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PREVALENCE OF ANTICOAGULANT RODENTICIDES IN FECES OF WILD RED FOXES (*VULPES VULPES*) IN NORWAY

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ABSTRACT: High occurrence of anticoagulant rodenticides (AR) in wildlife is a rising concern with numerous reports of secondary exposure through predation. Because of widespread distribution of the red fox (*Vulpes vulpes*), they may act as sentinels for small mammal-hunting predators in rural, suburban and urban areas. No AR surveillance in wild mammals with analyses of residues in feces has been conducted throughout a country. We collected 163 fecal samples from presumed healthy red foxes from 18 out of 19 counties in Norway. The foxes were shot during regular hunting between January and December 2016, and samples collected directly after death. Fecal samples were analysed for six AR: brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, flocoumafen. We detected AR in 54% (75/139) of the animals. Brodifacoum was most frequently detected (46%; 64/139), followed by coumatetralyl (17%; 23/139), bromadiolone (16%; 22/139), difenacoum (5%; 7/139), difethialone (1%; 2/139) and flocoumafen (1%; 2/139). More than one substance was detected in 40% (30/75) of the positive foxes, and 7% (5/75) of these animals were exposed to four different AR. There were no statistically significant seasonal, age or sex differences in foxes after exposure to one AR compound. We found a significant difference in occurrence of brodifacoum and coumatetralyl in foxes from different geographical areas. These findings demonstrate fecal analyses as a valuable method of detecting AR exposure in red foxes. We suggest using direct fecal sampling with analyses as a method to evaluate the occurrence of AR in live endangered wildlife in connection with radio tagging or collaring operations.

Key words: Carnivores, fecal analyses, non-target animal, predators, rat poison, secondary exposure, wildlife

INTRODUCTION

Use of anticoagulant rodenticides (AR) for urban and agricultural rodent control has been extensive the past 60 yr. These rodenticides inhibit vitamin K epoxide reductase, and are designed to induce lethal hemorrhage (Watt et al. 2005). First-generation anticoagulant rodenticides (FGAR), including warfarin, diphacinone, coumatetralyl and chlorophacinone, were developed in the 1950s. Extensive use of FGAR led to resistance against these rodenticides both in brown rats (*Rattus norvegicus*) and house mice (*Mus musculus*) resulting in their acquired and inherited tolerance and cross-resistance between compounds (Rowe and Redfern 1965; Greaves and Rennison 1973; Hadler and Shadbolt 1975). This prompted the development of second-generation anticoagulant rodenticides (SGAR), such as brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen. Compared to FGAR, SGAR have higher toxicity and prolonged liver half-life, and are effective after a single exposure (Watt et al. 2005). The SGAR can cause mortality after several days, allowing animals to ingest multiple doses and accumulate high concentrations in their body (Daniels 2013).

Predators can accumulate AR through ingesting bait (primary exposure), by consuming poisoned prey (secondary exposure) or by ingesting prey secondarily exposed to AR (tertiary exposure; Daniels 2013; Gabriel et al. 2018). Wildlife studies in Europe and North America have shown 23-100% AR occurrence in liver samples from predators such as American mink (*Neovison vison*; Ruiz-Suárez et al. 2016), bobcats (*Lynx rufus*; Riley et al. 2007; Serieys et al. 2013), stoats (*Mustela erminea*) and weasels (*Mustela nivalis*; McDonald et al. 1998; Elmeros et al. 2011), red foxes (*Vulpes vulpes*; Tosh et al. 2011; Tjus 2014), polecats (*Mustela putorius*; Shore et al. 2003) and stone martens (*Martes foina*; Elmeros et al. 2018). In Norway SGAR have been detected in raptors found dead in the wild, like the golden eagle (*Aquila chrysaetos*) and

eagle owl (*Bubo bubo*; Langford et al. 2013). To our knowledge, there are no publications investigating AR occurrence in wild mammals in Norway.

