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Accumulation of anticoagulant rodenticides (chlorophacinone, bromadiolone and brodifacoum) in a non-target invertebrate, the slug, *Deroceras reticulatum*

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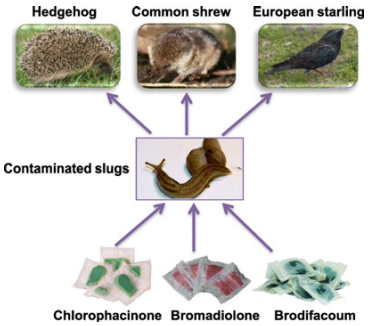
HIGHLIGHTS

- Chlorophacinone, bromadiolone and brodifacoum accumulate in slugs.
- In the field, brodifacoum was detected in >90% of analyzed slugs.
- Brodifacoum baits represent a high risk of secondary poisoning for slug predators.

GRAPHICAL ABSTRACT



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ABSTRACT

Anticoagulant rodenticides (ARs) are used worldwide to control populations of agricultural and urban rodents, but these pesticides may be accumulated in and poisoned non-target species of wildlife. Slugs may feed on rodenticide bait following field applications. Thus, it can be assumed that their predators are exposed to rodenticides through food chain transfer. However, AR exposure in the slugs has not been systematically studied. We investigated the accumulation of three ARs (chlorophacinone, bromadiolone or brodifacoum) in the slug *Deroceras reticulatum* exposed for a period of 5 days followed by depuration time of 4 days in the laboratory. Moreover, we studied the exposure of slugs to brodifacoum in the field. In the laboratory exposure, the slugs consumed rodenticide baits, but no mortality was observed. After 1 day, their concentrations were stable over the time and no differences were detected between the concentrations of the three ARs. After 5 days of exposure, mean concentrations in slugs were 1.71, 1.91 and 0.44 mg/kg wet weight for chlorophacinone, bromadiolone and brodifacoum respectively. A significant decrease of bromadiolone and brodifacoum in slugs was observed in the post exposure period. In the field study, brodifacoum was detected in >90% of analyzed slugs after application of brodifacoum baits. Then, based on a toxicity-exposure ratio approach, we found that slug consumption may represent a risk of secondary poisoning for three of their predators under acute, repeated or subchronic exposure scenarios. These results suggest that the slugs are not only the potential subject to primary exposure, but also the source of secondary exposure for their predators following application of rodenticide baits.

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1. Introduction

Anticoagulant rodenticides (ARs) are highly toxic compounds and widely used for pest control of rodents (Eason et al., 2002; Ruiz-Suárez et al., 2014). They act by inhibition of vitamin K epoxide reductase, and as a result block the vitamin K cycle, which is essential for the production of blood-clotting factors (Pelfrène, 2010).

The first generation anticoagulant rodenticides (FGARs) such as warfarin, chlorophacinone and diphacinone were introduced in the late 1940s. The second generation anticoagulant rodenticides (SGARs) such as bromadiolone, brodifacoum, difenacoum, difethialone and flocoumafen were developed in the 1970s, they generally are more effective, toxic and persistent for animals than FGARs (Thijssen et al., 1989; Eason et al., 2002; Pelfrène, 2010). Non-target species may be affected by ARs, either directly through consumption of poisoned baits or indirectly through consumption of contaminated preys (secondary poisoning) (Berny et al., 1997; Lambert et al., 2007).

Since many years, numerous studies have reported the secondary exposure of predatory birds or mammals due to the consumption of poisoned rodents (Berny et al., 1997; McDonald et al., 1998; Shore et al., 1999, 2003a; Sánchez-Barbudo et al., 2012; Langford et al., 2013; Stansley et al., 2014; Poessel et al., 2015). Moreover, the exposure of insectivorous birds and mammals to ARs was reported (Borst and Counotte, 2002; Dowding et al., 2006; Dowding et al., 2010) and invertebrates, including insects, snails and/or slugs, were suggested as a potential route of contamination (Spurr and Drew, 1999; Elliott et al., 2014). Indeed, a variety of invertebrates, including slugs, were observed on and around baits after the application of ARs in New Zealand, Spain or Hawaiian forests (Morgan et al., 1996; Spurr and Drew, 1999; Dunlevy et al., 2000; Elliott et al., 2014; Hernández-Moreno et al., 2013). The accumulation of ARs in invertebrates, exposed potentially by direct consumption of baits, and/or rodent carcasses and faeces and/or by ingestion of soil-bound residues, was shown in arthropods, earthworms and gastropods, including slugs (Spurr and Drew, 1999; Dunlevy et al., 2000; Craddock, 2003; Eason et al., 2002; Hernández-Moreno et al., 2013; Liu et al., 2015). The diet of many mammals and birds, such as European hedgehog or European starling, consists of slugs that could therefore represent an important pathway of AR transfer for their predators. However, exposure of slugs to ARs was rarely studied and the occurrence of AR residues in slugs was not well characterized.

