Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Companion Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2020:61

Prevalence of anticoagulant rodenticides in dogs and red foxes

Forekomst av antikoagulerende rotte- og musegifter hos hund og rødrev

Kristin Opdal Seljetun

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“There are poisons that blind you, and poisons that open your eyes”

August Strindberg

TABLE OF CONTENTS

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SUMMARY

Anticoagulant rodenticides (AR) are used worldwide in urban and agricultural rodent pest control. Ingestion of ARs is a major cause of poisoning in dogs and secondary exposure (ingestion of poisoned prey) in wildlife is a global problem. Despite this, few studies have examined the excretion of ARs in dogs, and elimination time is largely unknown in this species. In addition, no studies of AR prevalence in mammals have been conducted in Norway. Furthermore, no previous studies have determined the prevalence of ARs in faeces from presumed healthy animals across a country. Previous estimations of AR exposure in wildlife may be overestimated, as prevalence is assessed by analyses of livers from opportunistically sampled dead animals, not including possibly unexposed healthy living animals. Increased knowledge of exposure in living wildlife and the healthy dog population is an important part of the assessment of the impact of ARs. Furthermore, a better understanding of the elimination of ARs in dogs could generate ideas for new treatment options and optimize individual therapy in poisoned dogs.

This study was initiated to determine the occurrence of ARs in red foxes in Norway by reversed phase ultra-high performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analyses of faeces. Faecal samples from 163 presumed healthy wild red foxes from most counties in Norway were collected and analysed for six different ARs, and residues were detected in 54% of the animals. Brodifacoum was most frequently detected, followed by coumatetralyl, bromadiolone, difenacoum, difethialone and flocoumafen. More than one substance was detected in 40% of the positive foxes with several exposed to up to four different ARs. There were no statistically significant seasonal, age or sex differences in foxes exposed to ARs. These results indicate a high unintended exposure from ARs in healthy wild foxes throughout Norway.

This study also compared AR levels between faeces and liver from 40 of the same wild red foxes to determine the value of assessing AR exposure by faecal analysis. Residues of ARs were detected in 53% of the faecal samples and 83% of the liver samples. We found good concordance between AR residues in faeces and liver for coumatetralyl, difenacoum, and difethialone. Bromadiolone occurred in significantly greater frequency in livers compared to faecal samples, but no significant difference in concentration between faeces and liver could be detected. However, brodifacoum displayed a significant difference in concentration and occurrence of positive samples between liver and faeces.

The AR concentrations were analysed in accidentally exposed dogs, displaying biphasic elimination of ARs in faeces. Long terminal half-lives in faeces of 81, 190, 200-330 days were detected for coumatetralyl, difenacoum and brodifacoum, respectively. Comparatively shorter terminal half-life of 30 days was detected for bromadiolone. One of the poisoned dogs gave birth to four healthy puppies several months after exposure, and low concentrations of brodifacoum were detectable in the puppies' faeces for at least one month after parturition.

When analysing blood and faeces from 110 healthy domestic dogs, we detected low prevalence of ARs in the healthy dog population. This suggests low exposure of ARs in healthy dogs in Norway.

Together, the present work provides important findings of AR prevalence in the red fox in Norway and the healthy dog population previously not described. Our research of prevalence and elimination in both blood and faeces contribute to broader knowledge of the long excretion of ARs in *Canidae*. This will improve the background for decision making regarding use of these rodenticides in Norway and internationally.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Antikoagulerende rotte- og musegifter (AR) brukes over hele verden til bekjempelse av gnagere. Inntak av AR er blant de vanligste årsakene til forgiftning hos hunder, og for rovdyr er sekundær eksponering som følge av inntak av forgiftet byttedyr et globalt problem. Til tross for dette er kunnskapen om utskillelse av AR hos hunder mangelfull, og eliminasjonstider er ikke nøye undersøkt. I tillegg er forekomsten av AR-eksponering hos rovdyr i Norge ikke studert tidligere, og ingen studier har undersøkt forekomsten av AR i avføringen fra tilsynelatende friske dyr i et helt land. Forekomsten hos ville dyr kan være overestimert i tidligere studier, fordi vurderingen er gjort på bakgrunn av leveranalyser fra opportunistisk samlede døde dyr, uten inkludering av mulig friske ueksponerte levende dyr. Det er viktig med økt kunnskap om hvor utbredt AR-eksponering er hos levende ville dyr og i den friske hundepopulasjonen, i risikovurderingen av AR. Videre vil økt kunnskap om eliminasjonen av AR hos hunder kunne gi bidra til bedre og nye behandlingsalternativer og optimalisere individuell behandling av forgiftede hunder.

Målet med studien var å anslå forekomsten av AR hos ville dyr i Norge ved å analysere avføring med ultraytelse væskekromatografi-tandem massespektrometri (UPLC-MS/MS). Avføringsprøver fra 163 antatt friske ville rødrev fra de fleste fylker i Norge ble analysert for seks forskjellige AR virkestoff, og AR-rester ble påvist i 54% av dyrene. Brodifakum ble påvist oftest, fulgt av kumatetralyl, bromadiolon, difenakum, difetialon og flokumafen. I 40% av de AR-positive revene ble det påvist mer enn et virkestoff, og flere dyr var eksponert for opptil fire forskjellige AR virkestoff. Det var ingen statistisk signifikant forskjell på sesong, alder og kjønn blant revene eksponert for AR. Disse resultatene indikerer en høy forekomst av AR hos friske rødrev i hele Norge.

I studien sammenliknet vi også AR-nivåer i avføring med lever fra 40 av de samme ville rødrevene for å vurdere om avføringsanalysene er en god metode for å beregne eksponeringen for AR. Rester fra AR ble påvist i 53% av avføringsprøvene og 83% av leverprøvene. Vi fant god sammenheng mellom AR nivåer i avføring og lever for kumatetralyl, difenakum og difetialon. Bromadiolon ble påvist i signifikant flere leverprøver enn avføringsprøver, men det var ikke signifikant forskjell i AR konsentrasjonen mellom avføring og lever. Brodifakum ble derimot påvist i signifikant forskjellig konsentrasjon og forekomst mellom lever og avføring.

AR-konsentrasjonen ble analysert hos ufrivillig eksponerte hunder og viste en bifasisk utskillelse av AR i avføringen. Vi påviste lange terminale halveringstider i avføringen på 81, 190, 200-330 dager for kumatetralyl, difenakum og brodifakum. Bromadiolon hadde derimot en kortere terminal halveringstid på 30 dager. En av de forgiftede hundene fødte fire friske valper flere måneder etter eksponering, og lave konsentrasjoner av brodifakum ble påvist i valpenes avføring i minst en måned etter fødsel.

Ved analyse av blod og avføring fra 110 friske hunder, var forekomsten av AR lav. Dette tyder på lav eksponering for AR i den friske hundepopulasjonen i Norge.

Dette doktorgradsarbeidet gir ny kunnskap om forekomsten av AR hos rødrev i Norge og i en del av den friske hundepopulasjonen som ikke tidligere er beskrevet. Våre undersøkelser av forekomst og utskillelse i blod og avføring bidrar til økt kunnskap om den lange utskillelsen av AR fra dyr i hundefamilien. Dette vil kunne bedre beslutningsgrunnlaget for myndighetenes vurdering vedrørende regelverket for bruk av rotte- og musegifter i Norge og ellers i verden.

ABBREVIATIONS

ACN	Acetonitrile
AC	Activated charcoal
aPTT	Activated partial thromboplastin time
ASPCA	American Society for the Prevention of Cruelty to Animals, Animal Poison Control Center
AR	Anticoagulant rodenticides
BMBT	Buccal mucosal bleeding time
BPR	Biocidal Products Regulations
bw	Body weight
CBC	Complete blood count
CYP	Cytochrome P450
DIC	Disseminated intravascular coagulation
ECHA	European Chemical Agency
ESI	Electrospray ionization
EU	European Union
FGAR	First generation anticoagulant rodenticides
FFP	Fresh frozen plasma
HPLC	High performance liquid chromatography
IC_{50}	Median inhibitory concentration
IS	Internal standard
k_{el}	Elimination rate constant
k_i	Inhibition rate constant
LC-MS/MS	Liquid chromatography– tandem mass spectrometry
LD50	Median lethal dose
LOD	Limit of detection
Log P_{ow}	Log of the octanol-water partition coefficient
LOQ	Limit of quantification

MRM	Multiple reaction monitoring
NMBU	Norwegian University of Life Sciences
NOAEL	No observed adverse effect level
NVI	Norwegian Veterinary Institute
pRBC	Packed red blood cells
PT	Prothrombin time
SGAR	Second generation anticoagulant rodenticides
SULT	Sulfotransferase
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UHPLC	Ultra-high performance liquid chromatography
UPLC	Ultra performance liquid chromatography (Waters trademark for UHPLC)
V_d	Volume of distribution
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase complex subunit 1
vWF	von Willebrand factor

LIST OF PAPERS

Paper I

Seljetun KO, Eliassen E, Karinen R, Moe L, Vindenes V.

Quantitative method for analysis of six anticoagulant rodenticides in faeces, applied in a case with repeated samples from a dog.

Acta Vet Scand 2018; 60:3

Paper II

Seljetun KO, Eliassen E, Madslien K, Viljugrein H, Vindenes V, Øiestad EL, Moe L.

Prevalence of anticoagulant rodenticides in feces of wild red foxes (*Vulpes vulpes*) in Norway.

J Wildlife Dis 2019; 55: 834-843

Paper III

Seljetun KO, Sandvik M, Vindenes V, Eliassen E, Øiestad EL, Madslien K, Moe L.

Comparison of anticoagulant rodenticide concentrations in liver and feces from apparently healthy red foxes.

J Vet Diagn Invest 2020; 32: 560-564

Paper IV

Seljetun KO, Vindenes V, Øiestad EL, Brochmann G, Eliassen E, Moe L.

Determination of anticoagulant rodenticides in faeces of poisoned dogs and in a healthy dog population.

Acta Vet Scand 2020; 62: 30

1. INTRODUCTION

1.1 Rodenticides

Rats and mice have a worldwide distribution and are found in all climatic zones and on every continent except Antarctica (Wood and Singleton, 2014). In Norway, the main commensal rodents are brown rats (*Rattus norvegicus*), wood mice (*Apodemus sylvaticus*), yellow-necked mice (*Apodemus flavicollis*), bank voles (*Myodes glareolus*), northern red-backed voles (*Myodes rutilus*), grey red-backed vole (*Myodes rufocanus*), field vole (*Microtus agrestis*), tundra vole (*Microtus oeconomus*) and also European water voles (*Arvicola amphibius*) (Folkehelseinstituttet, 2018). House mice (*Mus musculus*) are less common in Norway but may occur in greater frequency at some farms (Soleng, 2020). Most of the rodents occur in all counties in Norway, but the range of the bank voles extend from south to Salten in Nordland county, while the northern red-backed voles only occurs north of Salten (Artsdatabanken, 2020).

1.1.1 Rodent control

Despite their worldwide distribution, knowledge of occurrence of rat populations remains scarce. Rats are widespread across Norway, occurring most frequently in urban ecosystems, but data of their prevalence is lacking (Soleng, 2020).

Commensal rodents are considered pests by humans as they destroy crops and stored produce causing huge economic losses worldwide (Stenseth et al., 2003). In Asia, rats eat agricultural produce that could feed 200 million people for one year, and annual agricultural losses due to rodents in the USA alone have been estimated to $900 billion (Mason and Littin, 2003). In addition, rodents chew cables and cause damage to infrastructure and buildings. Their borrow systems reduce crop yields, decrease forest productivity and damage irrigation ditches (Witmer et al., 2012). Rats and

mice transmit diseases to livestock and humans through contamination of food with urine and faeces and are considered a risk to human health. Rodents act as reservoirs of zoonotic diseases such as leptospirosis, salmonellosis, campylobacteriosis, hepatitis E, listeriosis, toxoplasmosis and various endo- and ectoparasites (Matuschka et al., 1997; Quy et al., 1999; Meerburg and Kijlstra, 2007; Kanai et al., 2012; Yan et al., 2014; Boey et al., 2019; Cao et al., 2019; Pellizzaro et al., 2019). Rats are opportunistic and pose a threat to native endangered species and ecosystems through predation and competition, especially in New Zealand, Australia and on tropical islands (Duron et al., 2017). As a result of these concerns, rodent control is necessary.

1.1.2 Chemical rodent control methods

A variety of methods are used to control rodents, including physical (live traps, snap traps, electrocution traps), chemical (fumigants, toxic baits), biological (chemosterilants) and others (non-toxic baits, deterrence, proofing) (Mason and Littin, 2003). A limited number of chemical rodenticides are approved for use in the European Union (EU) (European Chemicals Agency, 2020). Fumigants allowed by professionals include aluminium phosphide, hydrogen cyanide and carbon dioxide. Approved toxic baits include alphachloralose, cholecalciferol and anticoagulant rodenticides (ARs). ARs are by far the most common chemical agents in rodent control (Mcdonald and Harris, 2000; Janković et al., 2019).

1.1.3 History of anticoagulant rodenticides

The history of ARs originates in the 1920s where previously healthy cattle and sheep grazed on mouldy sweet clover hay (*Melilotus alba* and *Melilotus officinalis*). The animals developed a sickness known as “sweet clover disease” characterized by internal bleeding and high mortality. Two veterinary surgeons, Schofield and Roderick, discovered in 1924 that the

coagulation disorder could be reversed by removing the mouldy clover and transfusing fresh blood in the bleeding animals (Roderick, 1929).

Later the cause of the disease was identified as natural occurring coumarin in the plants being oxidised to dicoumarol by mould (Stahmann et al., 1941). In 1945 a compound was synthesised from natural occurring coumarin and named warfarin after Wisconsin Alumni Research Foundation (WARF), who funded the research, and "arin" from coumarin (Link, 1959). Warfarin was introduced as a rodenticide in 1948. The synthesising of warfarin opened for development of other anticoagulants; diphacinone (1952), coumatetralyl (1956) and chlorophacinone (1961). These were later called first generation anticoagulant rodenticides (FGARs) or short acting anticoagulants (Hadler and Buckle, 1992). After 1975 more compounds were manufactured with increased potency and length of action; bromadiolone (1975), difenacoum (1975), brodifacoum (1978), flocoumafen (1984) and difethialone (1987) (Lund, 1988; King and Tran, 2015). These agents were called second generation anticoagulant rodenticides (SGARs), superwarfarins or long acting anticoagulant rodenticides.