Large amounts of AR may cause bleedings and death in animals. Even small amounts of rodenticides in the liver are suspected to cause a variety of sublethal effects. Residues of AR affect reproduction by reducing sperm motility, increasing embryonic mortality, causing teratogenic effects and neonatal death (Greaves 1993; Munday and Thompson 2003; Robinson et al. 2005). Vidal et al. (2009) suggested an association between chlorophacinone residues in voles (*Microtus arvalis*) and increased susceptibility to the bacteria *Francisella tularensi*s. Additionally, a correlation between increased parasite load and AR residues was found in bobcats and fishers (*Martes pennant*) suggesting a chronic weakening of the animal (Gabriel et al. 2012; Serieys et al. 2013). Furthermore, sublethal AR exposure is suggested to increase mortality when the animals are subjected to environmental stressors (Jaques 1962). Finally, rodenticides can reduce body condition of poisoned animals (Elmeros et al. 2011), impairing hunting ability and making them more susceptible to accident, injury, and predation.

The AR have an enterohepatic circulation and accumulate in the liver (Huckle et al. 1988; Watt et al. 2005). Non-target animal exposure to AR is usually measured by analyses of residues in the liver. The major elimination route is through bile and feces (Huckle et al. 1988; WHO 1995). An experiment in foxes demonstrated prolonged excretion of bromadiolone in feces for 2-19 d after no AR residues could be detected in plasma. Fecal residues were still detectable at the conclusion of the experiment (Sage et al. 2010). Because of long fecal elimination of AR, we suggest fecal analysis as a suitable method to investigate this unintended exposure.

The aim of our study was to estimate the occurrence of AR in feces of presumed healthy red foxes throughout a country. In addition, AR exposures were compared between age groups, seasons, and geographical regions with different human population densities.

MATERIALS AND METHODS

Population and study area

We collected 163 fecal samples from red foxes shot by experienced hunters in 2016 (January throughout December) in a project monitoring the parasite *Echinococcus multilocularis* commissioned by the Norwegian Food Safety Authority (Madslien et al. 2017). The samples were collected from 56 municipalities (ranging in size from 7,000-310,600 ha), representing 18 out of 19 counties in Norway and including areas surrounding three major cities in Norway (Oslo, Bergen, Trondheim). The municipalities were divided in groups based on human population density. Population density per square kilometre for each municipality in 2016 was obtained from Statistics Norway (StatisticsNorway 2018).

Sample collection

The hunter removed feces directly from the rectum immediately after death, and submitted fresh samples to the Norwegian Veterinary Institute (NVI) within 2 d. In the statistical analyses, 24 of the 163 samples consisted of mostly hair and were omitted. The foxes were shot during the licensed hunting season from January to mid-April and mid-July to late December, and grouped according to sampling season: winter (n=66) from January-February and December, spring (n=30) from March-May, summer (n=20) from June-August, and autumn (n=23) from September-November. Most samples were collected during the winter, due to preferred tracking conditions in the snow. The hunters provided information on sex (male or female), estimated age (juvenile, <1 yr-old, or adult), together with the municipality and date when the fox was killed.

The hunters estimated age according to foxes' size and the presence of deciduous teeth, and determined the sex based on presence or absence of a penis. Of the 139 foxes analysed, 65 were male, 64 female and the sex of 10 were not determined. The samples were immediately frozen at -80 C upon arrival at NVI and kept frozen at this temperature for 3 d, before being stored at -20 C until preparation. One sample per fox was analysed.

Sample analysis

The samples were lyophilized to dryness before analyses at the laboratory at the Department of Forensic Sciences at Oslo University Hospital. We have previously described and validated procedures for fecal extraction and analysis of AR (Seljetun et al. 2018). In brief, AR were extracted from feces by liquid-liquid extraction with acetonitrile and dichloromethane followed by separation using a Waters Acquity ultra performance liquid chromatography (UPLC) BEH C18 column (Waters Corporation, Milford, MA, USA) with a mobile phase consisting of 5mM ammonium formate buffer (pH 10.2) and methanol. Positive electrospray ionization (ESI+) MS/MS detection was performed on a triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA), using two multiple reaction monitoring transitions. Limits of quantification (LOQs) were set at the level of the lowest calibrators; brodifacoum 2.6 ng/g, coumatetralyl 1.5 ng/g, bromadiolone 2.6 ng/g, difenacoum 2.2 ng/g, difethialone 2.7 ng/g, and flocoumafen 2.7 ng/g. Criteria of signal-to-noise ratios were above 10 as well as precision and accuracy within ±20%. The extraction recovery ranged from 18 to 69%. Concentrations of AR above LOQ were classified as positive, while detectable AR concentrations below quantitation limits were labelled as trace concentrations. The AR analysed in this study were brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone and flocoumafen, which are all registered for use in Norway.