The aim of this study is to estimate (i) the accumulation of three anticoagulant rodenticides (chlorophacinone, bromadiolone and brodifacoum) in the slug *Deroceras reticulatum* exposed to these ARs in laboratory conditions and (ii) the accumulation of brodifacoum in the field. Then, in order to test whether invertebrates may cause lethal or sublethal poisoning of some of their predators by ARs, we calculated the dose of brodifacoum ingested daily by three carnivorous, including the Common shrew, the European starling and the Hedgehog, eating slugs randomly among those collected in the field trial. Then, based on a toxicity-exposure-ratio approach, we determined the subsequent risk of secondary poisoning under acute, repeated and subchronic exposure scenarios.

2. Materials and methods

2.1. Laboratory exposure study

This experiment was conducted in the laboratory of Vetagro Sup in Lyon, France. Slugs for control analyses and laboratory exposure tests were collected in April 2016 from some areas near the city of Lyon without history use of ARs. After collection, slugs were kept in an incubation chamber under controlled conditions of temperature (18 ± 1 °C) and moisture content in soil (30–35%, local agricultural soil). After acclimatization for three days, 120 healthy slugs weighing 100–1000 mg were selected and placed randomly in individual petri box for testing with wax block baits. These slugs received chlorophacinone (green bait Rozol pat 0.005%), bromadiolone (red bait Maki®pat 0.005%) or brodifacoum (blue bait Saphir pasta® 0.004%), 40 individuals for each rodenticide during an exposure period from 1 to 5 days and a post exposure period. Fifteen individuals were collected each day ($n = 5$ for each rodenticide). Blocks were cut to administer them to slugs. A total of 5 g of rodenticide bait per slug was offered, as the only food during exposure period. Daily bait consumption was checked by the visual presence of bait color in the faeces. It was not possible to determine the exact amount of bait ingested, because of the high hydrophilicity of the bait associated to the high humidity in the enclosure, which was maintained to keep slugs active and in good conditions. At the end of the exposure, the remaining slugs received only salad during a post exposure period of 4 days during which 45 slugs were collected at 1 d (15 slugs), 3 d (15 slugs) and 4 d (15 slugs).

The slug samples were finally collected, fast frozen at −80 °C and stored at −20 °C until use. The concentration of active ingredient in the Block baits was confirmed by HPLC analysis.

2.2. Field exposure study

Field study was carried out in a local area near Lyon (France), in November 2016. Tamper-resistant plastic bait stations were placed on the ground at 10 m intervals along rat trails. A total of 12 bait stations were placed on site. Bait stations were checked daily for food consumption, slug presence and slug collection during 3 weeks. We found twenty-three slugs on day 3 (7 slugs), day 14 (8 slugs) and day 15 (8 slugs) after the distribution of brodifacoum baits.* These slugs were collected, identified and stored at −20 °C until use. Brodifacoum concentration in bait, 0.0024%, was confirmed by HPLC analysis.

2.3. Residue analyses

Each slug sample was analyzed separately, homogenized and blended in 10 ml of acetone using Ultraturrax® (Ika,Werke, Germany). The whole slug was used for analysis (total weight 100–1000 mg). The obtained extract was centrifuged at 4000*g* for 10 min and evaporated to dryness under a stream of nitrogen at 60 °C. The residues were reconstituted to 2 ml of methanol, mixed in vortex 30 s, and centrifuged at 4000*g* for 10 min. Reconstituted extracts were transferred to clean test tubes, and evaporated to dryness. The dry extract was reconstituted with 2 ml of acetonitrile, vortexed for 30 s, and cleaned with 2 ml of hexane. After agitation for 20 s in vortex, the upper hexane phase was removed using a Pasteur pipette. The extract was evaporated to dryness under a stream of nitrogen at 60 °C and reconstituted to 0.5 ml of mixture of 70% methanol and 30% phosphate buffer (*v*/*v*) for analysis by HPLC. The concentration of these ARs is given in mg/kg wet weight. Individuals collected in an area with no AR treatment were used as a negative control. The recovery level on spiked samples was always >92%.

