0.037
Chlorophacinone	17.4 ± 7.5	0.031	0.4 ± 1.0	0.004	0.7 ± 1.6	0.013	0.6 ± 1.8	0.024
Coumatetralyl	0.9 ± 1.5	0.009	0.4 ± 1.0	0.002	0.3 ± 0.8	0.009	1.0 ± 3.0	0.063
Difenacoum	2.0 ± 1.0	0.063			0.6 ± 1.6	0.088		
Difethialone	3.1 ± 3.5	0.013			0.6 ± 1.1	0.013		
Flocoumafen	0.8 ± 1.4	0.013	0.2 ± 0.6	0.057	0.3 ± 0.8	0.050		
Warfarin	3.2 ± 2.1	0.041	4.2 ± 3.9	0.034	0.8 ± 1.7	0.133		

when trapped during than after baiting. It is likely that those individuals would have died before the second trapping session, explaining lower residue concentrations after baiting. As high residue concentrations were less present after baiting it seems that there was no immediate immigration within the three weeks of baiting. Residue occurrence in non-target small mammals (22.6%) after three weeks of baiting is well in the range of earlier findings of 15 to 48.6% using AR-residues or bait markers (Brakes and Smith, 2005; Tosh et al., 2012; Townsend et al., 1995). Exposure rates as well as concentrations could be underestimated because only trapped individuals were analyzed. During baiting the potential to ingest highly poisoned prey is highest for predators, but through accumulation of lower BR-amounts, the risk could stay high for several weeks.

Before the first application of BR bait in autumn 2011 only 4.7% of small mammals carried BR-residues. This most likely indicates residues from earlier BR-baiting conducted by farmers on some farms. Before seasonal BR applications, more BR-residues were found in winter than in autumn where BR had been used previously, whereas residues of other AR-substances were rare. BR, like other SGARs, persists for a long time in the liver of vertebrates; half lives in the liver of mice are 307.4 days (Vandenbroucke et al., 2008) and 113.5 days in rats (Fisher et al., 2003). Persistence could explain BR-residues before baiting in winter, because the interval between autumn and the following winter was shorter than between winter and autumn. The fraction of farms untreated in the previous season differed somewhat among trapping events. This may have increased BR-residues during and after baiting in winter. Tosh et al. (2011b) suggested remaining bait to be responsible for residues in non-target species months after application, but we found residues in small mammals months after baiting although all baits had been removed. This indicates that BR persistence caused long lasting occurrence of AR residues in small mammals in our study. It seems unlikely that small mammals with BR residues moved in from neighboring farms because 90% of residues occurred in animals trapped within 50 m of bait stations. This distance is less than half the minimum distance to the next farm (160 m).

Exposure rates as well as concentrations varied among small mammal taxa. Beside habitat preferences, AR toxicology could explain differences in AR residue concentrations but data on AR toxicology are lacking for non-target small mammals. *Apodemus* species inhabit a wide range of habitats (Benhamou, 1988; Braun and Dieterlen, 2005) and can have large home ranges (Benhamou, 1988; Crawley, 1969; Korn, 1986). Accordingly, *Apodemus* species were trapped on all farms at all distances, and in large numbers (Fig. 1). Behaviorally mediated access to ARs was reflected by the highest maximal concentration of BR-residues in *Apodemus*. AR-occurrence in *Apodemus* species in our study is consistent with findings from Brakes and Smith (2005), Townsend et al. (1995) and Cox and Smith (1990), where bromadiolone or coumatetralyl was used combined with bait markers. In our study, most concentrations >1 μg/g (1.724 μg/g when recovery corrected) occurred in *Apodemus* species, which may have had lethal consequences as mentioned before. This may account for the considerable decrease in the number of *Apodemus* trapped <15 m from bait stations after baiting, whereas there was no decline at other distances from bait stations. Furthermore high BR-residue concentrations in *Apodemus* indicate high risk to predators, when they prey on these animals.

BR-residues in *Microtus* voles were rare (9 out of 130) confirming Brakes and Smith (2005), where residue occurrence in *Microtus agrestis* was lowest, and bait uptake did not (Cox and Smith, 1990) or rarely (Elliott et al., 2014) occur. Residues in *Microtus* species >1 μg/g occurred only on farms, where grassland bordered farm buildings. This could possibly be explained by the smaller niche of habitat and food pattern of *Microtus* voles compared to *Apodemus* mice and *M. glareolus* (Braun and Dieterlen, 2005; Hansson, 1971a,b). Thus, the risk of primary poisoning for *Microtus* seems lower than for *Apodemus*.

Five out of 168 analyzed *M. glareolus* had higher residues than 1 μg/g, suspecting little lethal impact on this species. However, BR-residues occurred regularly in bank voles indicating considerable potential to disperse BR through the environment. AR-residue occurrence and concentrations in *M. glareolus* were lowest in autumn 2011, although trap success for *M. glareolus* was highest. In 2011, there was an especially high abundance of acorns and nuts (beech mast; Falkenried, 2012). It is likely that *M. glareolus* rarely fed on bait in autumn 2011 due to plentiful alternative food supply, which ultimately reduced AR-residues.