Statistical analysis

After rejecting the 24 of 163 fecal samples that were mostly hair, the 139 remaining samples were grouped according to age, sex, season and human population density. Data from cases where information on age or sex was lacking were excluded in the corresponding proportion estimates. In order to test the sensitivity of the specific categorisation of rural, suburban and urban from human population density, we included variants of population measures. Municipalities with less than 10 inhabitants per km^2 were first categorized as rural, 11-200 inhabitants as suburban, and more than 200 inhabitants as urban. We then reduced the definition of rural municipalities to less than five inhabitants per km^2, and altered suburban municipalities to 6-200 inhabitants. Finally, we categorised municipalities based on population only with rural area (1,000-10,000), suburban area (10,000-50,000) and urban area (50,000-180,000).

Estimated prevalence of foxes positive for AR was calculated for the total of all samples (n=139) and within groups. Differences between prevalence of AR substances were tested using McNemar's Chi-squared test, while significant differences in AR exposure between groups were tested using Pearson's Chi-squared tests. *P*-values of the Pearson's Chi-squared test were obtained by Monte Carlo simulations using 10,000 replicates. Single AR exposure was classified as a sample being positive for one AR compound, and multiple AR exposure was specified as samples being positive for at least two AR compounds.

The relationship between AR exposure and the covariates age, sex and seasons were investigated by multiple logistic regression analyses. The full model included age, sex, and season. However, results from simple regressions were reported if one or the two other covariates did not improve the model according to AIC-value. In order to emphasize possible

confounding effects, potential dependency between samples from the same county was tested for by including a random effect of county (variance of random effect=0), however, the inclusion of a random effect did not influence the results. All analyses were performed using R (version 3.5.0, R Development Core Team). Results were considered significant when *P* values were below 0.05.

RESULTS

Prevalence of anticoagulant rodenticides

At least one AR compound analysed was detected in 54% (75/139) fecal samples (Table 1). Brodifacoum was most frequent and was identified in 46% (64/139) of the foxes, significantly more than coumatetralyl (17%, 23/139; χ^2=30.56, *P*<0.0001, df=1) and bromadiolone (16%, 22/139; χ^2=33.92, *P*<0.0001, df=1; Fig. 1). In contrast, difenacoum was found in only seven foxes (5%), and difethialone and flocoumafen in two samples each (1%). Among the AR positive fecal samples, most samples (60%; 45/75) contained a single AR, but multiple substances were detected in 40% (30/75), with two (27%; 20/75), three (7%; 5/75) and four (7%; 5/75) compounds respectively.

Seasonal variance

Exposure of foxes varied by season with 61% (14/23) foxes positive for AR in the autumn, 53% (35/66) in the winter, 57% (17/30) in the spring and 45% (9/20) in the summer (Fig. 2). There were no significant seasonal differences in exposure to a single AR (χ^2 =1.20, *P*=0.759). In exposure to multiple AR, season tended to be significant (χ^2 =7.17, *P*=0.065); exposures to more than one AR was slightly more common in the autumn compared to spring (Wald test, *P*=0.037) and winter (Wald test, *P*=0.031).

Sex and age differences

Of the 139 foxes analysed, 65 were male, 64 female and the sex of 10 were not determined (Table 1). Fecal residues of at least one AR was detected in 49% (32/65) males, 59% (38/64) females and 50% (5/10) of unknown sex. There was no significantly different in AR exposure between sexes (χ^2 =1.34, *P*=0.299). Exposure to AR between ages ranged from 58% (45/78) adults, 48% (24/50) juveniles and 55% (6/11) of unknown sex. Positive findings were not significantly different between ages for either single nor multiple AR exposure (*P*>0.437). Logistic regression indicated a tendency of positively association between sex and exposure to AR when combined with age. In adult female foxes, 68% (23/34) were positive to AR, compared to 49% (46/93) in a combined group of juveniles and adult male foxes (*P*=0.066).