1.1.4 Chemical properties of anticoagulant rodenticides

ARs are divided in three classes based on their chemical structure; hydroxycoumarins, indanediones and thiocoumarin (Figure 1). The hydroxycoumarins have a 4-hydroxycoumarin ring with a side-chain

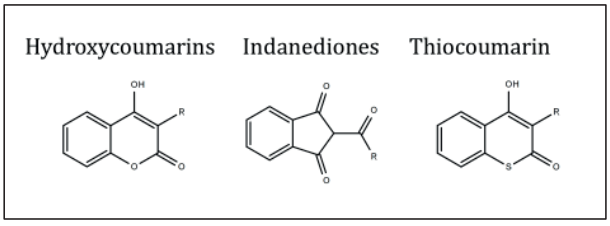


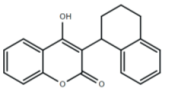
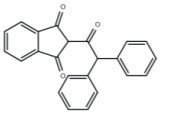
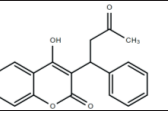
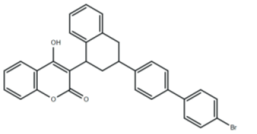
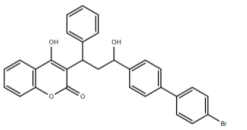
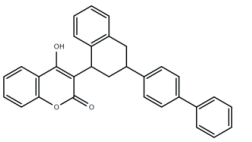
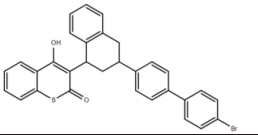
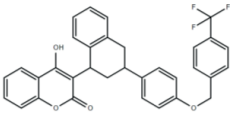
Figure 1. Molecular structures of the three classes of anticoagulant rodenticide compounds. R denotes varying functional groups.

substituent at the 3-position. Bromadiolone, brodifacoum, coumatetralyl, difenacoum, flocoumafen and warfarin are included in this group (Murphy, 2018). The indanediones have a 1.3-indanedione structure with a side-chain substituent at the 2-position and include chlorophacinone and diphacinone (Murphy, 2018). The thiocoumarin group differs from the hydroxycoumarins in the replacement of oxygen by sulphur in the 4-hydroxycoumarin ring (Lechevin and Poche, 1988). This group consists of difethialone.

The chemical structure of ARs affects their fat solubility and pharmacokinetic properties (Table 1). The FGARs are moderately lipophilic, with a log of the octanol-water partition coefficient, log P_{OW}, between 2.7 (warfarin) and 4.3 (diphacinone) (PubChem, 2020). The SGARs generally have a higher lipophilicity, with log P_{OW}-values between 4.0 (bromadiolone) and 6.1 (flocoumafen). The water solubility of FGARs (0.3 – 425 mg/L) is correspondingly higher than for SGARs (0.0025 – 0.48 mg/L). In contrast to FGARs, the SGARs have an additional, hydrophobic phenyl ring in the side chain, resulting in increased lipophilicity of SGARs. The larger structure and higher lipophilicity of the molecules increase tissue accumulation and consequently retention time in the body, resulting in long biological half-lives and higher toxicity of the SGARs (Thijssen, 1995; Dolmella et al., 1999). The association between lipophilicity (log P_{OW}), the median lethal dose (LD_{50}) and the elimination rate constants (k_{el}) for mouse plasma and liver are listed in Table 2 (Vandenbroucke et al., 2008; PubChem, 2020). The FGARs have a lower lipophilicity and consequently higher k_{el} in plasma and partly liver, compared to SGARs.

The ability of ARs to bind to and inhibit VKOR enzymes is essential for their effect on clotting. The kinetics of enzyme inhibition is described by the parameters IC_{50} (median inhibitory concentration) and k_i (inhibition rate constant). The IC_{50} is dependent on the concentrations of enzyme and substrate in the assay, while the k_i is an intrinsic equilibrium constant.

Table 1. Chemical properties of anticoagulant rodenticides.

Substance	Group	Mol. formula	Mol. weight (g/mol)	Log P_{ow}	Water solubility (mg/L)	Structural formula
Coumatetralyl	FGAR	$C_{19}H_{16}O_3$	292.3	3.46§	425 at 20°C, pH 7.0	
Diphacinone	FGAR	$C_{23}H_{16}O_3$	340.4	4.27§	0.3§	
Warfarin	FGAR	$C_{19}H_{16}O_4$	308.3	2.7§	17* at 20°C	
Brodifacoum	SGAR	$C_{31}H_{23}BrO_3$	523.4	4.9 at 20°C, pH 7.0	0.24 at 20°C, pH 7.0	
Bromadiolone	SGAR	$C_{30}H_{23}BrO_4$	527.4	3.95 at 20°C, pH 7.0	0.00248 at 20°C, pH 7.0	
Difenacoum	SGAR	$C_{31}H_{24}O_3$	444.5	6.11 at 20°C, pH 6.5	0.483 at 20°C, pH 6.5	
Difethialone	SGAR	$C_{31}H_{23}BrO_2S$	539.5	5.17§	0.39* at 25°C	
Flocoumafen	SGAR	$C_{33}H_{25}F_3O_4$	542.5	6.12* at 20°C	0.114* at 20°C	

*pH not stated

§Temperature and pH not specified

Table 2. Lipophilicity (log P_{OW}) for the anticoagulant rodenticides. The oral median lethal dose (LD_{50}) and the elimination rate constants (k_{el}) for plasma and liver in mice and oral median lethal dose (LD_{50}) and the VKOR inhibition rate constant (k_i) for susceptible rat liver microsomes.

Anticoagulant rodenticide	log P_{OW}	LD_{50}, mice (mg/kg)	k_{el}, plasma, mice (1/d)	k_{el}, liver, mice (1/d)	LD_{50}, rats (mg/kg)	k_i, VKOR, rat liver microsomes (µM)
Coumatetralyl	3.46	>1000	1.345	0.043	15-30	NA
Warfarin	2.70	374	0.046	0.010	1.60	0.72
Brodifacoum	4.90	0.40	0.008	0.002	0.16-0.26	0.04
Bromadiolone	3.95	0.99-1.75	0.021	0.025	0.56-0.84	0.13
Difenacoum	6.11	0.80	0.034	0.011	1.8	0.07
Difethialone	5.17	1.29	0.018	0.024	0.56	0.16
Flocoumafen	6.12	0.80	0.026	0.007	0.46	NA

NA: not analysed; d: day

The association between lipophilicity (log P_{OW}), the median lethal dose (LD_{50}) and the VKOR inhibition rate constant (k_i) for susceptible rat liver microsomes are listed in Table 2 (Hodroge et al., 2011; PubChem, 2020). Halogenation of the side chain (bromine and fluorine) in the structures of bromadiolone, brodifacoum, difethialone and flocoumafen increase the potency of SGARs (Lechevin and Vigie, 1992). This is best seen for brodifacoum and difenacoum, which are identical except for the bromination in the side chain of brodifacoum. Although difenacoum is more lipophilic than brodifacoum, it has lower toxicity and is less effective as a VKOR inhibitor.

SGARs have two chiral centres, hence four enantiomers; S/S, S/R, R/S and R/R. These can be configured in two diastereomeric forms; *cis* (1R,3S and 1S,3R) isomers and *trans* (1R,3R and 1S,3S) isomers (Lefebvre et al., 2017; Feinstein et al., 2019). The *cis*-isomer constitutes the largest part in baits of brodifacoum, difenacoum, difethialone and flocoumafen, while *trans* is the major isomer in bromadiolone baits (Lattard and Benoit, 2019). The *cis*- and *trans*-isomers have different chemical properties which influence biological half-lives and toxicity.

Cis-brodifacoum had a slower elimination and displayed higher toxicity than *trans*-brodifacoum in studies in rats and white rabbits (Damin-Pernik et al., 2017; Feinstein et al., 2019). Furthermore, a study in red kites (*Milvus milvus*) detected *cis*-brodifacoum in all livers, in contrast to *trans*-brodifacoum (Fourel et al., 2017a). However, in a study of wild boars (*Sus scrofa*) the isomers of brodifacoum were detected in similar proportions in the livers (Alabau et al., 2020). These differences could indicate a variation in metabolism and/or excretion of *cis*- and *trans*-brodifacoum between species.

On the other hand, *cis* and *trans*-difenacoum display a good accordance in liver accumulation and elimination between species. *Cis* was the main isomer detected in livers of both wild boars, red kites and wild rats, and displayed a slower elimination compared to *trans*-isomer (Damin-Pernik et al., 2017; Fourel et al., 2017a; Fourel et al., 2017b; Alabau et al., 2020).

A single study has examined stereoisomers in red foxes (*Vulpes vulpes*). Fourel and colleagues (2018) detected mainly *trans*-bromadiolone in livers from wild red foxes with *cis*-bromadiolone only in 10%. Furthermore, *trans*-bromadiolone was the only detected isomer in 50% of the faecal samples. However, this study has several shortcomings, e.g. were faeces collected from the ground, and might have been affected by degradation. In addition, analysis was based on few samples from red foxes and multiple defecations by the same fox cannot be excluded and would skew the results.

The volatility of ARs is low, hence concentrations in the air will be negligible (World Health Organisation, 1995). The SGARs have low water solubility (specified in Table 1) and are unlikely to be a source of direct water contamination (MacBean, 2012). However, low concentrations of SGARs were detected in fish liver from freshwater environments from several locations in Germany, with brodifacoum in 88% of the liver samples (Kotthoff et al., 2019). In addition, ARs were detected in low concentrations

in wastewater treatment plants in Spain (Gómez-Canela et al., 2014). In the soil the ARs appear to remain stable with slow degradation from microorganism and no leaching, and brodifacoum displayed a half-life of 157 days under aerobic conditions (World Health Organisation, 1995). Brodifacoum was detected in soil from all sample sites 60 days after an island eradication at Palmyra Atoll (Pitt et al., 2015), but climate and soil type affect soil persistence (Sage et al., 2007). Hence, environmental persistence may influence exposure in invertebrates, birds and mammals.

1.1.5 Mechanisms of anticoagulant rodenticides

The coagulation cascade is in a simplistic model divided in an intrinsic and an extrinsic pathway (Figure 2). In the intrinsic pathway all the coagulation

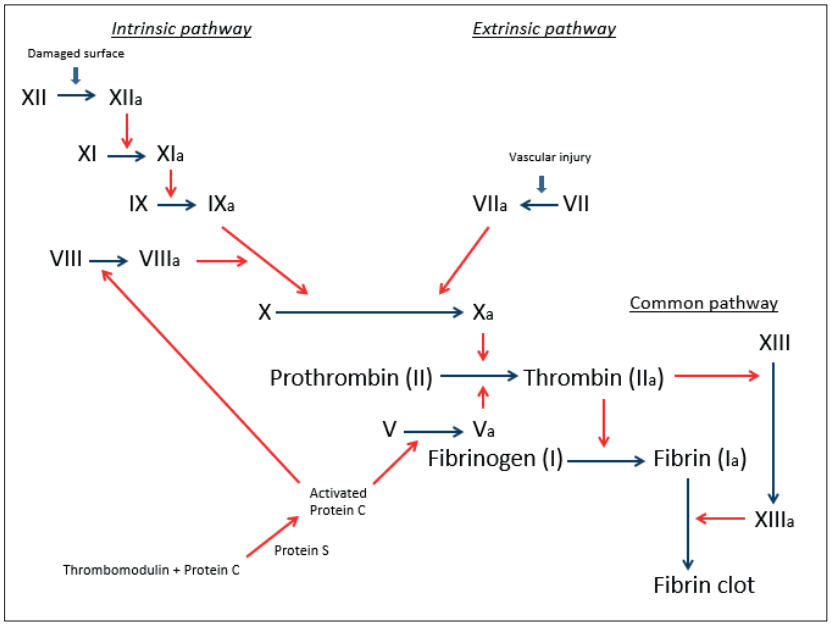


Figure 2. Blood coagulation cascade. Clotting factor X is activated through intrinsic and extrinsic pathways, resulting in conversion of fibrinogen to fibrin and stabilization of the fibrin clot.

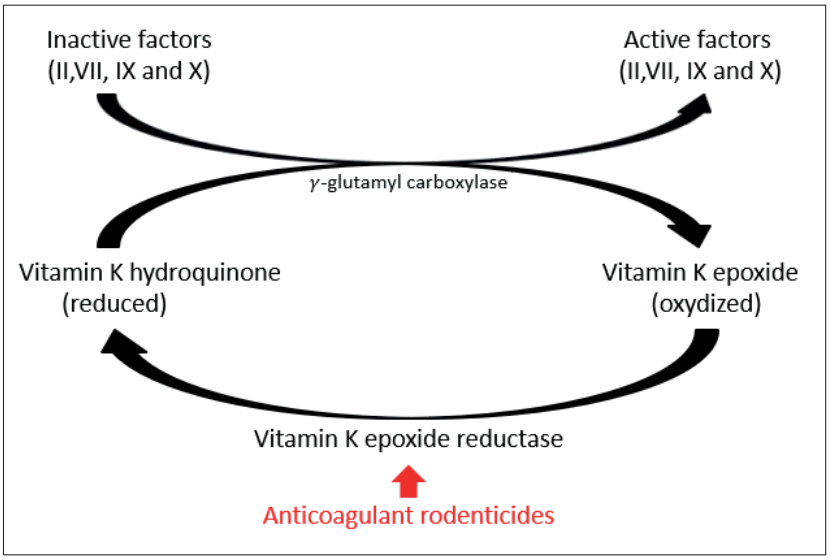


Figure 3. The vitamin K cycle and the influence of anticoagulant rodenticides with vitamin K recycling and activation of clotting factors.

factors are present in the blood, while the extrinsic pathway is activated by clotting factor III (tissue factor), present in the vessel wall (Palta et al., 2014). Damage to the vessel wall will activate both intrinsic and extrinsic pathways simultaneously. Clotting factors I, II, V and X are common for both pathways. Factor Xa activates prothrombin to thrombin, subsequently converting fibrinogen to fibrin and activating factor XIII (Furie and Furie, 1988; Palta et al., 2014). Factor XIIIa creates a fibrin network stabilizing the haemostatic clot. Mediated by thrombomodulin, protein C binds with protein S to form the activated Protein C, which in turn inhibits factors V and VIII (Furie and Furie, 1988). Coagulation factors II, VII, IX and X, together with proteins C and S are produced in the liver in inactive forms and require gamma carboxylation for their biological activity (Hirsh et al., 2001). Concomitant with this activation, the active form of vitamin K, vitamin K hydroquinone, is converted to an inactive vitamin K 2,3-epoxide (Figure 3). The inactive form is recycled to active vitamin K by vitamin K

epoxide reductase (VKOR). ARs interfere with the VKOR enzyme, inhibiting the activation of vitamin K and impairing the synthesis of gamma carboxylated coagulation factors (Suttie, 1990; Thijssen, 1995). SGARs have a higher binding affinity with VKOR compared to FGARs, resulting in a prolonged length of action (Thijssen, 1995). Consequently, concentrations of active clotting factors decrease, resulting in coagulation disorder and haemorrhages.