2.4. High performance liquid chromatography (HPLC)

Concentrations of ARs (chlorophacinone, bromadiolone and brodifacoum) were quantified by high-performance liquid chromatography (HPLC). The used HPLC system is equipped with an isocratic pump (L6000), an automatic sampler (AS2000), UV detector, a fluorimetric detector (F1000) and integration software. 15 μl of each sample or standard solution (Pestanal® Sigma-Aldrich, purity 99.5%) was injected in a C18 column (10 nm pores, 5 μm granule size), 250 × 4 mm (Chromcart Nucleosil, Macherey–Nagel, Strasbourg, France). The elution solution was prepared with 70% methanol and 30% of phosphate buffer (*v*/*v*) (pH = 6.5). The phosphate buffer solution was prepared from disodium hydrogen phosphate dehydrate and potassium

* Confidential field trial: Only the bait type, active substance and concentration were known.

dihydrogen phosphate (both > 99% purity). Bromadiolone and brodifacoum concentrations in samples were determined fluorimetrically (excitation wavelength of 250 nm and emission wavelength of 350 nm). Chlorophacinone was detected under UV light at 286 nm. Linearity was determined with 5-points calibration curves ($R^2 > 0.99$) on standard solutions and spiked samples (standard solutions added to control slugs before extraction from 0.02 to 0.5 mg/kg). Detection limit was 0.02 mg/kg for all ARs tested in spiked slugs.

2.5. Statistical analysis

Values were expressed as mean ± standard error of mean (SEM) for all the experiments. Coefficients of variation were also computed (CV = SEM * 100 / Mean). Chlorophacinone, bromadiolone and brodifacoum residue data and residuals were examined for normality using the Shapiro-Wilk test. Non parametric test (Mann-Whitney Wilcoxon or Kruskal-Wallis) were used in case of lack of normality to compare concentrations between days during the exposure period for each rodenticide. Pooled data for each AR rodenticide exposure were compared to determine if there was a difference between groups in terms of accumulation (Kruskal-Wallis). The Spearman correlation test was used to analyze residue concentration over time (absorption, elimination) comparing concentrations in slugs each day for the accumulation or elimination period to identify a significant positive or negative trend. Significance level was set at $p \leq 0.05$ and statistical analysis was computed using the R 3.1.1 computer program (http://R-project.org).

2.6. Risk assessment for slug predators

We used a toxicity-exposure ratio (TER) approach to assess the risk that slugs exposed to brodifacoum in the field may represent for three of their predators, two mammals, the European hedgehog (*Erinaceus europaeus*) and the Common shrew (*Sorex araneus*) and a bird, the European starling (*Sturnus vulgaris*). This methodology is specifically developed for birds and mammals exposed to pesticides (European Food Safety Authority, 2009). The characteristics of the predators used for risk assessment are presented in Table 1. The exposure factor was calculated as the daily dose of brodifacoum ingested by each predator (expressed in mg brodifacoum/kg body weight/day) based on the brodifacoum concentration in slugs measured in the field. We assumed that the slugs constitute 4%, 20% and 6.5% of the biomass of diet ingested daily by the three predators, respectively (Table 1). For each predator, predation was simulated by randomly sampling slugs among all the individuals collected in the field and assuming that, in a treated site, slug population may mix individuals exposed for 3 days or for 14–15 days. When the biomass of slugs sampled reached the biomass ingested daily by a predator (Table 1), the sampling was stopped and the quantities of brodifacoum contained in predated slugs were summed and divided by the body mass of the predator. For each predator, the dose of brodifacoum was calculated using Monte Carlo simulations based on 500 random samplings in slug population. The toxicity factors were retained in order to simulate different scenario of exposure: acute single dose exposure corresponding to acute oral LD50 for rat (0.4 mg/kg bw/day) or for bird (mallard duck, 0.31 mg/kg bw/day), repeated 5-days exposure corresponding to oral LD50 5d for rats (0.06 mg/kg bw/day) or for birds (laughing gull *Leucophaeus atricilla*, 0.07 mg/kg bw/day) and/or subchronic exposure (repeated 90 days) corresponding to the no-adverse-effect level (NOAEL) determined for rats (0.001 mg/kg bw/day) or for birds (calculated from toxicity test of difenacoum on Japanese quail *Coturnix japonica*, 0.000385 mg/kg bw/day). All these toxicity factors were proposed by the Standing Committee on Biocidal Products (2009). Then, the risks for predators were assessed as TER = LD50 or NOAEL/daily dietary dose. The acute and repeated-5d risks were evidenced when TER < 10 while a subchronic risk corresponded to TER < 5 (European Food Safety Authority, 2009). For each exposure scenario, risk was expressed as the probability that the TER < 10 or 5 and was calculated using Monte Carlo simulations as described above. The elimination half-life for AR residues in slugs was determined from the regression of log [AR] over time (Riviere, 2011).