We found BR-residues in *Sorex* species although they are insectivores (Churchfield, 1986; Corbet and Southern, 1977). Shrews are reported to consume difenacoum and coumatetralyl bait (Brakes and Smith, 2005; Townsend et al., 1995). Residues in *Sorex* shrews may have occurred through secondary exposure via invertebrates, which are known to feed on BR bait (Booth et al., 2003; Elliott et al., 2014; Ogilvie et al., 1997), like suggested for European hedgehogs (Dowding et al., 2010). Residues in *Sorex* shrew were generally <1 μg/g, which supports the exposure path via invertebrates. In contrast to this, 5 out of 25 *C. russula* carried BR-residue concentrations >1 μg/g, suggesting primary poisoning in this species.

Before the baiting campaign "high risk" species were not equally distributed among farms. Species distribution and relative abundance may affect the overall occurrence of BR residues and explain high variability in this regard among farms. This makes risk prediction for primary exposure of small mammals as well as secondary exposure of predators more complicated. Nevertheless, secondary poisoning seems particularly likely, if predators prey on species with regular BR-residue occurrence and high concentrations such as *Apodemus* and *M. glareolus*; both of which were highly prevalent on most farms.

Coumatetralyl, difethialone and flocoumafen were not used in our baiting campaigns and very rarely occurred within small mammals, and concentrations were always low. The occurrence of these substances could suggest small mammals consuming bait outside the farm area or obtained secondary exposure via animals that have carried compounds into the farm area. The occurrence of warfarin and difenacoum residues was related to the presence of BR-residues and mainly occurred when bait was used from a particular lot. Bait analysis confirmed the presence of warfarin and difenacoum in these bait lots suggesting that animals consumed warfarin and difenacoum from the BR bait used in the study and not from neighboring farms. Chlorophacinone was present in several liver samples (17%) in autumn 2011 and occurred on all farms in all sessions but was not related to BR occurrence. Chlorophacinone was registered in Germany for plant protection until July 2010 (BVL, 2010), thus residues could have been due to applications of remaining products, especially because residues occurred in <1% of samples after autumn 2011.

Based on the broad range of species and partly high BR-residue concentrations for animals trapped in the direct farm surrounding, there is a high risk for secondary poisoning of predators in this area via non-target small mammals. In target small mammal species SGAR-concentrations vary from about 1 to >10 μg/g in the liver (Littin et al., 2000; Sage et al., 2008; Winters et al., 2010). In our study the median BR-residue concentration in Norway rats was 6.461 and 25.282 μg/g in house mice. We detected similar concentrations in some non-target species, which adds significant AR-exposure for predators. Nevertheless, on average, residue concentrations in target species were much higher than in non-target small mammals. This was expected to be the case because target species were trapped in the baiting area and both trapped animals and carcasses of target species were used for analysis.

In feeding experiments with barn owls in the laboratory the transfer from ARs in poisoned small mammals to owls was measured and death occurred after sufficiently feeding on poisoned prey (Mendenhall and Pank, 1980; Newton et al., 1990, 1994). For example, Newton et al. (1990) investigated barn owls which were fed with BR poisoned mice for one, three or six days. Mice allowed to feed on bait for one day, died 2–11 days later, and showed liver residues of 2.127 μg/g. Three

poisoned mice per day were fed to barn owls. Four of 6 barn owls from the one day treatment died. The other two owls survived the 3 and 6 day treatments but showed prolonged bleeding. Our results suggest that such secondary poisoning could occur during the use of BR for rodent control, when barn owls regularly hunt closely around the baited farm area, because high residue concentrations were restricted to that area.

To derive detailed risk assessments for secondary poisoning of predators such as barn owls from AR-residue concentrations in non-target small mammals it is necessary to assess diet components of predators and the relative occurrence of BR in prey species.

5. Conclusions and outlook

The use of ARs on farms can result in primary exposure of non-target small mammals. Some of them are protected in Germany. The protected *Apodemus* species were trapped most often around farms. *Apodemus* had easy access to bait stations set around farm buildings, which resulted in regular and partially high brodifacoum (BR) residue concentrations in this species. Thus, primary exposure of non-target small mammals is a problem associated with rodenticide. Our findings add to the concerns expressed from the ample studies of secondary exposure of predators to ARs.

We were able to define spatial and temporal patterns in primary exposure of non-target small mammals, with the main exposure occurring close to the baited area. Most high residue concentrations occurred shortly after baiting campaign started, whereas more residues but with lower concentrations occurred three weeks after commencement of baiting. Thereby we established an important base to develop risk mitigation strategies to reduce risk for non-target small mammals according to biocidal AR usage. Furthermore, the fact of primary exposure in non-target small mammals results in a considerable risk of secondary exposure of predators via non-target small mammals. This risk is additional to those associated with ingesting the rodent pest species targeted by the poisoning regime. As some predators like barn owls (Görner, 1979) mainly prey on non-target small mammals this route of exposure seems even more likely and has possibly been underestimated until now.

Many factors seem to affect the occurrence of AR residues in non-target small mammals because we found considerable variability between farms. Factors could be habitat structure around farms, alternative food supply for small mammals, rodenticide sensitivity of non-targets, and the behavior of poisoned animals. Further research on this topic would improve risk assessment and strengthen risk mitigation strategies.

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