Diagnosis of AR poisoning in the dog is based on history of exposure and prolonged coagulopathy. In small animal veterinary medicine, the most common tests of the coagulation system are prothrombin time (PT) and activated partial thromboplastin time (aPTT). PT is most sensitive to depletion of factor VII in the extrinsic pathway, and less sensitive to depletion of factors I, II, V and X in the common pathway (Kamal et al., 2007). On the other hand, aPTT is sensitive to depletion of coagulation factors in the intrinsic and common pathways. The plasma half-lives of the vitamin K-dependent clotting factors II, VII, IX and X in the dog are 41.0; 6.2; 13.9 and 16.5 hours, respectively (Hellemans et al., 1963). Increase in coagulation parameters occur after the circulating active clotting factors are depleted. Factor VII is the coagulation factor with shortest half-life, hence PT is the first parameter to increase (Baker et al., 2004). This increase is not associated with clinical signs of AR poisoning. Antithrombotic effect due to AR ingestion is mostly through depletion of factors II and X, thus bleeding is not expected until both PT and aPTT are prolonged (Baker et al., 2004). After minimum two half-lives of clotting factor II, 3 to 5 days, clinical signs of poisoning are expected (Murphy and Talcott, 2012).

1.1.6 Metabolism of anticoagulant rodenticides

ARs are well absorbed from the gastrointestinal tract with poorly or no degradation by sheep ruminal microflora (World Health Organisation, 1995; Berny et al., 2006). Previous studies in pigs and rats have detected highest

concentration of ARs in liver, and in decreasing order of concentration in kidney, muscle, fat and blood (Huckle et al., 1988; Fisher, 2006). Similar results were detected in sheep and dogs (Laas et al., 1985; Maršálek et al., 2015). Hence, the main accumulation is in the liver.

Difenacoum together with the FGARs (coumatetralyl, chlorophacinone, diphacinone, warfarin) undergo substantial hepatic metabolism and are excreted mostly as metabolised compounds (Yu et al., 1982; European Commission, 2009a, c, b). These ARs are metabolized in the liver by cytochrome P450 (CYP) (Sutcliffe et al., 1990). Most extensive studies have been performed of warfarin, and the stereoisomers are metabolized by different phase 1 enzymes. Hydroxylation of S-Warfarin is mainly by CYP2C9, with minor contributions from CYP2C8, CYP2C18 and CYP2C19 (Zielinska et al., 2008; Miller et al., 2009). R-Warfarin is predominately hydroxylated by CYP1A2, CYP3A4, CYP2C9, CYP2C18 and CYP2C19 (Zielinska et al., 2008). The resulting hydroxywarfarins are more water soluble and are excreted as hydroxy metabolites or may undergo conjugation into sulfates by sulfotransferases (SULTs) or glucuronides by uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronyltransferases; UDP) (Miller et al., 2009; Jones et al., 2010). Significant differences of protein binding and intrinsic hepatic clearance of R- and S-Warfarin have been demonstrated between humans, rats, cats and rabbits (Smith et al., 2000a). Furthermore, inter-individual and intra-individual variation in metabolism of warfarin have been described (Park, 1988; Smith et al., 2000b). Fewer studies have been undertaken for difenacoum and the other FGARs; however, metabolism of difenacoum is through hydroxylation and glucuronide conjugation (European Commission, 2009c). Coumatetralyl is hydroxylated, resulting in four metabolites in urine and faeces and only traces as unchanged compound (Committee for Risk Assessment, 2014). On the other hand, metabolism of chlorophacinone

results in two hydroxylated metabolites and 20% as unchanged compound (European Commission, 2009a).

Most SGARs (brodifacoum, bromadiolone, difethialone, flocoumafen) are subject to little hepatic metabolism and are excreted largely as unchanged parent compounds (Huckle et al., 1988; European Commission, 2007, 2010a, b). Bromadiolone undergoes some hydroxylation, but the metabolites are not toxicologic significant (European Commission, 2010b). The anticoagulant effect of SGARs is mainly caused by the unchanged compound and not by their metabolites (Bachmann and Sullivan, 1983).

After excretion through bile to the duodenum, the ARs may be excreted through faeces, but the majority is absorbed into the portal circulation and removed from systemic circulation by hepatic uptake, followed by excretion into the bile; enterohepatic circulation (Sjaastad et al., 2003). Several factors affect the absorption from the duodenum, both formulation and characteristics of the specific AR, together with aspects in the animal such as presence of food in the gastrointestinal tract, gastric emptying time, pH of duodenal contents, intestinal transit time and mesenteric blood flow (Roberts et al., 2002). Digestion differs between species, with intermittent fat digestion and fluctuation in bile flow in dogs and foxes, in contrast to other species such as ruminants and rats with little fluctuation (Sjaastad et al., 2003; Cattley and Cullen, 2017). The enterohepatic circulation in dogs and foxes, together with the high lipophilicity of ARs and their strong binding to liver enzymes result in extensive accumulation in the liver and prolonged duration of action (Hadler and Shadbolt, 1975; Watt et al., 2005).

1.1.7 Elimination of anticoagulant rodenticides

ARs are mainly excreted through bile to faeces with little or no urinary excretion (Erickson and Urban, 2004). However, 10% of diphacinone and

20-44% of coumatetralyl are eliminated in the urine (Yu et al., 1982; European Commission, 2009b).

In the literature a number of elimination half-lives have been listed, but many are not comparable between studies due to different species, different matrixes (e.g. plasma, serum, liver), different models (e.g. non-compartmental, two-compartmental, not given), different administration routes (e.g. intravenously, orally), different dosage regimens (single dose, multiple doses) and others. Clearance, another elimination parameter, is generally not listed at all. However, as there are limited studies of AR exposure in dogs and literature on elimination of ARs in dogs and foxes is sparse, a brief description and discussion of other studies are done (Table 3).

A biphasic exponential decay in plasma is suggested after peroral administration of ARs in mice and rats (Bachmann and Sullivan, 1983; Vandenbroucke et al., 2008). Similar result was detected in plasma of warfarin in dogs; however, this was after intravenous injection (Neff-Davis et al., 1981). In liver, the initial elimination phase is rapid with a half-life of approximately two days in rats for all ARs, followed by a slow terminal elimination phase with varying half-lives of the different substances (Parmar et al., 1987). There are no studies of liver elimination in dogs. As the main elimination of ARs is from liver to faeces, increased knowledge of faecal residues in this species will provide important information for veterinarians in monitoring and assessing treatment length of poisoned dogs.

There are limited studies of plasma elimination half-life for coumatetralyl, and no studies have been performed in dogs or foxes. In mice, plasma elimination half-life was estimated to 0.52 days (Vandenbroucke et al., 2008). Another study determined the plasma elimination half-life to 1.9-3 days (46-71 hours) in rats (European Commission, 2009b). In Scottish red

Table 3. Elimination half-life values ($t_{1/2}\beta$) of anticoagulant rodenticides in blood (plasma unless specified) and liver following a range of single oral exposure levels in different species.

Anticoagulant rodenticide	Species	Blood t½ (days)	Liver t½ (days)	Reference
Coumatetralyl	Rat		55	Parmar et al., 1987
	Rat	1.9-3		European Commission, 2009b
	Mouse	0.52	15.8	Vandenbroucke et al., 2008
	Deer		14	Eason et al., 2011
	Deer		19	Crowell et al., 2013
Brodifacoum	Rat		130	Parmar et al., 1987
	Rat		282	Hawkins et al., 1991*
	Rat		150-200	Bratt and Hudson, 1979*
	Rat		350	Batten & Bratt, 1987*
	Rat	6.5 s		Bachmann and Sullivan, 1983
	Mouse	91.7	307.4	Vandenbroucke et al., 2008
	Dog	6 ±4[§] s		Woody et al., 1992
	Dog	6		Mount et al., 1986
	Dog	0.9-4.7 (mean 2.4)		Robben et al., 1998
	Horse	1.22±0.22		Boermans et al., 1991
Bromadialone	Rat		318	Hawkins et al., 1991*
	Rat		170	Parmar et al., 1987
	Mouse	33.3	28.1	Vandenbroucke et al., 2008
	Deer		6.0	Crowell et al., 2013
Difenacoum	Rat		128	US Environ Protect Agency, 2007
	Rat		120	Parmar et al., 1987
	Mouse	20.4	61.8	Vandenbroucke et al., 2008
Difethialone	Rat	2.3	126	European Commission, 2007
	Mouse	38.9	28.5	Vandenbroucke et al., 2008
	Dog	2.2-3.2		Robben et al., 1998
Flocoumafen	Rat		220	Huckle et al., 1989
	Mouse	26.6	93.8	Vandenbroucke et al., 2008

s: serum
*Unpublished report, cited in Erickson and Urban, 2004
[§]dosed three consecutive days

deer (*Cervus elaphus scoticus*), mean liver elimination half-lives were estimated to 14 days and 19 days in two studies (Eason et al., 2011; Crowell et al., 2013). Comparatively in mice, the liver elimination half-life was 15.8 days (Vandenbroucke et al., 2008).

Brodifacoum has been analysed in a few studies in dogs. A median plasma elimination half-life was estimated to 2.4 days (range 0.9-4.7 days) in seven poisoned dogs (Robben et al., 1998). An experiment in four dogs demonstrated that the depletion curve in plasma was biphasic (Woody et

al., 1992). However, in this study brodifacoum was studied after repeated ingestions for three consecutive days and modelled by a two-compartment model without listing the generated parameters. In comparison, serum elimination half-life was 6.5 days (156 hours) and after a single ingestion in rats (Bachmann and Sullivan, 1983). A longer plasma half-life was found in mice with 91.7 days (Vandenbroucke et al., 2008). In six horses, the elimination phase in plasma was estimated to 1.22±0.22 days (Boermans et al., 1991). In liver, elimination half-life of 307.4 days was observed in mice after a single ingestion, while rats displayed a variation of 130-350 days (Parmar et al., 1987; Erickson and Urban, 2004; Vandenbroucke et al., 2008).

There are no accessible published data of elimination of bromadiolone in dogs. In other species, acute oral ingestion of bromadiolone resulted in liver elimination half-life of 170-318 and 28.1 days in rats and mice, respectively (Parmar et al., 1987; Erickson and Urban, 2004; Vandenbroucke et al., 2008).

The elimination half-life of difenacoum in dogs has not been studied. In mice, a half-life of 61.8 days in liver was estimated after a single ingestion (Vandenbroucke et al., 2008). Several experiments have been conducted in rats, and a liver decay half-life of 120-128 days has been suggested (Parmar et al., 1987; United States Environmental Protection Agency, 2007).

Difethialone plasma elimination half-life was estimated to 2.2 and 3.2 days in two dogs (Robben et al., 1998). A study in mice estimated half-life in plasma to 20.4 days and liver to 61.8 days (Vandenbroucke et al., 2008). An experiment in rats suggested a plasma elimination half-life of 2.3 days and detected approximately 10% residues in liver six months after a single ingestion, giving an estimated half-life of 126 days (about 18 weeks) (European Commission, 2007).

Elimination half-life of flocoumafen has not been estimated in dogs. However, liver residues of 8% of the administered dose floucomafen was observed 43 weeks after a single ingestion in eight dogs (Veenstra et al., 1991). Liver decay half-lives of 93.8 and 220 days were assessed in mice and rats, respectively (Huckle et al., 1989; Vandenbroucke et al., 2008).

These studies suggest very low elimination rates of all ARs from the liver, although there are large discrepancies between the estimated results. In addition, there are substantial differences between species. There are; however, no information of liver decay half-lives in dogs after acute ingestions and no studies assessing elimination in faeces. Elimination half-lives in plasma are considerably shorter than decay half-lives in liver. Furthermore, ARs below detectable limits in blood does not exclude intoxication in dogs as accumulated ARs in liver still may be sufficient to cause coagulation disturbances (Travlos et al., 1985; Robben et al., 1998). Thus, more information is needed of elimination of ARs in dogs.

1.1.8 Anticoagulant rodenticide resistance

Extensive use of warfarin and diphacinone in the 1950s led to resistance in brown rats first discovered in the United Kingdom in 1958 (Boyle, 1960). Resistance to warfarin was found in house mice shortly thereafter (Dodsworth, 1961). Since then, resistance to warfarin has been reported in Europe, USA, Australia, Japan and New Zealand (Lund, 1964; Jackson and Kaukeinen, 1972; Saunders, 1978; Naganuma et al., 1981; Cowan et al., 2017). Increasing resistance led to development of SGARs. Resistance to SGARs followed, possible as a result of cross resistance between compounds (Greaves et al., 1982). Tolerance to ARs has been demonstrated to be inherited, causing selection pressure in the rodents (Rowe and Redfern, 1965; Greaves and Ayres, 1967). Resistance is caused by of one or more mutations at the VKOR complex subunit 1 gene (VKORC1), altering the VKOR activity (Rost et al., 2004; Pelz et al., 2005). The most commonly

detected mutations in Europe change wild-type tyrosine at codon 139 in the VKORC1 gene to cysteine (Tyr139Cys), phenylalanine (Tyr139Phe) or serine (Tyr139Ser) (Meerburg et al., 2014). Several different genotypes associated with resistance have been detected in both rats and mice (Rost et al., 2009; Buckle, 2013). Another mechanism for resistance in rodents is increased degradation of ARs by CYP (Sutcliffe et al., 1990; Ishizuka et al., 2007). Resistance is widespread across Europe and has been reported to various degrees against all ARs, resulting in reduced effect in rodents (Murphy and Lugo, 2015; Desvars-Larrive et al., 2017). Hence, the use of rodenticides escalates.

1.1.9 Regulatory status of anticoagulant rodenticides

The use of ARs is important in agriculture and to protect human health, but numerous reports have documented adverse effects to non-target wildlife. Regulations are necessary to balance the need for rodenticides against impact on wildlife and the environment. In Europe, ARs are registered as a biocide and are regulated by the Biocidal Products Regulations (BPR). In 2012 the BPR introduced new regulations (EU 528/2012) for biocidal substances harmonized at the EU level (European Chemicals Agency, 2012). This was implemented in Norway through Biocidforskriften (FOR-2014-04-10-548) and applied in 2014 (Lovdata). The main change was restrictions on non-professional use, with a maximum quantity of bait per pack and tamper-resistant bait stations. In this thesis I will focus on the ARs used in Norway; brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone and flocoumafen. AR substances and concentrations allowed in Norway corresponds to the substances allowed in EU, but the Norwegian Environment Agency (2014) have applied stricter national AR restrictions than the ones implemented in the EU. The Norwegian public are restricted to AR products for indoor use against mice only, and grain and pellet formulations are prohibited. The concentration of most substances in

Norway (brodifacoum, bromadiolone, difenacoum, difethialone, flocoumafen) in commercial baits were predominantly 0.005%, while coumatetralyl had a higher concentration of 0.0375% (Haraldsen, 2018). However, in 2016 (applied in 2018) the European Chemical Agency (ECHA) reclassified AR concentrations of 0.003% and higher as "toxic to reproduction" and restricting higher concentrations for professional use only (Frankova et al., 2019). This regulation with lower concentration of ARs, has resulted in fewer products on the market.