Table 1
Ecological characteristics of the 3 slug predators used in risk assessment.

Slug predator	Body mass[a] (g ww)	Daily food intake (g ww/d)	% slug in diet
Hedgehog *Erinaceus europeus*	1100	100[b]	4[b]
Common shrew *Sorex araneus*	12	18.75[c]	20[e]
European starling *Sturnus vulgaris*	80	50[d]	6.5[f]

[a] Crocker et al., 2002.
[b] Yalden, 1976.
[c] Estimated from Churchfield, 1982.
[d] Estimated from Feare and McGinnity, 1986.
[e] Estimated from Whitaker et al., 1983.
[f] Cited in South, 1992.

3. Results and discussion

3.1. Accumulation of rodenticides in slugs in the laboratory experiment

Our laboratory and field exposure trials showed that all slugs consumed chlorophacinone, bromadiolone and brodifacoum baits, and AR residues were detected in these slugs, as previously reported in field studies (Spurr and Drew, 1999; Dunlevy et al., 2000).

The results we obtained indicate that chlorophacinone, bromadiolone and brodifacoum are accumulated in slugs rapidly as residues were detected from the first day of exposure in 100% of the sampled individuals. Then, the concentrations were stable over time and were not significantly different between the 3 ARs (KW $p > 0.05$). The highest average chlorophacinone residues were observed on the third day followed by the fourth day, with 2.05 and 1.92 mg/kg wet weight, respectively. The maximum average residues of bromadiolone was found on the fifth day followed by the first day, with 1.91 and 1.78 mg/kg, respectively. The maximum average values of brodifacoum was measured on the third day followed by the second day, with 0.77 and 0.65 mg/kg, respectively (Fig. 1). This can be explained by the fact that the amount of bait ingested was different between slugs. Moreover, as evidenced by the lack of bait color in faeces, some slugs did not continuously consume bait throughout the period of exposure. It should be noted, however, that the obtained results were highly variable (CV > 30%), which may be attributed to the variable food or bait intake as well as variable body mass/water content. The measured concentrations in slugs in laboratory exposure correspond to what is accumulated and also to what is found in the digestive tract.

Previous researches have also concluded that snails feeding on the bait also accumulated brodifacoum (Booth et al. 2001, 2003; Craddock, 2003; Bowie and Ross, 2006). According to Booth et al. (2003), the maximum brodifacoum residue level found in both the body and foot tissue of snails was 3.9 and 1.2 mg/kg wet weight, respectively, whereas the highest residues of brodifacoum in earthworms were 0.90 mg/kg ww. Moreover, Liu et al. (2015) observed in a laboratory experiment that bromadiolone in soil can be accumulated in earthworms, with maximal residue 0.44 mg/kg ww, and they showed that bromadiolone is toxic for earthworms at 1 mg/kg soil, which is a likely concentration in the field following application of bromadiolone baits. Primus et al. (2006) studied the exposure of two species of slugs (*Deroceras laeve* and *Limax maximus*) to baits containing diphacinone (FGAR), and this rodenticide was measured in whole slug, at concentrations ranging from 1.3 to 4.0 mg/kg dw for *D. laeve* and <LOD to 1.8 mg/kg dw for L. *maximus*.