Prevalence of AR in foxes correlated to human population densities

Foxes in suburban areas had an occurrence of AR of 61% (39/64), compared to rural (48%; 21/44) and urban (48%; 15/31) foxes (Table 2). However, this difference in AR exposure was not statistically significant (χ^2=2.55, *P*=0.285). In order to determine if a change in classification of human population density might influence the results, we repeated the analyses with the alternative measures of rural, suburban and urban category. There was no significant difference between different human population densities in the total exposure, individual compounds differed significantly between population areas. Coumatetralyl was increased in urban compared to rural areas (*P*=0.032), while brodifacoum was increased in suburban compared to urban areas (*P*=0.010). Significant differences were also independent of the specific choices of urban, suburban and rural population densities.

DISCUSSION

Sources of AR exposure

The high prevalence of 54% foxes exposed to AR in our study was most likely due to ingestion of rodents. Rodents dominate their diet, with 26-47% of consumed food volume (Contesse et al. 2004; Kidawa and Kowalczyk 2011). In Norway, season and rodent cycles influence the quantity of rodents fox ingest (Jensen and Sequeira 1978; Panzacchi et al. 2008). Another factor contributing to increased rodent ingestion and hence, rodenticide exposure, is the clinical signs of AR poisoned animals displaying slow movements and abnormal activity (Cox and Smith 1992; Brakes and Smith 2005). Predators will selectively hunt such vulnerable prey, thus increasing the risk of secondary poisoning. Additional important food items for foxes are mammals such as cervids, mountain hares (*Lepus timidus*), and carnivores, and wild birds (Kidawa and Kowalczyk 2011). Carnivores secondary exposed to AR could have contributed to the high occurrence of residues found in red foxes. Furthermore, foxes as facultative carnivores consume plants, berries and invertebrates depending on season (Larivière and Pasitschniak-Arts 1996; Panzacchi et al. 2008). Invertebrates constitute a minor percentage of food volume in foxes, but AR have also been detected in cockroaches, beetles and gastropods (Howald 1997; Craddock 2003; Alomar et al. 2018). Thus, rodenticide exposure through invertebrates is possible.

Previous studies in red foxes demonstrated AR in 60-95% of liver samples (Tosh et al. 2011; Daniels 2013; Geduhn et al. 2015;), which is higher than our findings. One reason for this difference is probably due to high lipid solubility and affinity binding sites for AR in the liver that results in it being the organ with highest tissue concentration (Huckle et al. 1988; WHO 1995). In addition, AR are not homogenously dispersed in feces, lowering the recovery

compared to liver analysis. A low dose study of flocoumafen in rats demonstrated a mean fecal elimination of 28% (Huckle et al. 1988). Differences between countries in the availability of AR may also be a factor. Furthermore, these previous studies were multi-year studies, compared to our single year study. This could affect the results, as rodent population and AR use can vary between years. Lastly, collection of material in some of the previous studies were restricted to roadkill, sick, or dead foxes discovered in the field, in contrast to our presumed healthy foxes. Sometimes, AR can decrease fitness and cause abnormal behaviour of exposed animals (Erickson and Urban 2004, Elmeros et al. 2011), which may predispose them to vehicular strikes. In addition, AR exposure is a possible cause of illness and mortality; this will increase the likelihood of positive findings in samples from sick or dead animals. Excluding possibly unexposed healthy animals in studies, may introduce a bias that leads to an overestimate of the AR prevalence in wildlife.

We detected brodifacoum more frequently (46%) than other AR, significantly higher than coumatetralyl and bromadiolone. Langford et al. (2013) presented similar findings in raptors in Norway with brodifacoum and bromadiolone occurring most frequently. However, coumatetralyl was not analysed in that study. In Sweden and Finland, bromadiolone and coumatetralyl were the most common residues found in foxes (Tjus 2014; Kiovisto et al. 2016). We suspect the difference between the countries in occurrence of these AR is caused by higher sale of brodifacoum in Norway compared to other Scandinavian countries. The Norwegian Environment Agency has currently no data of sales volume or use of AR in Norway, making these comparisons difficult. Since 2014, Norway's regulatory framework restricts AR use for both public and licensed professionals (Lovdata 2018). Tamper-proof bait stations are mandatory for

both FGAR and SGAR, and the public is restricted to use indoors only. However, our results demonstrated continued exposure to non-target wildlife despite these legislative measures.