Similar restrictions of AR products have been implemented in other countries. In the United States, consumers are restricted to AR bait blocks in bait stations only since 2008 (United States Environmental Protection Agency, 2008). In Canada, comparable restrictions to the EU legislation have been implemented, in addition to prohibiting use of SGARs for non-professionals (Government of Canada, 2012). In contrast, there has been no changes in regulations on ARs in New Zealand or Australia (Eisemann et al., 2018).

1.2 Anticoagulant rodenticide poisoning in dogs

1.2.1 Sources of poisoning

Ingestion of ARs is among the most frequent causes of poisoning in dogs worldwide. In 2019, ARs were the most commonly reported pet toxins in six of the American states to the American Society for the Prevention of Cruelty to Animals, Poison Control Center (ASPCA, 2020). A total of 800 calls to the Veterinary Poisons Information Service in Great Britain in 2017 were regarding ARs (Veterinary Poisons Information Service, 2018). In Norway, ARs are of the top 5 reported enquiries in animals to the Norwegian Poison Information Centre (Norwegian Poison Information Centre, 2020).

Exposure of ARs in dogs is most common through direct ingestion of the bait from bait stations or refill boxes (primary exposure). Consumption of

poisoned rodents (secondary exposure) seldom causes acute toxicosis in dogs because the amount of ARs in one rodent is small and dogs rarely ingest several rodents at the same time. Dogs can; however, be exposed to subtoxic levels. In 2014, AR prevalence was examined at the Norwegian University of Life Sciences (NMBU) in deceased dogs (Olerud et al., 2014). Liver samples were taken from the dogs, irrespective of cause of death, illness or clinical signs. The majority of the dogs were euthanized due to different illnesses. ARs were detected in the liver in 13 of the 63 dogs included in the study (20%), and in 8% of the necropsied dogs more than one type of AR was present. The source of the rodenticide in these dogs could not be determined. A previous investigation of 115 domestic pets revealed two dogs with trace amounts of diphacinone in the liver (United States Environmental Protection Agency, 2018). However, clinical status of the dogs was not specified in the study.

1.2.2 Toxicity of anticoagulant rodenticides

Toxic doses of ARs in dogs are not well established. Acute toxicity is usually estimated by LD_{50} values (median lethal dose). There is a wide variation between the acute oral LD_{50} values (single dose studies) in the literature and between the different ARs in dogs (Table 4). In general, treatment is recommended if dogs ingest more than 1/10 of the LD_{50} of the AR (Merola, 2002). However, due to the difference between the LD_{50} in the literature and to prevent clinical signs in more sensitive dogs, 0.02 mg/kg body weight (bw) is recommended as a dosage of concern for all SGARs (Dunayer, 2011). There are large anatomic, physiologic and metabolic differences between birds and mammals. Birds have therefore not been included in the scope of this project, since the focus is on carnivores such as dogs and foxes.

The FGAR coumatetralyl has a LD_{50} of 35 mg/kg bw in dogs (European Commission, 2009b). However, ingestion as low as 1 mg/kg bw is reported to have caused disturbance in blood coagulation (Berzins, 1993).

Table 4. Acute LD50 of anticoagulant rodenticides in mg/kg body weight determined after single oral administration in dogs.

Anticoagulant rodenticide	Oral LD_{50} (mg/kg bw)	Reference
Coumatetralyl	35	European Commission, 2009
Brodifacoum	0.25-3.5	Mount et al., 1986
	0.25-1	ICI-United States, 1976*
	3.5	Murphy, 2018
	1.09-3.56	Godfrey et al., 1981
Bromadialone	11-15	Mount et al., 1986
	10	Murphy, 2018
	8.1	European Commission, 2010
Difenacoum	50	Murphy, 2018
Difethialone	4	MacBean, 2012
	11.81	European Commission, 2007
Flocoumafen	0.075-0.25	Eason et al., 2001

bw: body weight

*Unpublished report, cited in Godfrey et al., 1981

Surprisingly, several reports state >1000 mg/kg bw as LD_{50} in mice for this rodenticide (European Commission, 2009b; PubChem, 2020). The toxicity of coumatetralyl in other species corresponds, being one of the less toxic ARs after a single oral ingestion (Table 5).

Brodifacoum is one of the more toxic ARs in dogs spanning in reported LD_{50} values from 0.25 to 3.56 mg/kg bw. The lowest dose is suggested by Mount and colleagues (1986), but no information is given of the number of dogs, doses or time frame of the experiment. The low value is also given by the manufacturer (I.C.I. United States Ltd) but is questioned in two later experiments by other researchers (Godfrey et al., 1981; Travlos et al., 1985). The use of pure-bred beagles only by the manufacturer is suggested to have influenced the results. Godfrey and colleagues (1981) performed two experiments with 20 and 59 dogs resulting in LD_{50} of 1.09 and 3.56 mg/kg bw, respectively; hence, a variation in LD_{50} was observed under similar experimental conditions. In other species, brodifacoum is likewise the most toxic AR. Bromadiolone with a LD_{50} of 8.1-15 mg/kg bw is less toxic than

Table 5. Acute LD_{50} of anticoagulant rodenticides in mg/kg body weight determined after single oral administration in different species.

Anticoagulant rodenticide	Rat	Mouse	Rabbit	Pig	Cat
Coumatetralyl	15-30	>1000	10	NA	50
Brodifacoum	0.16-0.26	0.40	0.30	0.5-10	25
Bromadialone	0.56-0.84	0.99-1.75	1	3	>25
Difenacoum	1.80	0.80	2	80-100	100
Difethialone	0.56	1.29	0.75	2-3	>16
Flocoumafen	0.46	0.80	0.70	60	>10

NA: Not available
Reference: PubChem, 2020

brodifacoum in dogs, and this corresponds with the findings in other animals. Difenacoum, on the other hand, displays a larger variation between species with a high LD_{50} in dogs, pigs and cats, but comparatively low values in rats and mice (PubChem, 2020). Difethialone demonstrates a large variation in LD_{50} values in dogs with 4 and 11.81 mg/kg bw (European Commission, 2007; MacBean, 2012). However, details of the two studies are not accessible. In contrast, difethialone exhibits high toxicity comparable to flocoumafen in most other species. Flocoumafen is highly toxic to dogs with LD_{50} of 0.075-0.25 mg/kg bw (Eason and Wickstrom, 2001). In rats, rabbits and cats, flocoumafen has similar toxicity to bromadiolone and brodifacoum.

Reviewing reported LD_{50} of all ARs in the different animals, substantial variations within species and between species are observed. In dogs, the range in LD_{50} estimates is large between some of the substances. Some could be due to differences in experimental conditions, analytical methodology and sampling; however, this is not possible to verify because the studies are not published or are confidential by the manufacturer. Individual differences in metabolism may also contribute to the broad range in LD_{50}. Furthermore, difference in sensitivity is not consistent between species. Flocoumafen displays higher toxicity in dogs compared to rats;

however, the opposite is observed for difenacoum. Hence, comparisons of acute toxicity after single oral ingestion between species remain challenging.

In the evaluation of ARs by government authorities, experimental data on repeated dose effects on organs and tissues are required, as are data on genotoxicity, carcinogenicity, reproduction and developmental toxicity. In general, the observed subtoxic and chronic effects of ARs can be explained by their pharmacological mode of action (European Commission, 2007, 2009a, b, c, 2010a, b, 2016). There are few chronic toxicity studies published in dogs (Table 6). Several secondary exposure experiments with feeding of poisoned rodents to other predatory mammals have been performed. However, the exact dose ingested by the predator is seldom estimated. Furthermore, several of the experiments are conducted by the manufacturer with confidential details, further limiting the usefulness of the studies. In dogs, coumatetralyl displayed high toxicity in a study by the manufacturer with death within seven days after ingestion of 1-2 mg/kg bw per day (European Commission, 2009b). The number of dogs in this study was not given. Chronic toxicity of brodifacoum was estimated in a 6 weeks dog study (European Commission, 2016). After ingestion of 0.01 mg/kg bw per day, two of the dogs had to be euthanized day 36 due to prolonged coagulation time. Details of the study are unpublished and confidential by the manufacturer. Repeated ingestions of 0.02 mg bromadiolone/kg bw in dogs resulted in death after 64 to 85 days; however, no observed adverse effect level (NOAEL) was determined to 0.008 mg/kg bw per day (European Commission, 2010b). Both studies were conducted by the manufacturer without further information of the experiments. Only one chronic toxicity study in foxes with estimated exposure dose has been performed (Sage et al., 2010). Three red foxes ingested 0.15 mg bromadiolone/kg bw per day for five days through spiked water voles, resulting in moderate to severe haemorrhages. Difenacoum and difethialone displayed similar toxicity in the

Table 6. Chronic toxicity studies with repeated oral ingestions of anticoagulant rodenticides in dogs and foxes.

Anticoagulant rodenticide	Oral dose (mg/kg bw/d)	Effect	Reference
Coumatetralyl	1-2	Death within 7d	European Commission, 2009
Brodifacoum	0.01	Death d 36	European Commission, 2016
Bromadialone	0.02	Death after 64-85d	European Commission, 2010
	0.008	NOAEL	European Commission, 2010
	0.15 (f)	Moderate to severe haemorrhages	Sage et al., 2010
Difenacoum	0.01	Changes in blood coagulation d 30	US Environmental Protection Agency, 2007
Difethialone	0.02	Haemorrhagic effects after 90 d	European Commission, 2007
	0.01	NOAEL	European Commission, 2007
Flocoumafen	NA		

bw: body weight; d: day, NA: Not available; NOAEL: No observed adverse effect level; f: foxes

dog with clinical signs after ingestion of 0.01-0.02 mg/kg bw per day (European Commission, 2007; United States Environmental Protection Agency, 2007).

These chronic toxicity studies mostly estimate toxicity based on lethal outcome or haemorrhages. Subtoxic or sublethal quantities of ARs could be defined as amounts ingested without causing prolonged coagulation time, as this is the object of the rodenticides (Erickson and Urban, 2004). Nevertheless, subtoxic levels of ARs have not been estimated in the dog or red fox. Further research of subtoxic effects of ARs in both dogs, foxes as well as other wild animals is needed. However, to estimate the extend of this problem, knowledge of prevalence of ARs in the population is necessary. In the current project the focus was thus on prevalence of ARs.

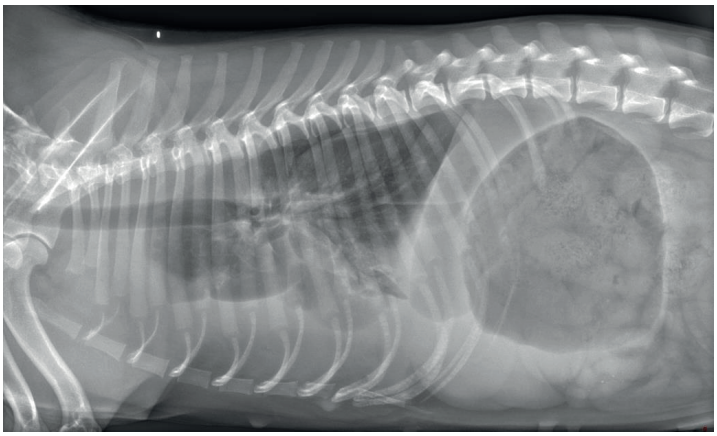


Figure 4. Lateral thoracic radiograph of a dog poisoned with anticoagulant rodenticides. Note the mixed pattern of alveolar infiltrates and pleural effusion (haemorrhage).

1.2.3 Clinical signs of acute poisoning

The ARs cause external bleeding or internal haemorrhage after ingestion of toxic doses, but clinical signs and findings of AR poisoning in dogs are often unspecific. The most common presenting complaints are lethargy, pale mucous membranes, inappetence, dyspnoea and coughing (Figure 4) (Sheafor and Guillermo, 1999; Haines, 2008). Other clinical signs depend on where the bleeding occurs and can include anaemia, vomiting, tachycardia, tachypnoea, haematuria, subcutaneous hematoma and lameness.

Diagnosis of AR poisoning is based on history of exposure and prolonged coagulopathy (PT and aPTT). The main laboratory finding is low number of red blood cells and low haematocrit. Other laboratory findings frequently include hypoproteinaemia, thrombocytopenia, hyperglycaemia and hypoglobulinaemia (Sheafor and Guillermo, 1999; Haines, 2008).

1.2.4 Bleeding disorders in dogs

Differential diagnoses of AR poisoning in dogs include other causes of anaemia. Three areas of the haemostatic system may be affected and cause

coagulopathies; disorders of primary haemostasis, secondary haemostasis and fibrinolysis. Hence, in diagnosing the dog with AR poisoning other coagulopathies need to be ruled out.

Primary haemostasis is the formation of the initial platelet plug. Disorders of the primary haemostasis may be caused by defective platelet production or function, reduced platelet survival, loss of platelets from the systemic circulation or be drug induced (Herring and McMichael, 2012). Von Willebrand's disease is the most common inherited bleeding disorder in dogs and causes a deficiency in the amount of a specific protein (von Willebrand factor; vWF), a carrier for factor VIII. Dysfunctional platelets lead to thrombocytopathy, and vWF disease is regarded as a variant of this (Kohn, 2011). Other diseases cause reduced number of platelets; thrombocytopenia. These can be immune-mediated or caused by underlying conditions such as ehrlichiosis (loss of blood platelets), drugs like estrogen (suppress production of platelets in the bone marrow) or acetylsalicylic acid (destroy circulation platelets) (Dodds, 2005). In primary haemostatic disorders, PT and aPTT are generally normal. Thrombocytopenia is diagnosed by reduced platelet count ($<180x10^9$/L). In dogs with normal coagulation panel and normal/slightly reduced platelet count, test of buccal mucosal bleeding time (BMBT) is performed (Kohn, 2011). Prolonged BMBT (>4 minutes) suggests platelet dysfunction, deficiency of vWF or vasculitis. Further diagnostics include platelet function tests and vWF testing.

Secondary haemostasis is a disturbance of the blood coagulation system resulting in generation of thrombin and formation of a stable fibrin clot. Deficiencies in the secondary haemostasis are caused by mutations in genes responsible for synthesis or processing of active coagulation factors or by acquired coagulopathies (Kohn, 2011). In dogs, the most common mutations are in the intrinsic pathway factors (Figure 2). Factor IX (Haemophilia B) and Factor VIII (Haemophilia A) cause increased aPTT (>102 seconds), but PT remains normal. Deficiencies in the extrinsic pathway factors are less

common. Factor VII deficiency is suspected with increased PT (>17 seconds) and normal aPTT. Acquired coagulopathies like aflatoxicosis, hepatic failure and disseminated intravascular coagulation (DIC) cause increase in both PT and aPTT and additional testing is needed for differentiation from AR poisoning (Herring and McMichael, 2012). Thrombin time is a test for fibrinogen, independent of vitamin K coagulation factors, and can be useful in this differentiation (Kohn, 2011). Further diagnostics of deficiencies in secondary haemostasis include evaluation of liver values, complete blood count (CBC), D-dimers and factor assays.