Blood-clotting mechanisms in invertebrates are different to those found in vertebrates, so invertebrates are less sensitive to ARs than

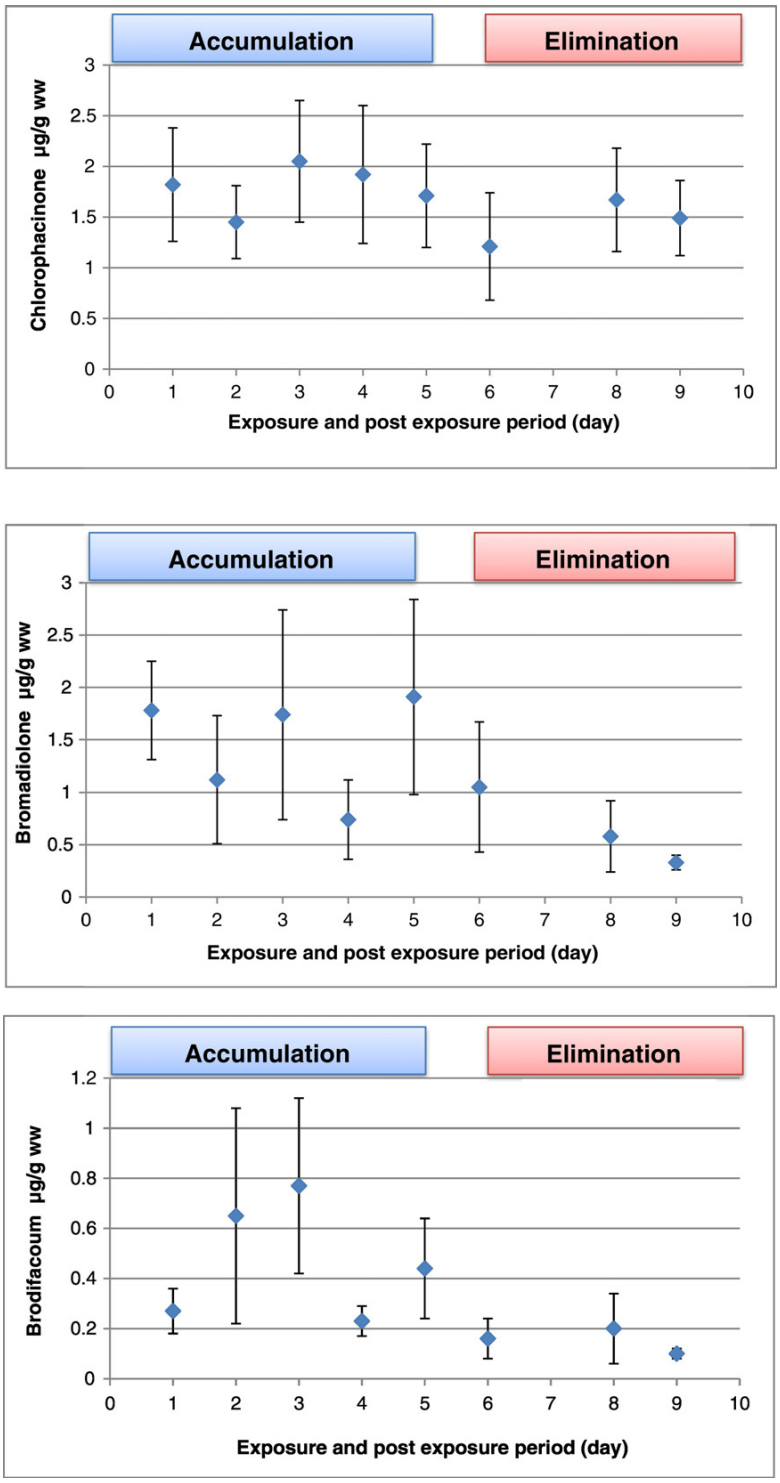

Fig. 1. Mean accumulation and elimination of chlorophacinone, bromadiolone and brodifacoum in slug (µg/g wet weight) (for each day, 5 slugs were analyzed) (The bars represent the SEM).

mammals and birds (Pain et al., 2000; Craddock, 2003; Johnston et al., 2005a, 2005b). In our study, no mortality was observed in any of the slugs exposed to the three used ARs during the course of the study, similar tolerance to ARs was also reported in previous studies (Shirer, 1992; Eason and Spurr, 1995). This observation indicates that the acute primary toxicity of chlorophacinone, bromadiolone or brodifacoum to the slug *D. reticulatum* is probably low even if it cannot be quantified from the present results.