More than one AR were detected in 22% of the foxes. Only one commercial product contains a combination of two AR (bromadiolone and difenacoum) out of 46 government approved AR products in Norway, which does not fully explain the occurrence of multiple compounds in the foxes. Another possible explanation could be migratory birds and wildlife that come to Norway are exposed to combination products in other countries. However, products with combinations of AR are not commercially sold in other European countries (López-Perea et al. 2015). We believe that accumulation of AR in wildlife is more likely due to multiple exposures to contaminated prey over time.

Seasonal variance

We did not find a significant difference in seasonal variance of AR residues in foxes, consistent with a previous study in Northern Ireland and Great Britain (Tosh et al. 2011). In contrast, Elmeros et al. (2011) found the highest AR occurrences throughout winter in weasels and stoats in Denmark. In France a higher occurrence of AR poisoning in European mink (*Mustela lutreola*) was identified during autumn and late winter (Fournier-Chambrillon et al. 2004). Differences in diet and climatic conditions are probable explanations of this variation. In addition, winter food hoarding has been documented in foxes, making seasonal comparisons of AR exposure in this species difficult (Sklepkovych and Montevecchi 1996). Furthermore, SGAR have long persistence in the body. For compounds like brodifacoum, with an estimated liver half-life of 282-350 d (European Commission 2010), detection of possible seasonal variances is of limited value.

Sex and age differences

We did not find association between AR exposure and sex, which is in accordance with previous studies in red foxes (Tosh et al. 2011) and other wild predators (Shore et al. 2003; Elmeros et al. 2011; Ruiz-Suárez et al. 2016). However, sex differences in the extent of territory usage, with single male foxes having a larger home range than females have been observed (Larivière and Pasitschniak-Arts 1996). This could have influenced our study results, as male foxes may have preyed on rodents from different geographical areas, which would not necessary reflect the human population density of the municipality where they died.

We found no correlations between AR exposure and age groups in our study. A similar lack of associations was observed in other carnivores, such as bobcats, weasels, and stoats (McDonald et al. 1998; Serieys et al. 2015). However, a correlation between AR exposure and increased age was found in American mink (Ruiz-Suárez et al. 2016) and European polecats (*Mustela putorius*; Sainsbury et al. 2018).

Habitat influence

The red fox is widely distributed, living in both rural habitats and in proximity to residential areas (Adkins and Stott 1998). Different population densities can influence AR exposure in non-target animals due to varying rodenticide use and differences in the foxes' diet. Wildlife in urban areas is considered to be at greater risk of exposure to AR, due to frequent rodent control in residential areas. However, a higher consumption of rodents in agricultural landscapes is suggested by Kidawa and Kowalczyk (2011). We did not find a significant relation between prevalence of AR in foxes and human population density. This is in accordance with a study in Finland with no significant relationship between overall AR concentration and environmental variables like farm density and industrial surroundings (Koivisto et al. 2018). In

contrast, San Joaquin kit fox (*Vulpes macrotis mutica*) demonstrated the highest AR exposure in low-density development areas (Nogeire et al. 2015). These regions generally included single-family housing units, which is similar to our suburban areas. Our AR findings with correlation to human population density are in contrast to previous studies in bobcats (Serieys et al. 2015), hedgehogs and birds of prey (López-Perea et al. 2015; Lohr 2018; López-Perea et al. 2019), but variation in species' consumption of rodents and diversity of AR use between countries could explain the differences. A more precise landscape analysis with geographical situation of each sample would have improved our study, as building density, landscape elements, agricultural lands and livestock density affect rodent population and AR use. This was, however, not possible with our data.

Fecal analysis

Fecal analysis is a valuable method of monitoring AR residues in the body, because fecal excretion persists after residues are no longer detectable in plasma (Sage et al. 2010). Fox feces is inhomogeneous and contains plant material and hair, which influences the extraction recovery and AR concentration. Nevertheless, our fecal analyses demonstrated a high occurrence of AR residues in the presumed healthy foxes. Prat-Mairet et al. (2017) observed a decline in AR concentration when feces were exposed to natural decomposition outdoors, indicating the necessity to collect feces within 5 d to produce reliable results. However, fecal samples in our study were collected from the fox immediately after death, reducing natural degradation in the feces. Sampling scats from the ground lead to a risk of species misclassification, and studies report 18-25% erroneous identification of presumed fox feces according to DNA analysis of the scats (Jacquot et al. 2013; Fourel et al. 2018). In addition, the direct fecal sampling method assures that only one sample is collected from each individual animal. A previous study of the

fecal analysis in a poisoned dog demonstrated transferability to other live AR exposed animals (Seljetun et al. 2018).