Fibrinolysis is the breakdown of the fibrin clot by plasmin, resulting in fibrinogen split product and D-dimer formation (Kohn, 2011). In dogs, fibrinolysis may be caused by diseases such as DIC, immune mediated haemolytic anaemia, neoplasia and hepatic disease. Diagnostics include fibrinogen assays, fibrin degradation products, D-dimers and thrombin time (Herring and McMichael, 2012).

1.2.5 Treatment of acute poisoning

Recommended treatment for dogs with a known recent ingestion of a toxic amount of ARs is decontamination by induction of emesis followed by administration of a single dose of activated charcoal (DeClementi and Sobczak, 2018). Coagulation should be monitored 48 and 72 hours after ingestion to assess if vitamin K_1 therapy is necessary (Pachtinger et al., 2008). Decontamination is not indicated in dogs presenting with prolonged coagulation due to risk of haemorrhage.

Vitamin K_1 is recommended in dogs with prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT). Oral administration together with food is preferred, due to four to five times enhanced bioavailability of vitamin K_1 when ingested with canned food (Murphy and Talcott, 2012). When oral vitamin K_1 is unavailable, second choice of

administration is by injection. Intramuscular administration is contraindicated due to risk of haematoma and unpredictable absorption of vitamin K_1 (Soedirman et al., 1996). Subcutaneous injection is no more effective than placebo and is not recommended (DeZee et al., 2006; Tran et al., 2013). Hence, second choice of administration is intravenous injection (Long et al., 2016). Intravenous vitamin K_1 normalises coagulation within hours and can be used in the acute phase until oral vitamin K_1 is obtained (Mooney et al., 2020). Previously, intravenous administration of vitamin K_1 increased the incidence of anaphylactoid reaction, caused by polyethoxylated castor oil used as a dispersant (Mi et al., 2014). This solvent was removed from the vitamin K_1 medications, and anaphylactoid reaction from intravenous injection is now quite rare (Martin, 1991; Fiore et al., 2001). Treatment with vitamin K_1 is recommended to last until toxic amounts of ARs are no longer present in the dog and varies depending on kinetics of the substance and amount ingested. As knowledge of kinetics of ARs in dogs is sparse, administration of vitamin K_1 is recommended for 3-4 weeks (Murphy and Talcott, 2012).

Blood transfusion is often recommended, and current blood products include fresh frozen plasma (FFP), fresh whole blood and packed red blood cells (pRBC) (Haines, 2008). FFP lowers haematocrit, hence lowers oxygen-carrying capacity and replaces coagulation factors and antithrombin. It is most commonly used as it can be stored up to one year (Cornell University, 2020). Fresh whole blood includes RBC, white blood cells, platelets, coagulation factors, albumin and immunoglobulins, but has a short shelf-life with depletion of coagulation factors V and VIII after six hours of storage (Godinho-Cunha et al., 2011). pRBC contains a significant reduced volume compared to whole blood but constitutes higher amount of RBC, hence a higher oxygen-carrying capacity (Haldane et al., 2004). The smaller volume is advantageous in dogs at high risk of volume overload, but it does not replenish coagulation factors.

Additional supportive treatment includes oxygen therapy for dyspnoea, intravenous fluids for cardiovascular support and cage rest to prevent trauma.

1.3 Anticoagulant rodenticide exposure in wildlife

1.3.1 Sources of exposure

Wildlife can accumulate ARs through ingestion of bait or by consumption of poisoned prey. In addition, wildlife can potentially be exposed by tertiary exposure; ingestion of mammals and birds secondarily exposed to ARs (Gabriel et al., 2018). Rodents constitute the main part of red foxes' diet with 48% of consumed food volume (Kidawa and Kowalczyk, 2011). Previous studies have found that poisoned rodents display 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. In addition, death in rodents occur 3-14 days after initial ingestion of a lethal dose (Elias and Johns, 1981). During this period, they may continue to eat bait, resulting in a high total dose in subsequent predators. Carnivores and wild birds are other important food items for foxes (Kidawa and Kowalczyk, 2011). Tertiary AR exposure could contribute to the occurrence of residues in red foxes. European polecats (*Mustela putorius*) and stone martens (*Martes foina*) are both known to constitute a part of the wild red fox' diet (Kidawa and Kowalczyk, 2011), and studies from Denmark detected ARs in 95-99% of liver samples from these species (Elmeros et al., 2018). Another thinkable route of exposure is through faeces from AR exposed mammals and birds. Moreover, ARs have been detected in slugs after feeding on poisoned bait in field applications (Dunlevy et al., 2000; Alomar et al., 2018). Furthermore, ARs have been detected in cockroaches, beetles and gastropods (Howald, 1997; Craddock, 2003). ARs are not known to affect invertebrates due to their different blood clotting system (Cerenius and Söderhäll, 2011), and invertebrates

constitute a minor percentage of food volume in foxes. However, a study detected invertebrates in 18% of foxes' stomachs (Contesse et al., 2004), and consumption of contaminated slugs and beetles could contribute to the total AR burden in the animals (Spurr and Drew, 1999). In addition, studies have detected ARs in livers in 63-67% of European hedgehogs (*Erinaceus europaeus*) (Dowding et al., 2010; Booth et al., 2012). Foxes are known to eat hedgehogs (Contesse et al., 2004; Kidawa and Kowalczyk, 2011), and insectivorous mammals could be another source of secondary exposure to foxes.

1.3.2 Analytical methods

Exposure to ARs in wildlife is normally assessed by analysing AR residues in liver. Few studies have assessed exposure in healthy wildlife, as animal welfare considerations preclude killing healthy animals for research. Liver analyses are often restricted to roadkill, sick or dead animals opportunistically sampled in the field. AR exposure could affect fitness and behaviour, hence increase the risk of being killed by a car (Cox and Smith, 1992; Brakes and Smith, 2005; Elmeros et al., 2011). Furthermore, AR exposure is a possible cause of illness and mortality, increasing the likelihood of positive findings in samples from sick or dead animals. AR prevalence in wildlife could be overestimated by not including possibly unexposed healthy animals in the studies.

Analyses of plasma and assessment of coagulation have been used to assess AR exposure in animals (Braselton et al., 1992; Hindmarch et al., 2019). This may; however, underestimate exposure in wildlife, as ARs have a shorter half-life in plasma compared to liver. Thus, a different method of verifying exposure in animals is needed. Due to the faecal elimination of ARs from the liver via the bile, ARs can be detected in faeces when residues are no longer detectable in plasma (Huckle et al., 1988; Sage et al., 2010). Nevertheless,

there are currently no studies of AR prevalence based on faecal analyses conducted throughout a single country.

Detection of ARs requires sensitive and specific methods. Warfarin and its metabolites are regularly analysed by gas chromatography or HPLC. The SGARs have lower volatility and larger mass; hence, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been considered a more suitable method with a more sensitive detection system (Feinstein et al., 2016).

1.3.3 Validation of analytical methods

Method validation is important in order to offer accurate and consistent data. Validation parameters include accuracy, precision, linearity and range, specificity, limit of detection (LOD)/limit of quantitation (LOQ), robustness and stability (Ravichandran et al., 2010), and are described in guidelines and internal documentation in accredited laboratories (European Medicines Agency, 2011; U.S. Food and Drug Administration, 2018; European Medicines Agency, 2019).

Accuracy is the closeness of agreement between the test results and the true value and is determined by using certified reference materials or a spiked sample (Peris-Vicente et al., 2015). In a spiked sample a known amount of the analyte is added to a blank sample, and the accuracy is determined as recovery or bias from the theoretical value. Accuracy can be estimated by analysing intra-day and inter-day variations.

Precision is the closeness of agreement between individual test results obtained by repeated measurements of a homogeneous sample under stipulated conditions (Ravichandran et al., 2010). Hence, precision is a measure of the uncertainty of the analytical method by considering the repeatability and reproducibility of the test results. It is usually specified as the standard deviation or the relative standard deviation.

To quantify the amount of a given analyte in a sample a calibration curve is made to establish a relationship between known concentrations of the target analyte and the instrument response. In addition, a blank sample, a processed matrix sample without analyte or internal standard (IS), and a zero sample, a processed matrix sample spiked with IS, are evaluated. Calibration standards should ideally be prepared in an identical matrix to the study samples, e.g. negative faeces for faeces samples. The linearity of the resulting calibration curve is evaluated. This is the capability of the analytical method to produce test results that are directly proportional to the analyte concentration in the sample (Peris-Vicente et al., 2015). The range is the interval between the upper and lower levels of the analyte in samples determined with precision, accuracy and linearity in the method (Ravichandran et al., 2010). Especially for methods with a wide range, weighting of the calibration curve might be important and other curve fitting models than linear, e.g. quadratic, might be more appropriate.

The specificity is another important parameter in validation of analytical methods. It determines the ability of the method to differentiate and quantify the analyte from other components in the mixture (Peris-Vicente et al., 2015). Other components may in addition interact with the analyte by increasing or decreasing the signal, known as matrix effects. The matrix effect is a common concern in LC-MS/MS analysis, affecting detection capability, accuracy and precision (Sell et al., 2018). The effect can be assessed quantitatively by the common method for a quantitative assessment of matrix effects developed by Matuszewski and colleagues (2003).

Sensitivity of an analytical method is the association between size of the measured signal and analyte concentration (González and Herrador, 2007). LOD and LOQ are indicators of the sensitivity of an analytical method. LOD is the lowest detectable concentration of an analyte, reliably differentiated from the background noise but not necessarily quantifiable. LOQ is the

lowest concentration of an analyte that is quantifiable with an acceptable level of precision. LOD and LOQ can be determined by signal-to-noise ratio, standard deviation and calibration curve (Peris-Vicente et al., 2015). Signal-to-noise ratio compares measured signals from the samples with known low analyte concentrations with those of blank samples, to establish the minimum level at which the analyte can be reliably detected (Ravichandran et al., 2010). By this method, LOD can be determined by a ratio of three times the background noise, and the ratio for LOQ is 10 to 1. In addition, the LOD and LOQ can be calculated as 3.3 times and 10 times, respectively, the standard deviation of a blank matrix sample divided by the slope of the calibration curve. The slope of the calibration curve is the mathematical equation relating the area of the peak to the concentration of the analyte. As most guidelines require a certain precision and accuracy at LOQ, LOQ must not only fulfil a signal to noise ratio of 10 to 1, but in addition have e.g. precision within 20% and accuracy within ± 20 %.

Robustness describes the ability of a method to remain unaffected by deliberate deviations from the experimental conditions, indicating its stability during normal usage (Peris-Vicente et al., 2015). Parameters influencing robustness could be pH, temperature, composition of the mobile phase, extraction time, etc.

1.3.4 Prevalence in wildlife

AR exposure in wildlife is a worldwide concern with residues detected in livers from a large number of predators. Predators can be exposed through primary, secondary or tertiary exposure. In addition, exposure through contaminated water has been suggested in wildlife living in marine environment (Serieys et al., 2019). However, most wildlife studies consist of opportunistically collected dead animals, hence route of exposure is generally not investigated.

In South Africa, 45 livers from seven different predator species were examined for ARs (Serieys et al., 2019). A prevalence of 81% were found in the animals. Blood was analysed in 10 of the caracals (*Caracal caracal*) examined in the study, but ARs were not detected in the blood. In California, livers from bobcats (*Lynx rufus*) displayed an AR prevalence of about 90%, and 77-87% of these presented with more than one AR (Riley et al., 2007; Serieys et al., 2015). In Denmark, 95% of 69 examined livers from polecats (*Mustela putorius*) had detectable levels of ARs, and 73% were exposed to more than one AR (Elmeros et al., 2018). In Scotland, AR prevalence was examined in livers from 99 presumed healthy, trapped American mink (*Neovison vison*) (Ruiz-Suárez et al., 2016). ARs were detected in 79% of the animals, and 50% were exposed to multiple compounds. In Norway, AR prevalence has only been examined in raptors, and 67% of opportunistically collected golden eagles (*Aquila chrysaetos*) and eagle owls (*Bubo bubo*) were exposed to ARs (Langford et al., 2013). The results from these studies point to a widespread problem of AR exposure in a large variety of wildlife predators worldwide. There is; however, no investigation of the prevalence of ARs in wild mammals in Norway.

Studies of AR exposure in wildlife can consist of comparison of the analytical methods used, investigation of parallels between species and prevalence in predators. The focus in this thesis is the prevalence of ARs in the red fox. All previous studies of prevalence in the red fox have been conducted by analysing livers from opportunistically collected animals. Details of prevalence studies in red foxes are given in Table 7. In Spain, two studies examined AR prevalence the red fox, and detected residues in 39% and 60% of the animals (Sánchez-Barbudo et al., 2012; López-Perea et al., 2019). In Northern Ireland, 84% of the foxes were exposed to ARs, with multiple substances detected in 59% of the animals (Tosh et al., 2011). In Sweden, residues of ARs were found in all 10 examined foxes (Tjus, 2014).

Table 7. Prevalence of anticoagulant rodenticides in livers from red foxes in different countries.