During laboratory exposure period, chlorophacinone, bromadiolone and brodifacoum residues in slugs ranged 0.6 to 3.3 mg/kg, 0.2 to 4.24 mg/kg and 0.07 to 1.65 mg/kg, respectively. The decrease of bromadiolone and brodifacoum concentrations in slugs was significant

during the post exposure period (Spearman test $p = 0.002$ and 0.02, respectively), whereas this decrease was not significant for chlorophacinone (Spearman test $p = 0.26$) (Fig. 1). It can be suggested that these ARs were eliminated quickly (some days) from slug through metabolism and/or fecal excretion. We estimated the elimination half-life for bromadiolone and brodifacoum at 1.9 (±0.6) and 2.5 (±0.9) days respectively. For chlorophacinone, the elimination half-life was estimated at 4.0 (±1.2) days. Prolonged persistence of chlorophacinone residues may result in prolonged exposure of predators. These elimination half-lives are considerably shorter than those observed in rodents (for instance: Brodifacoum and bromadiolone >6 months, chlorophacinone >3 months) (Erickson and Urban, 2004).

3.2. Accumulation of brodifacoum in slugs in the field and risk assessment for slug predators

In our field study, no invertebrates except slugs were observed on the rodenticide baits, may be because of the low activity of most of the invertebrates during the field trial in November. Similarly, we did not detect slugs every day, but it should be noted that the period was cool and dry and only 2 days of rain were recorded during the exposure period (on day 2 and day 14). However, previous studies identified a broad range of invertebrate species visiting bait stations (Spurr and Powlesland, 1997; Sherley et al., 1999; Spurr and Berben, 2004; Bowie and Ross, 2006). Brodifacoum was detected in 21 slugs from a total of 23 samples. The concentrations ranged from <LOD to 10.6 mg/kg wet weight (Fig. 2). It means that >90% of analyzed slugs were contaminated with brodifacoum during the field trial. These concentrations are quite similar or even higher than those measured in the laboratory experiment, but we need to mention that we do not know how long these slugs have been exposed (from 1 to 15 days). It should also be mentioned that there were only two days of rain during the field trial, and it may well be that slugs were active and fed more actively during these two days. We could not detect evidence of mortality in slugs in the field. Insectivorous birds and mammals could be exposed to ARs in baiting areas via consumption of gastropods, including contaminated slugs. According to different scenario of exposure, we showed that the European hedgehog, the Common shrew and the European starling can be exposed to high doses of brodifacoum via slug consumption. The hedgehog is exposed to the lowest median doses, 0.007 mg brodifacoum/kg bw/d, whatever the scenario, while the European starling ingested doses that are approximately 10 times higher (Table 2). With a median exposure of 0.576 to 0.580 mg brodifacoum/kg bw/d, the Common shrew is the most exposed predator (Table 2). The calculated toxicity-exposure ratios suggest that such levels of exposure could be at risk in most of the cases (Fig. 3). The absence of risk was determined in only one scenario corresponding to a hedgehog consuming slugs for one day. In this case, the doses ingested were from 40 to 100 times lower than the LD50 for rats (Fig. 3). We calculated that the dose of brodifacoum would lead to a TER < 10, the threshold retained for a risk, if contaminated slugs constitute 23.4% of the biomass ingested by a hedgehog. Assuming a repeated exposure of a hedgehog for 5 days, the TER were <10 in 80% of the simulations (Fig. 3), suggesting that, under this realistic scenario, slugs could be a pathway leading to lethal poisoning of hedgehog. The frequent exposure of hedgehog to rodenticides has been previously shown in UK (Dowding et al., 2010). Residues were detected in the liver of 66.7% of the individuals studied ($n = 120$), difenacoum and bromadiolone being the most frequently detected. The authors did not investigate the main routes of exposure of hedgehogs to ARs but they assumed that predation of contaminated invertebrates is likely to be a major pathway. Moreover, although no evidence of lethal poisoning was found, this cannot be excluded because animals with fatal doses may become lethargic some hours before death and die in cryptic locations (Dowding et al., 2010). The modeling approach developed in this work reinforces these hypotheses about the possible role of invertebrates in lethal or sublethal poisoning of hedgehog by AR. For the Common shrew feeding on slugs, the risk posed by brodifacoum is high even in the acute exposure scenario in which the TER < 1 in 99% of the simulations (Fig. 3). We also calculated that the repeated consumption of slugs during 5 days would probably kill shrews (TER < 0.16, Fig. 3) and that subchronic exposure is also at high risk (TER < 0.003, Fig. 3). In a site treated with brodifacoum for rat control, a high residue occurrence was measured in the White-toothed shrew *Crocidura russula* (66% of individuals) and *Sorex* spp. (28%), but neither the exposure pathway nor effects were investigated (Geduhn et al., 2014). It has been proposed that shrew exposure could be due to both primary (direct bait consumption) and secondary poisoning caused by invertebrate predation (Brakes and Smith, 2005). Finally, feeding on contaminated slugs would be detrimental also for the European starling in all scenarios. In the case of acute and/or repeated exposure scenario, our assessment is consistent with previous zoo case reporting that the insectivorous birds died from eating ants and cockroaches that had fed on brodifacoum baits (reported by Godfrey, 1985). Moreover, in the cultivated crops areas, populations of slugs are strongly variable according to weather conditions of the soil and culture conditions. Studies carried out in UK indicate that the populations varied from 10 to 100 slugs per m^2 with peaks at 500 slugs per m^2 (Glen et al., 1996). Despite our limited sampling period, we could determine that, after the end of exposure, the calculated half-life for AR in slugs is around 2–2.5 days for brodifacoum and bromadiolone and up to 4 days for chlorophacinone. It can be estimated that slugs would need 10–15 days (i.e., 5 half-lives) to eliminate completely bromadiolone or brodifacoum residues and up to 20 days to eliminate chlorophacinone (Riviere, 2011).