Our study demonstrated that more than half of the wild red fox population in Norway is exposed to AR. Because of widespread distribution of the red fox, they may act as sentinels for other mammal-hunting predators, including endangered species such as arctic fox (*Vulpes lagopus*), gray wolf (*Canis lupus*) and Eurasian lynx (*Lynx lynx*), since they feed on some of the same resources as the red fox (Shirley et al. 2009; Wikenros et al. 2017).

Government radio tagging under sedation is performed in surveillance of free-ranging gray wolves, wolverines, brown bears (*Ursus arctos*) and Eurasian lynx in Norway (Arnemo et al. 2017). Using our method and sampling feces directly from animals during these radio tagging or collaring operations will enable authorities to monitor the occurrence of AR in live endangered wildlife.

In conclusion, our fecal analyses revealed widespread AR exposure in presumed healthy red foxes throughout Norway. Red foxes were susceptible to AR exposure both as scavengers in urban areas and as opportunistic predators with a diet of rodents, birds, small carnivores and invertebrates potentially exposed to AR. Despite government restrictions implemented in 2014, our results demonstrated that AR is a continuing hazard in non-target wildlife. Monitoring AR residues in wildlife is challenging. Studies are often based on liver analyses from necropsied animals found opportunistically, which may overestimate the prevalence in wildlife as healthy unexposed animals are not included in the sampling. Our study showed fecal analyses to be a valuable method for evaluating AR exposure in wildlife, which could be a useful method of AR assessment in other wildlife studies.

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Table 1. Fecal samples from 139 wild red foxes (*Vulpes vulpes*) collected in Norway in 2016 for analysis of anticoagulant rodenticides, by sex, age, location and the occurrence of anticoagulant rodenticides within each group. Anticoagulant rodenticides were found in 54% (75/139) of the samples.

Fox classifications		Number	Percent positive
Sex	Female	64	59
	Male	65	49
	Unknown	10	50
Age	Juvenile	50	48
	Adult	78	58
	Unknown	11	55
Location	Rural	44	48
	Suburban	64	61
	Urban	31	48

Table 2. The percent (number) of fecal samples from wild red foxes (*Vulpes vulpes*) containing different anticoagulant rodenticides by geographical population areas in Norway in 2016. The location where the foxes were shot in Norway and the fecal samples collected were defined in terms of human population as rural (1,000-10,000), suburban area (10,000-50,000) and urban area (50,000-180,000).

Population	**Samples**	**Percent (number) fecal samples with anticoagulant rodenticides**					
		Any	Brodifacoum	Coumatetralyl	Bromadiolone	Difenacoum	Difethialone	Flocoumafen

Rural	44	48 (21)	41 (18)	11 (5)	11 (5)	5 (2)	0	2 (1)
Suburban	64	61 (39)	58 (37)	12 (8)	19 (12)	6 (4)	3 (2)	0
Urban	31	48 (15)	29 (9)	32 (10)	16 (5)	3 (1)	0	3 (1)
Total	**139**	**54 (75)**	**46 (64)**	**17 (23)**	**16 (22)**	**5 (7)**	**1 (2)**	**1 (2)**

Figures

Figure 1. Occurrence of different anticoagulant rodenticide compounds in 139 fecal samples collected from presumed healthy wild red foxes (*Vulpes vulpes*) in Norway in 2016.

Figure 2. Seasonal occurrence of anticoagulant rodenticide compounds in 139 fecal samples from red foxes (*Vulpes vulpes*) in Norway in 2016. Exposure varied by season with 61% (14/23) foxes positive for AR in the autumn (September-November), 53% (35/66) in the winter (January-February and December), 57% (17/30) in the spring (March-May) and 45% (9/20) in the summer (June-August).