Anticoagulant rodenticide	Country	% Animals exposed (number)	Method used	Conc. range (ng/g)	Reference
Prevalence of all substances	Germany	59.8 (198)	LC-MS/MS	1-2433	Geduhn et al., 2015
	N. Ireland	84.3 (97)	LC-MS/MS	2-1781	Tosh et al., 2011
	Scotland	68 (30)	LC-MS/MS	unknown	Tosh et al., 2011
	Spain	39 (12)	LC-ESI-MS	5-12300	Sánchez-Barbudo et al., 2012
	Spain	60 (6)	LC-ESI-MS	4-5810	López-Perea et al., 2019
	Sweden	100 (10)	unknown	0.9-1100	Tjus, 2014
	Wales/England	65.5 (19)	LC-Flsc	unknown	Tosh et al., 2011
Coumatetralyl	Germany	5.7 (19)	LC-MS/MS	1-891	Geduhn et al., 2015
	N. Ireland	14.5 (17)	LC-MS/MS	2-91	Tosh et al., 2011
	Scotland	6.8 (3)	LC-MS/MS	unknown	Tosh et al., 2011
	Sweden	70 (7)	unknown	0.9-520	Tjus, 2014
	Wales/England	6.9 (2)	LC-Flsc	unknown	Tosh et al., 2011
Brodifacoum	Germany	45.6 (151)	LC-MS/MS	10-2433	Geduhn et al., 2015
	N. Ireland	32.1 (37)	LC-MS/MS	3-654	Tosh et al., 2011
	Scotland	9.1 (4)	LC-MS/MS	unknown	Tosh et al., 2011
	Spain	16.1 (5)	LC-ESI-MS	5-4500	Sánchez-Barbudo et al., 2012
	Sweden	nd	unknown		Tjus, 2014
	Wales/England	10.3 (3)	LC-Flsc	unknown	Tosh et al., 2011
Bromadialone	Germany	37.8 (125)	LC-MS/MS	4-1574	Geduhn et al., 2015
	N. Ireland	74 (85)	LC-MS/MS	4-1781	Tosh et al., 2011
	Scotland	50 (22)	LC-MS/MS	unknown	Tosh et al., 2011
	Spain	25.8 (8)	LC-ESI-MS	5-12300	Sánchez-Barbudo et al., 2012
	Sweden	90 (9)	unknown	0.9-1100	Tjus, 2014
	Wales/England	51.7 (15)	LC-Flsc	unknown	Tosh et al., 2011
Difenacoum	Germany	11.2 (37)	LC-MS/MS	10-774	Geduhn et al., 2015
	N. Ireland	39.5 (46)	LC-MS/MS	2-305	Tosh et al., 2011
	Scotland	27.3 (12)	LC-MS/MS	unknown	Tosh et al., 2011
	Spain	3.2 (1)	LC-ESI-MS	78	Sánchez-Barbudo et al., 2012
	Sweden	30 (3)	unknown	1.7-4.8	Tjus, 2014
	Wales/England	24.1 (7)	LC-Flsc	unknown	Tosh et al., 2011
Difethialone	Germany	7.9 (26)	LC-MS/MS	17-327	Geduhn et al., 2015
	N. Ireland	NA			Tosh et al., 2011
	Scotland	NA			Tosh et al., 2011
	Spain	nd	LC-ESI-MS		Sánchez-Barbudo et al., 2012
	Sweden	NA			Tjus, 2014
	Wales/England	NA			Tosh et al., 2011
Flocoumafen	Germany	13.9 (46)	LC-MS/MS	8-838	Geduhn et al., 2015
	N. Ireland	21.7 (25)	LC-MS/MS	2-280	Tosh et al., 2011
	Scotland	nd	LC-MS/MS		Tosh et al., 2011
	Spain	nd	LC-ESI-MS		Sánchez-Barbudo et al., 2012
	Sweden	nd	unknown		Tjus, 2014
	Wales/England	nd	LC-Flsc		Tosh et al., 2011

Flsc: Fluorescence; NA: Not analysed; nd: Not detected

Bromadiolone was most commonly detected in most of the fox studies. However, in Germany, brodifacoum was most prevalent (Geduhn et al., 2015). In Northern Ireland, Scotland, Wales and England, the prevalence of the different ARs was similar with difenacoum detected second most livers, followed by brodifacoum (Tosh et al., 2011). In contrast, coumatetralyl was the second most common AR in examined foxes in Sweden, with brodifacoum not detected (Tjus, 2014). Difference in prevalence between the countries may be caused by difference in sales volume or use. These data are not available from Norway (Haraldsen, 2018). Data from the other Scandinavian countries; however, determines bromadiolone as the most commonly used AR in Finland, while coumatetralyl, bromadiolone and difenacoum occurs in the highest number of products in Sweden (Tjus, 2014; Koivisto et al., 2018).

The red fox has widespread distribution all over Norway; hence they may act as sentinels for mammal-hunting predators in rural, suburban and urban areas. The endangered species of arctic fox (*Vulpes lagopus*) and gray wolves (*Canis lupus*) have different habitats from the red fox. However, these wildlife species belong to the *Canidae* family and feed on some of the same nutrient resources the red fox (Shirley et al., 2009; Wikenros et al., 2017). Hence, the prevalence of ARs in the red fox in Norway can give an indication of the extent of the problem in these species.

1.3.5 Impacts on non-target species

Large amounts of ARs may cause bleeding and death in animals, but even small amounts of rodenticides in the liver are suspected to have a variety of impacts on non-target species. Sublethal AR amounts are suggested to increase mortality in the animals when subjected to environmental stressors. An older study in rabbits demonstrated increased mortality after administration of phenylindanedione (100 mg/kg, followed by 25 mg/kg three times a day for 5 days) combined with frostbite (Jaques, 1962).

Similar results were achieved in rats administered dicumarol (1 mg/100g/day for one week) and different type of stressors. Furthermore, residues of AR affect reproduction. Therapeutic use of warfarin in pregnant women causes teratogenic effects, spontaneous abortions and stillbirths (Ginsberg et al., 1989; Greaves, 1993). Furthermore, reduced sperm motility, embryopathy, embryonic mortality and neonatal death were demonstrated in merino sheep (*Ovis aries*) administered pindone (10 mg/kg, decreased to 3 mg/kg and 2 mg/kg over three days) (Robinson et al., 2005). In addition, neonatal death (8/13) was observed in a litter of puppies with brodifacoum detected in two out of three analysed livers (630 ng/g and 230 ng/g) (Munday and Thompson, 2003).

Negative correlation was observed between body condition and AR concentrations in livers from opportunistically collected stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) (Elmeros et al., 2011). Furthermore, lower body condition was detected in the animals found dead compared to animals that died following accidents, hence suggesting that ARs cause a weakened body condition. Weakened body condition may reduce hunting ability and increase susceptibility to accident, injury and predation. However, lethal doses of ARs cause rapid reduction of food intake in rats (Cox and Smith, 1992). The reduced body condition in the stoats and weasels could thus be caused by ingestion of lethal amounts of ARs prior to death, in contrast to subtoxic amounts.

Subtoxic AR levels are suspected to increase susceptibility to pathogens and parasites in mammals. The common vole (*Microtus arvalis*) is suggested to have increased susceptibility to chlorophacinone when infected with the bacterium *Francisella tularensis* (Vidal et al., 2009). The study demonstrated lower levels of chlorophacinone in voles positive for *F. tularensis* (geometric mean 418 ng/g), compared to levels in voles negative for the pathogen (geometric mean 1084 ng/g). In another study, two fishers (*Martes pennanti*) dead from AR poisoning (concentration not given) displayed high

levels of tick infestations (Gabriel et al., 2012). However, whether AR exposure predisposes fishers to parasites cannot be concluded based on two animals. Significant association between AR exposure and notoedric mange caused by the mite *Notoedres cati* was suggested in bobcats and mountain lions (*Puma concolor*) (Riley et al., 2007). In the study, all 19 animals that died from severe mange were exposed to ARs (arithmetic mean 390 ng/g). In most of these animals, no evidence of AR toxicity was demonstrated on necropsy. Furthermore, as notoedric mange is an epizootic disease in Californian bobcats and other studies from this area have demonstrated AR residues in 89-100% of the animals, these exposures are probably unrelated (Serieys et al., 2013; Serieys et al., 2015).

Several studies have examined AR-related effects on the immune system in different species. Blood from apparently healthy bobcats with detectable levels of ARs (concentrations not given) displayed changes in the expression of immune-related genes (Fraser et al., 2018). Furthermore, in another study, blood from wild bobcats with detectable AR levels were compared to bobcats without detectable AR residues (Serieys et al., 2018). The exposed animals were apparently healthy with normal clotting times, but AR concentrations were not given. Bobcats exposed to ARs demonstrated suppression of immune response and changes in inflammatory response with increased lymphocyte counts, suppression of neutrophils, higher B-cell counts and B-cell fractions. These studies indicated an association between changes in the immune system and AR levels, but the studies were not designed to detect causal relationship between the level of subtoxic ARs and the clinical effect on the immune system in the animal. Correlation does not equal causation, and some of these results could correlate to AR exposure simply by chance. In addition, as these studies were undertaken in wild endangered animals, information of ingested doses, time of exposure or liver concentrations could not be obtained. In order to investigate the impact of subtoxic AR concentrations on the immune system, other methods

such as delayed-type hypersensitivity tests and cell proliferation assays should be applied. Furthermore, the study design must aim to exclude other explanatory variables for the clinical effect on the immune system. In contrast, a randomized controlled study examined the effect of subtoxic brodifacoum exposure in domestic cats (Kopanke et al., 2018). The cats were administered 0.05 mg brodifacoum/kg bw once weekly for six consecutive weeks and had no observed changes in blood coagulation. A decreased production of cytokines was detected, otherwise there were minimal effect on the immune system. Felines; however, differ in pharmacokinetics from *Canidae* by lack of several UGT enzymes (Court, 2013), as seen by difference in LD_{50} doses (Table 5), and there is no information whether subtoxic levels in dogs or foxes have similar effect.

2. AIMS OF THE STUDY

The overall objectives of this study were to increase the knowledge about the extent of AR residues in wildlife and a healthy dog population in Norway and to determine the half-lives of specific ARs in exposed dogs.

In order to accomplish this, we pursued the specific aims:

- Establish a new and better method for detection and quantification of ARs in faeces by reversed phase ultra-high performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS; Paper I).

- Determine occurrence of AR exposure in wild red foxes in Norway by faecal analyses, in relation to age, sex, seasonal and geographical occurrence (Paper II).

- Assess the faecal analytical method by comparing faecal and liver residues of ARs from the same red foxes (Paper III).

- Determine occurrence of AR exposure in healthy dogs in Norway (Paper IV).

- Estimate elimination of ARs from exposed dogs by analyses of serially collected blood and faeces (Papers I and IV).

3. MATERIALS AND METHODS

3.1 Study design

Privately owned dogs and wild red foxes were included. The study in Paper IV was designed as a prospective case-series study of exposed dogs and a cross-sectional study of non-randomly selected healthy dogs. The studies of wild red foxes in Paper II and III were designed as cross-sectional studies.

3.2 Animal populations

Exposed dogs

Privately owned dogs admitted to NMBU with clinical signs consistent with AR poisoning (anaemia or suspected bleeding) or with witnessed ingestion of an AR from April 2016 to November 2019 were included in the project (n=19). Of these dogs, one died and 12 tested negative for ARs and did not develop any clinical signs and were excluded. Six dogs were included in Papers I and IV (Table 8); one dog ingested coumatetralyl and brodifacoum, two dogs ingested brodifacoum; two dogs ingested bromadiolone, and one dog ingested difenacoum. All dog owners gave written informed consent before participation. The blood samples were collected during a general

Table 8. Overview of the studies included in the PhD project

Species	Number of animals included in the study	Material analysed	Period of collected material (days)	Type of analysis	Paper
Dog	1	Blood Faeces	513	UPLC-MS/MS UPLC-MS/MS	I
Red foxes	139	Faeces	once	UPLC-MS/MS	II
Red foxes	40	Faeces Liver	once	UPLC-MS/MS HPLC-MS	III
Dogs	6	Blood Faeces	37-1376	UPLC-MS/MS UPLC-MS/MS	IV
Dog puppies	4	Faeces	86	UPLC-MS/MS	
Dogs	110	Blood Faeces	once	UPLC-MS/MS UPLC-MS/MS	

clinical examination, with the owners' permission. Additional eight dogs were eligible for inclusion in the project, but the owners declined to participate. The owners filled out a standardized questionnaire before the clinical consultation. The questionnaire included: (i) General information about the dog: age, breed, sex, neuter status, food source, exercise, living conditions; (ii) General information about clinical health of the dog: concurrent medications, previous clinical signs; (iii) More detailed information of the suspected exposure to ARs: suspected product, time of exposure, previous treatment, previous possible exposure to rodenticides.

Healthy dogs

The healthy dogs were all privately owned (n=110) and enrolled at routine visits to veterinary clinics and national dog shows (Paper IV). Dogs were included from all 18 counties in Norway with a variety of living conditions (rural, suburban and urban) between November 2017 and October 2018. The dogs were of 59 different breeds, with a mean age 5.2 years (range 1.5-13 years), mean weight 21 kg (range 2.9-70 kg) and both sexes (46 males and 64 females) were represented. According to owner's signed declaration and information obtained in a comprehensive questionnaire, the dogs were healthy with no previous known exposure to ARs.

Wild red foxes

The Norwegian *Echinococcus multilocularis* survey program includes examination of faecal samples from wild red foxes for this parasite. Annually, around 600 samples are analysed (Norwegian Veterinary Institute, 2020). Faeces from presumed healthy wild red foxes (n=163) were collected by experienced hunters as part of this governmental survey (Figure 5; Paper II). Of the 163 samples, 24 consisted of mostly hair and were omitted. The foxes were shot during the regular hunting season from

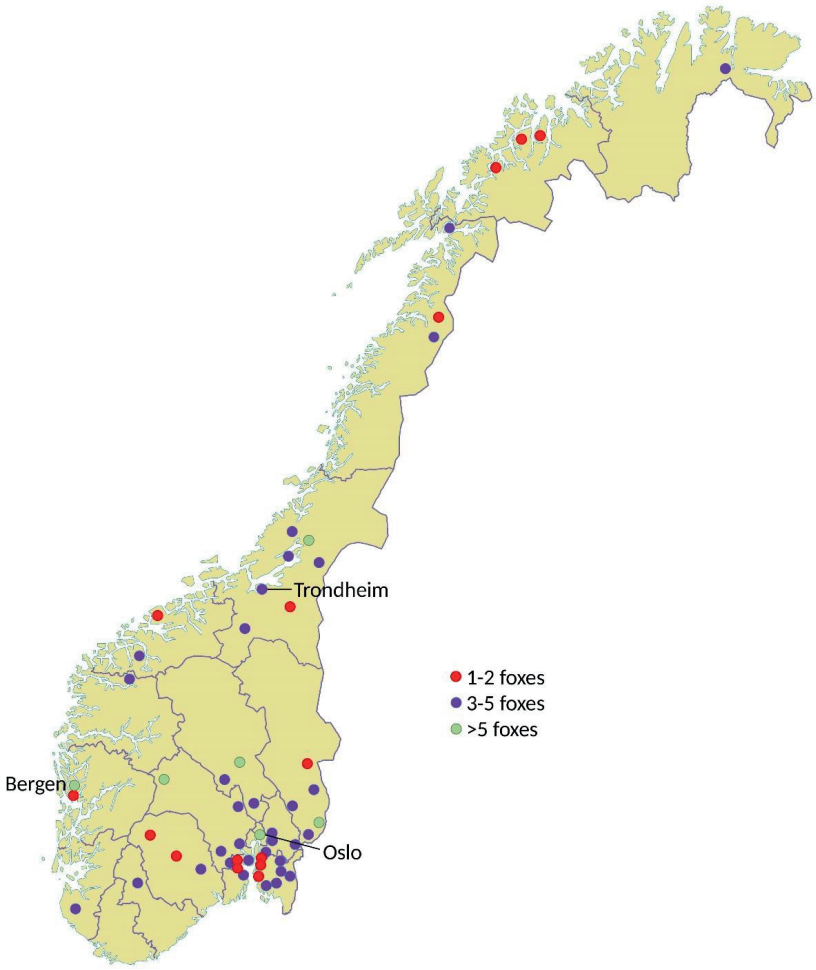


Figure 5. Sampling sites of faeces from red foxes collected in 2016, presented in Papers II and III. Colours of dots express the number of samples collected in the municipality. Map from Kartverket, Creative commons BY 4.0.