Table 2
Dose of brodifacoum ingested by three slug predators (in mg brodifacoum/kg bw/d) according to different scenario of exposure (values are median, 2.5 and 97.5 percentiles in parenthesis).

	Exposure scenario		
	Acute	Repeated	Subchronic
Hedgehog	0.0069 (0.0046–0.0090)	0.0069 (0.0052–0.0088)	0.0070 (0.0051–0.0091)
Common shrew	0.576 (0.401–0.799)	0.580 (0.420–0.760)	0.576 (0.444–0.781)
European starling	0.070 (0.045–0.096)	0.069 (0.049–0.091)	0.071 (0.051–0.095)

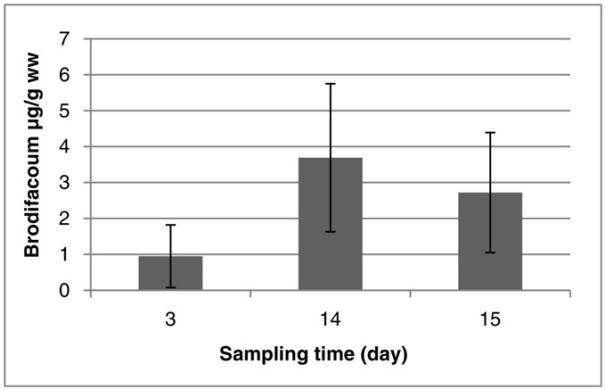

Fig. 2. Mean residues of brodifacoum in field-exposed slugs (for 3, 14 and 15 days of exposure, 7, 8 and 8 slugs were analyzed, respectively) (The bars represent the SEM).