January to mid-April and mid-July to late December in 2016. The year was divided in seasons; winter (January, February and December), spring (March to May), summer (June to August) and autumn (September to November). The hunters provided information on sex (male or female) and estimated age (juvenile, 1-year-old or adult), together with the municipality in Norway and date when the fox was killed. The hunters estimated age according to foxes' size and the presence of deciduous or permanent teeth. The sex was determined based on presence (intact male) or absence (intact female) of a penis. Most samples were collected during the winter months, due to preferred tracking conditions in the snow. The samples were collected from 56 municipalities (ranging in size from 7,000 to 310,600 hectares), representing 18 out of 19 counties in Norway and including areas surrounding the three largest cities in Norway (Oslo, Bergen and Trondheim). From a portion of the same animals (n=40) both liver tissue and faecal samples were collected, and the results from these animals were presented in Paper III.

3.3 Sampling procedures

Blood

Blood for AR analyses was collected once from the healthy dogs by a veterinarian (Paper IV) using vacuum tubes containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Blood samples were frozen (-20°C) shortly after collection and maintained frozen until analyses.

A blood sample from the exposed dogs was collected at first admittance followed by once a day for one week, then once a week for one month and once a month thereafter, until ARs were no longer detectable in blood or faeces or until the study was terminated in November 2019. In the exposed dogs, samples were collected by a veterinarian in vacuum tubes containing sodium fluoride as preservative and potassium oxalate as anticoagulant (Paper I and IV). In addition, blood samples were obtained with EDTA as

anticoagulant. The samples were frozen (-20°C) shortly after collection and maintained frozen until analyses of ARs. Details of blood sample preparation and analysis are described in Paper I, however as the focus of this work was development of analytical method to estimate prevalence of ARs in faeces, further discussion of the analytical method in blood is not included.

Blood samples for coagulation analyses (PT and aPTT) were obtained from the exposed dogs at each visit. These samples were collected into vacutainer tubes containing sodium-citrate (3.2%) and analysed by a Coag Dx Analyzer (IDEXX Laboratories Europe B.V., The Netherlands) within 2 hours of collection. Furthermore, serum was obtained 2-4 times from each exposed dog and analysed at Sentrallaboratoriet, NMBU. Analysis included full biochemistry with measurements of the following parameters; AST/aspartate aminotransferase, ALT/alanine aminotransferase, alkaline phosphatase, CK/creatine kinase, amylase, lipase, total protein, albumin, globulin, urea, creatinine, bile acids, total bilirubin, cholesterol, glucose, inorganic phosphate, calcium, sodium, potassium and chloride. In addition, haematology profiles with complete blood count (CBC) were obtained; RBC/total number of red blood cells, WBC/total number of white blood cells, HGB/haemoglobin concentration, HCT/haematocrit, MCV/mean corpuscular volume, MCH/mean corpuscular haemoglobin, MCHC/mean corpuscular haemoglobin concentration, MPV/mean platelet volume, RDW/red cell distribution width, total number of neutrophilic granulocytes, eosinophilic granulocytes, basophilic granulocytes, monocytes, lymphocytes, reticulocytes and platelets.

Faeces

Faeces from both healthy and exposed dogs were collected by the owners after natural defecation on the same day as blood was sampled (Papers I and IV). Sample sizes varied (5.6-51.4 g) as the dogs were of different

breeds and sizes (2.9-70 kg). However, due to poor clinical condition in two of the poisoned dogs, defecation was sparse during the first days after presentation and no samples were available for analysis (Papers I and IV). Faeces was collected in dark plastic bags, brought to NMBU, then stored frozen at -20°C until further processing.

Wild red foxes were shot during the licensed hunting season (January to mid-April and mid-July to late December) in 2016. Faeces was removed by the hunters directly from rectum immediately after death and submitted to the Norwegian Veterinary Institute (NVI) within 2 days (Papers II and III). One sample per fox (11.2-18.3 g) was collected. The samples were frozen at -80°C immediately upon arrival at the NVI and kept frozen at this temperature for 3 days, before being stored at -20°C until preparation and analyses.

Liver

A piece of the liver was removed from the wild red foxes immediately after death and submitted to NVI within 2 days (Paper III). The submitted samples were frozen at -80°C and kept frozen at this temperature for 3 days, before being stored at -20°C. ARs in livers were separated and analysed by HPLC-MS. The liver analyses were not the focus of this project, and details are described in Paper III. Wet liver tissue LOQ were 0.5 ng/g for coumatetralyl, 1.8 ng/g for brodifacoum and bromadiolone, 0.8 ng/g for difenacoum and 0.3% for difethialone and flocoumafen. The recovery rates of ARs were 87-95%.

3.4 Faecal sample analysis

Several methods for detection of ARs after poisoning using LC-MS/MS in different matrices have been established (Jin et al., 2008; Yan et al., 2012; Sell et al., 2018). In faeces, an analytical procedure for detection of

bromadiolone by HPLC-MS in foxes has been developed (Sage et al., 2010), but no methods for quantification of ARs in dog faeces by LC-MS/MS have been reported. In Norway, brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone and flocoumafen are registered for use. Therefore, a method using UPLC-MS/MS for detection and quantification of these ARs in faeces from dogs was developed and validated in this PhD project.

A brief overview of the analytical method in faeces is presented, with further details described in Paper I. The frozen faecal samples were lyophilized to dryness using a Christ Alpha 1-4 Isceon laboratory freeze dryer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). About 3 g were removed from the freeze-dried samples and homogenized, and aliquots of 100 mg were taken. There are three principles in sample preparation: Dissolution of the analyte in a suitable solvent, removal of any interfering compounds and preconcentration of the analyte. Matrix effect is a problem in faeces due to the high lipid content (Ho et al., 2003). To overcome this problem, sample purification by extraction was performed. In our studies we utilized liquid-liquid extraction, which separates compounds based on their relative solubilities in two different liquids. In the procedure, protein precipitation was done with acetonitrile (100 µL). Internal standard (50 µL; warfarin-d5) and borate buffer (400 µL; pH 11) were added to the samples followed by agitation. Then acetonitrile (1.0 mL) was added followed by agitation. Finally, dichloromethane (1.0 mL) was added followed by mixing and centrifugation, resulting in a thin, upper layer with some faecal residue in the tubes. This layer was carefully removed, and the dichloromethane phase was transferred to a clean glass tube, dried (N_2; 40°C) and reconstituted with methanol (100µL)/Type 1 water mixture (20:80 vol/vol). The tube was shaken, centrifuged and the contents transferred into auto sampler vials.

Different extraction solvent mixtures were tested in the development of the method. Ethyl acetate has previously been used for extraction of ARs from

serum (Guan et al., 1999; Jin et al., 2007), but preliminary experiments with ethyl acetate and heptane (4:1 vol/vol) resulted in poor extraction due to the high lipid content in faeces. A previous method with detection of bromadiolone in faeces used acetone for homogenization and protein precipitation, followed by drying of an aliquot of the supernatant, reconstitution in dichloromethane and hexane (50:50, vol/vol) and solid phase extraction on silica columns (Sage et al., 2010). We have more extensive experience with liquid-liquid extraction which is also a less expensive and simpler method compared to solid phase extraction; hence the method described by Sage and colleagues was not pursued further. Previous studies have shown that acetonitrile is one of the most efficient protein precipitants (Polson et al., 2003). We tested protein precipitation with acetonitrile alone and in combination with methanol (85:15 vol/vol), but extraction with only acetonitrile or combined with methanol did not produce sufficiently clean extracts. Clean extracts are important to reduce ion suppression and matrix effect. The best separation with good recoveries, high extraction efficiencies and minimal matrix effect was achieved using protein precipitation with acetonitrile and extraction with dichloromethane. Dichloromethane was used in extraction procedures, even though it is not optimal from a workplace health point of view due to suspected carcinogenicity. However, dichloromethane gave the best results of the different extraction solvents tested. All extracts were prepared in approved fume-hoods.

Separation was performed using a Waters Acquity UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm particles; Waters Corporation, Milford, Massachusetts, USA). In UPLC, an analyte in a solvent (mobile phase) is pumped through the column (stationary phase) at high pressure. The polarities, size and lipophilicities of the analytes result in different retention times (Table 9). In this project, a gradient elution with a mobile phase consisting of ammonium formate buffer (5 mM; pH 10.2) and methanol was

Table 9. Precursor ion-to-product ion transitions, retention time (RT) and limit of quantification (LOQ) for the anticoagulant rodenticides analysed in the studies.

Anticoagulant rodenticide	RT (min)	Precursor ion-to-product ion transitions (m/z)*	LOQ faeces/blood (ng/g or ng/ml)
Coumatetralyl	1.86	*239.1>107.1*/ 91.0	1.5
Warfarin-d_5	1.62	*314.2>163.1*/ 256.0	
Brodifacoum	3.27	*525.2>337.1*/ 178.2	2.6
Bromadiolone	2.73	*511.1>251.2*/ 173.0	2.6
Difenacoum	2.83	*445.3>179.1*/ 257.2	2.2
Difethialone	3.33	*539.1>178.1*/ 335.1	2.7
Flocoumafen	3.17	*543.2>159.1*/ 335.2	2.7

*Transitions in italic font were used for quantification

used. In the development of the method both acidic, neutral and basic mobile phases were tested. The compounds are weak acids with pKa from 5.6 to 6.1, values from Chemicalize.com. As shown in Table 1 several of the compounds are highly lipophilic with log P_{ow} between 5 and 6. With a basic mobile phase less retention and more efficient chromatography was achieved. This mobile phase has in addition been successfully applied for other applications in the laboratory (Berg et al., 2009; Valen et al., 2017; Kristoffersen et al., 2018). Ammonium formate has previously demonstrated to improve signal sensitivity (Choi et al., 2001), and in utilising this buffer we obtained good peak shape, best mass signal intensity and good peak separation for all analytes.

Positive electrospray ionization (ESI) MS/MS detection was performed on a tandem mass spectrometer (Waters), using two multiple reaction monitoring (MRM) transitions. ESI transfers the ions from a solution to highly charged droplets by electrical energy under atmospheric pressure (Ho et al., 2003). The droplets are passed through heated nitrogen to

evaporate the remaining solvent molecules and provide charged ions in a gaseous phase (Hoffmann and Stroobant, 2007). After the ionization in ESI, each ion has a particular mass-to-charge ratio (*m/z*). Several other studies have operated ESI in negative mode for ionization of ARs (Imran et al., 2015). We selected positive mode for our analysis, as experience in the laboratory has revealed an instrument dependent problem with stable signal intensity in negative mode, and comparable sensitivity was found for positive mode.

The ions are sampled and accelerated into the mass spectrometer for separation. In this project we used a tandem MS in which ions are separated in oscillating electric fields (Ho et al., 2003). The precursor ions are mass-selected by the first quadrupole and collide with a collision gas (argon) in the collision source, resulting in fragmentation. In the second quadrupole the fragmented ions are separated according to their *m/z* ratio (Table 9). In this project, two MRM transitions for each analyte and internal standards were used. In this method both first and second analysers focus on selected masses, hence focusing on the precursor and fragment ions over longer times (Hoffmann and Stroobant, 2007). This increases the sensitivity of the method, as well as the selectivity. Finally, a detector generates signals from the ions proportional to their abundance. Signals are recorded by a computer and displayed graphically (MassLynx 4.1 software; Figure 6).

In the chromatogram, bromadiolone displayed double peaks while flocoumafen was partly resolved (Figure 6), as the two diastereomers were separated by the LC-gradient. For the other compounds the diastereomers coeluted. In the project, the focus was not to identify diastereomers in plasma or faeces but gain knowledge of the prevalence of ARs in the population. Hence, further analyses of the diastereomers were not done.

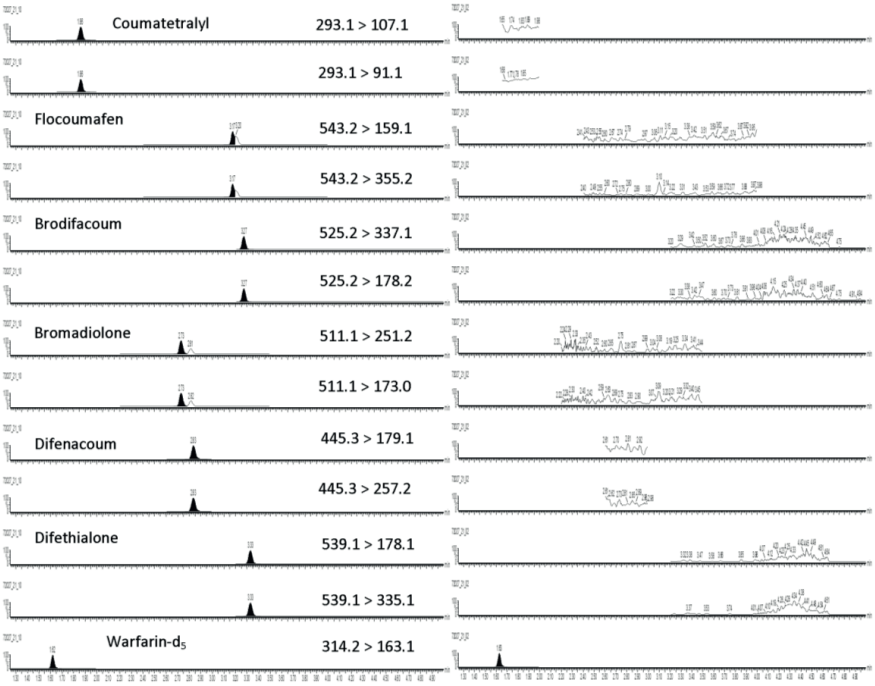


Figure 6. The chromatograms of the lowest quality control sample and the blank sample with the internal standards.

Estimations of AR elimination from the body (Paper I and IV) were performed by manual calculations of concentration versus time profile in the post-peak phase for the ARs. In first-order kinetics, an exponential decay is observed as a function of time ($dC/dT = -kC$), where dC is the change in concentration over a time interval (dT) and k is the elimination rate constant (Derendorf and Schmidt, 2020). The elimination half-life ($t_{½}$) was calculated directly from the rate constant; $t_{½} = \ln 2/k$ or $t_{½} = 0.693/k$. The results were based on few animals and few analyses, and the findings must thus be interpreted with caution.

3.5 Validation of analytical method

In validation of the faecal analytical method, we evaluated parameters previously described; accuracy, precision, linearity and range, specificity, LOQ, extraction recovery and matrix effects (Ravichandran et al., 2010; European Medicines Agency, 2011). The faecal analyses were performed using 3–6 parallels for each sample, and the average of the parallels used in further calculations.