4. Conclusion

The use of ARs in the field can result in primary exposure of non-target invertebrate such as slugs, which can consume AR baits and accumulate residues in their bodies. Therefore, several insectivorous birds

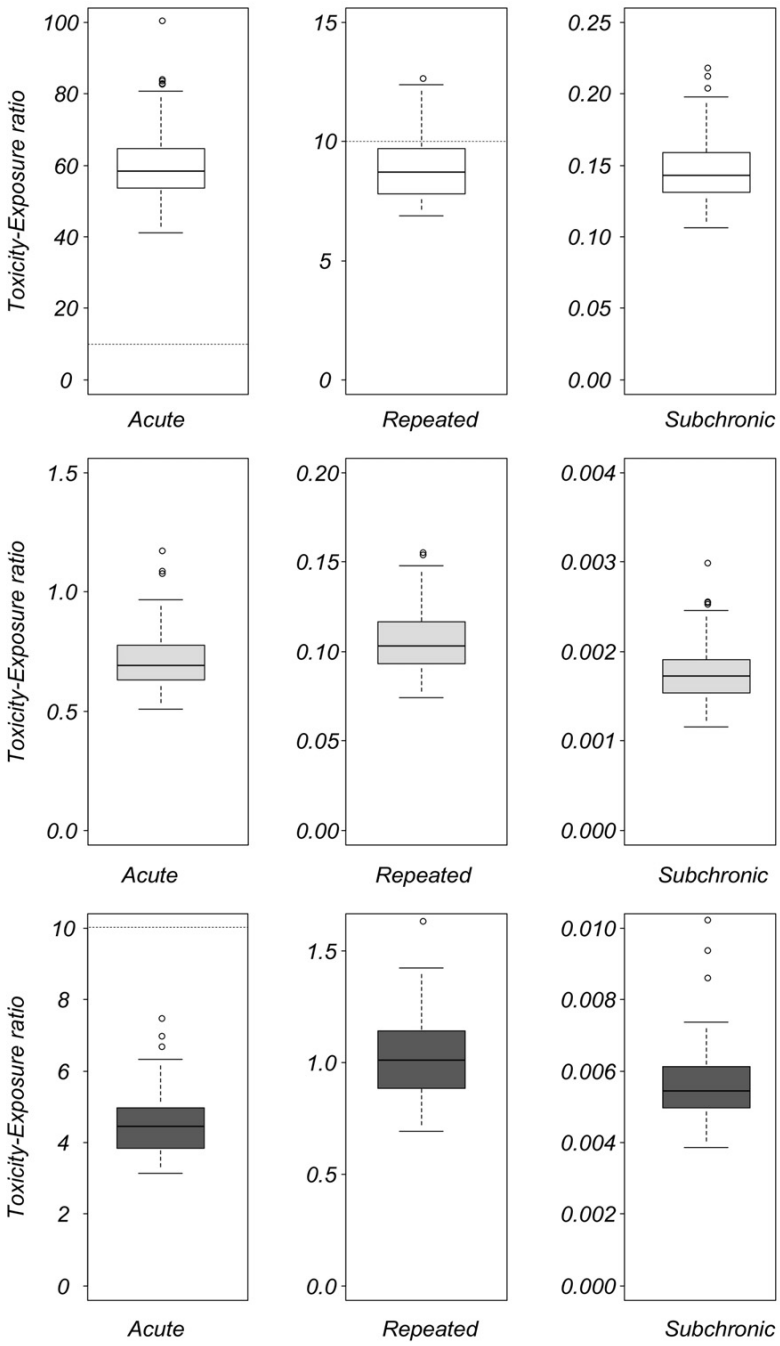

Fig. 3. Toxicity-Exposure Ratio (TER) calculated for three slug predators (hedgehog in white, Common shrew in light grey, European starling in dark grey). For both acute and repeated scenario, risk is evidenced when TER < 10 while TER < 5 is retained for subchronic scenario.

and mammals could be exposed to these rodenticides in baiting areas via consumption of contaminated slugs. The exposure can also continue during few days after removal of rodenticide baits. Our TER risk analysis based on brodifacoum residues in slugs collected from the field, shows that the slugs represent a high risk of secondary poisoning for 3 of their predators, the European hedgehog, the Common shrew and the European starling. It means that the contamination of slugs results a potential risk for non-target intoxication in terms of species and individuals. Therefore, it is recommended to implement a protocol of AR application to mitigate the risk of ARs baits for non-target invertebrates. For instance, (1) AR baits should not be applied during slug activity unless necessary, (2) after the application of AR baits, we have to examine the plastic bait stations every day to remove any presented slugs.

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