Accuracy was determined by analyses of spiked human whole blood and canine blank faeces samples. Warfarin d5 was chosen as internal standard because it has similar physicochemical properties to the other ARs. Extraction recoveries were studied at two concentration levels for blood samples and one level for faecal samples (Table 10). Extraction recoveries for SGARs were lower from faeces (18-32%) than from blood (43-90%). For coumatetralyl, extraction recovery was higher in faeces (69%) than blood (10-15%).

Precision of the analytical method were determined by relative standard deviations of within-day (n=6) and between-day (n=6) variations of spiked human whole blood and blank faeces samples analyses at three different concentration levels added for all compounds (Table 10). Precision and accuracy were determined as bias and were within ±20% for all substances. Working range extended from the upper to the lower concentration of the analyte in the sample.

Linearity was determined by evaluation of calibration curves. ARs in the faecal samples displayed wide concentration ranges (Table 10); hence, a 2nd order calibration curve ($y=ax^2+bx+c$) was used for quantification. Mean values of coefficient of determination (R^2) were above 0.995 for all substances in both blood and faeces. As R^2 was >0.990, adequate linearity was achieved (Peris-Vicente et al., 2015).

Table 10. Validation parameters of six anticoagulant rodenticides analysed in the studies.

Anticoagulant rodenticide	Calibration range (ng/ml or ng/g)	Blood		Faeces		QC-sample conc.	Blood					Faeces				
		Mean R^2	RSD (%)	Mean R^2	RSD (%)		Within-day precision RSD (%)	Between-day precision RSD (%)	Bias (%)	ME%	RE%	Within-day precision RSD (%)	Between-day precision RSD (%)	Bias (%)	ME%	RE%
Coumatetralyl	1.5 – 731 (0.0050 – 2.5 µM)	0.999	0.061	0.998	0.24	2.3	4.0	7.9	-3.5	90	10	2.8	15	-3.6	109	69
						88	3.6	8.7	5.2			5.7	13	1.6		
						585	6.0	11	-1.8	93	15	6.5	15	8.4	96	
Brodifacoum	2.6 – 1309 (0.0050 – 2.5 µM)	0.998	0.37	0.999	0.11	4.2	8.6	15	8.4	93	61	9.2	19	-1.7	32	25
						157	3.1	14	6.2			9.3	18	-2.1		
						1047	9.1	11	-3.1	90	78	6.4	19	-18	32	
Bromadiolone	2.6 – 1319 (0.0050 – 2.5 µM)	0.998	0.21	0.998	0.10	4.2	5.8	17	16	100	44	15	16	-7.7	63	32
						158	5.7	14	13			17	18	-3.1		
						1055	7.5	17	-4.1	97	52	16	13	-11	56	
Difenacoum	2.2 – 1111 (0.0050 – 2.5 µM)	0.997	0.33	0.999	0.10	3.6	9.1	13	5.5	101	43	13	18	-5.3	62	26
						133	2.2	15	7.8			14	17	-6.1		
						889	8.9	15	6.1	97	87	15	15	-11	60	
Difethialone	2.7 – 1349 (0.0050 – 2.5 µM)	0.997	0.37	0.998	0.17	4.3	8.9	14	16	86	69	10	17	1.3	31	22
						164	6.4	12	12			11	11	0.8		
						1079	13	17	3.1	81	84	5.1	16	-9.4	27	
Flocoumafen	2.7 – 1356 (0.0050 – 2.5 µM)	0.997	0.41	0.995	1.1	4.3	8.0	14	2.7	95	80	11	19	-5.5	45	18
						163	3.0	14	6.6			9.0	17	-2.8		
						1085	6.4	15	-4.6	92	90	6.3	19	-14	32	

In blood, no pronounced matrix effects were seen, while ion suppression was observed for all substances except for coumatetralyl for faeces. Preparation of standards in blank faeces will to a certain extent match the samples and compensate for the ion suppression, but inclusion of stable isotope labelled internal standards for all compounds would have been preferable. These were however not available in the laboratory.

LOQ was determined in the method validation based on a signal-to-noise approach, as the analytical procedure exhibited baseline noise (European Medicines Agency, 1995). Signal-to-noise ratio was determined by comparing measured signal from blank samples to samples with known low concentrations of analyte. Signal-to-noise ratios were above 10 for all compounds at the level of the lowest calibrators. This was deemed satisfactory, and no further work was done to determine a possible lower LOQ by dilution.

3.6 Statistical analysis

For Paper II, the clinical and laboratory data were collected into Microsoft Excel spreadsheet and transferred to R (version 3.5.0, R Development Core Team 2016) for statistical analysis. Estimated prevalence of foxes positive for ARs was calculated for the total of all samples (n=139) and within groups. The specific categorization of rural, suburban and urban of human population densities were tested by including 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. In further testing, the definition of rural municipalities was reduced to less than five inhabitants per km^2 and suburban municipalities were altered to 6-200 inhabitants. Finally, municipalities were categorised based on population only with rural area (1,000-10,000), suburban area (10,000-50,000) and urban area (50,000-180,000). The analyses of AR prevalence correlated to human population densities were

statistically independent of changes in the categories. Differences between prevalence of AR substances were tested using the McNemar χ^2 test, whereas significant differences in AR exposure between groups were tested using the Pearson χ^2 tests. The relationship between AR exposure and the covariates age (juvenile, 1-year-old or adult), sex (male or female) and seasons (winter, spring, summer or winter) were investigated by multiple logistic regression analyses. The full model included age, sex and season. However, results from simple regression analyses were reported if one or the two other covariates did not improve the model according to the Akaike information criterion value (Akaike, 1974). 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 significantly.

Frequencies of AR occurrence between compounds in faeces and liver (Paper III) were compared using contingency tables as Fisher exact test. Statistical comparisons were conducted using statistical software (Epi Info v7.2.3.1; Center for Disease Control and Prevention, Division of Health Informatics & Surveillance, Atlanta, GA). Nonparametric tests were used when data were not normally distributed and statistical associations of AR concentrations between faeces and liver were assessed by Wilcoxon signed rank test and conducted by JMP Pro (v14.2.0; SAS Institute). Results were considered statistically significant when p values of the statistical tests were ≤ 0.05.

4. SUMMARY OF RESULTS

Paper I

Quantitative method for analysis of six anticoagulant rodenticides in faeces, applied in a case with repeated samples from a dog

A new method for the quantification of the six ARs used in Norway (brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, flocoumafen) in canine blood and faeces was developed and validated using UPLC–MS/MS. Serially collected blood samples from an accidentally poisoned dog demonstrated an estimated initial α-elimination phase of coumatetralyl of 1.8 days, and residues detectable for 64 days after ingestion (Figure 7). Serially collected samples demonstrated coumatetralyl residues in faeces for 204 days after ingestion, with an estimated faecal elimination phase half-life of 81 days. A slow elimination of brodifacoum from faeces was found, with traces still detectable at day 513.

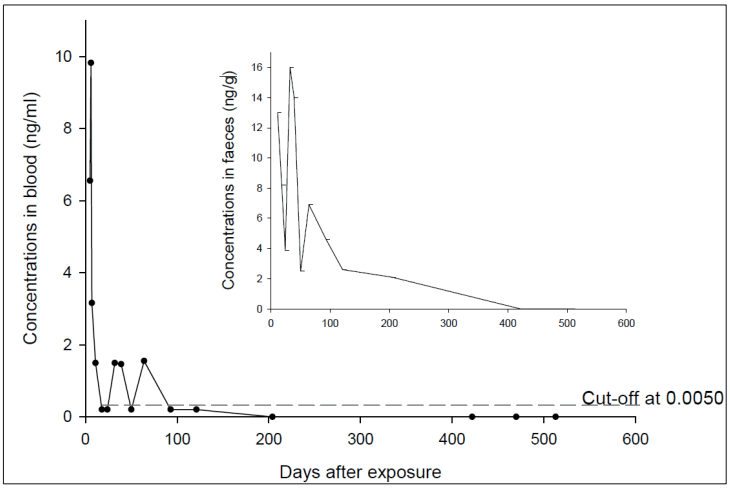


Figure 7. Concentrations of coumatetralyl in blood and faeces.

Paper II

Prevalence of anticoagulant rodenticides in feces of wild red foxes (*Vulpes vulpes*) in Norway

We analysed 139 faecal samples from presumed healthy red foxes for six ARs: brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone and flocoumafen. At least one AR was detected 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) (Figure 8).

Brodifacoum was detected in significantly more foxes than coumatetralyl (χ^2=29.09, p< 0.0001, df=1) and bromadiolone (χ^2=32.33, p< 0.0001, df=1). 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 ARs.

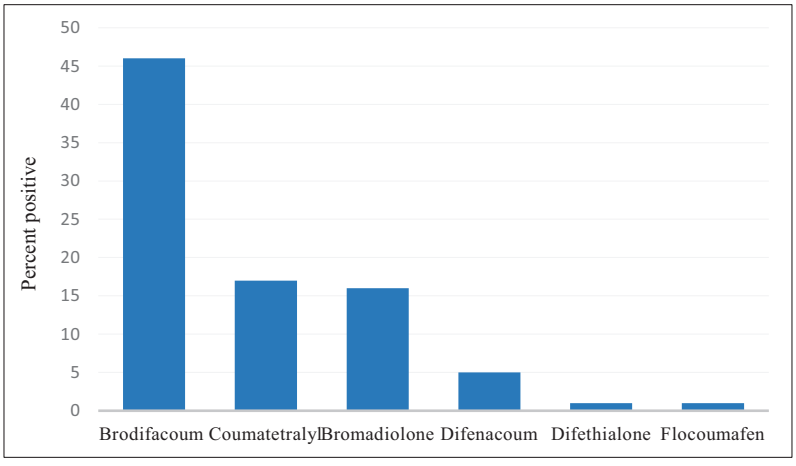


Figure 8. Occurrence of different anticoagulant rodenticide compounds in 139 faecal samples collected from presumed healthy wild red foxes in Norway.

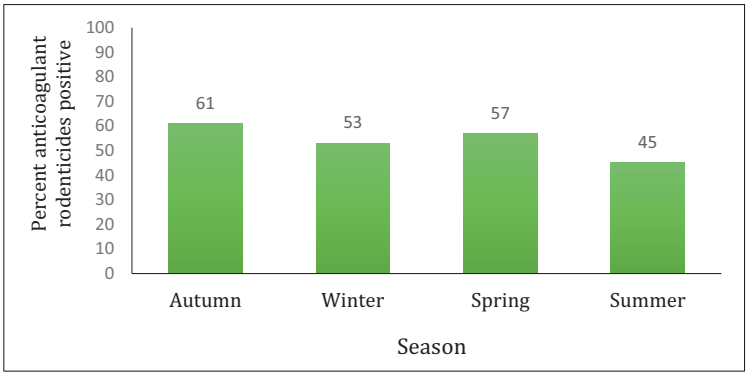


Figure 9. Seasonal occurrence of anticoagulant rodenticide compounds in 139 faecal samples from red foxes. Exposure varied by season; autumn (September-November), winter (December-February), spring (March-May) and summer (June-August).

Exposure 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 (Figure 9). However, there was no significant seasonal difference in exposure to a single AR (χ^2 =1.20, p=0.75, df=3).

Residues of at least one AR were detected in 49% (32/65) males, 59% (38/64) females and 50% (5/10) of those with unknown sex (Table 11). AR exposure was not significantly different between sexes (χ^2 =0.655, p=0.42, df=1). Exposure to AR differed between the age groups with exposure in

Table 11. Overview of the number (N) of faecal samples from wild red foxes by sex, age and location, and percent positive samples of anticoagulant rodenticides within each group.

		N	%
All foxes		139	54
Sex	Female	64	49
	Male	65	59
	Unknown	10	50
Age	Juvenile	50	48
	Adult	78	58
	Unknown	11	55
Location	Rural	44	48
	Suburban	64	61
	Urban	31	48

Table 12. Faecal samples (n) from wild red foxes containing different anticoagulant rodenticides (AR) (n, %) by geographical population areas. The location where the foxes were shot in Norway and the faecal samples collected are defined as rural (1,000-10,000), suburban area (10,000-50,000) and urban area (50,000-180,000) depending on population densities.

Population areas	**Faecal samples n**	**Faecal samples with AR n (%)**	**Anticoagulant rodenticides (AR)**					
			Brodifacoum n (%)	Coumatetralyl n (%)	Bromadiolone n (%)	Difenacoum n (%)	Difethialone n (%)	Flocoumafen n (%)
Rural	44	21 (48)	18 (41)	5 (11)	5 (11)	2 (5)	0	1 (2)
Suburban	64	39 (61)	37 (58)	8 (12)	12 (19)	4 (6)	2 (3)	0
Urban	31	15 (48)	9 (29)	10 (32)	5 (16)	1 (3)	0	1 (3)
Total	*139*	*75 (54)*	*64 (46)*	*23 (17)*	*22 (16)*	*7 (5)*	*2 (1)*	*2 (1)*

58% (45/78) adults, 48% (24/50) in juveniles and 55% (6/11) of unknown sex. However, positive findings were not significantly different between ages for neither single nor multiple AR exposure ($p>0.42$).

In examination of exposure to AR when sex was combined with age by logistic regression analyses, a tendency was indicated of positive association. In adult female foxes, ARs were detected in 68% animals, compared to 49% in a combined group of juveniles and adult male foxes ($p=0.07$).

Correlation of AR exposure to human population densities, revealed that foxes in suburban areas displayed a higher occurrence of ARs (61%; 39/64) compared to rural (48%; 21/44) and urban (48%; 15/31) areas (Table 12). The difference was however not statistically significant ($\chi^2=2.33$, $p=0.31$, $df=2$), but we found a significant difference in occurrence of brodifacoum and coumatetralyl in foxes from different geographical areas. Exposure to coumatetralyl was increased in urban compared to rural areas ($p=0.03$), while brodifacoum was increased in suburban compared to urban areas ($p=0.01$).

Paper III

Comparison of anticoagulant rodenticide concentrations in liver and feces from apparently healthy red foxes

This study compared faecal and liver residues of ARs in 40 red foxes to determine the value of assessing AR exposure by faecal analysis. Residues of ARs were detected in 53% of the faecal samples and 83% of the liver samples. Brodifacoum was most prevalent in both faeces and liver samples (Table 13).

Comparisons of AR concentrations between faeces and liver demonstrated no statistically significant difference for bromadiolone, coumatetralyl,

Table 13. Number of red foxes where anticoagulant rodenticides were detected.

	N	Occurrence (%)	Residues in positive individuals		
			Mean ±SE (ng/g)	Median (ng/g)	Min-max (ng/g)
Faeces					
Brodifacoum	21	53	35 ± 5.85	28	4-103
Bromadiolone	3	8	122 ± 88.82	44	23-299
Coumatetralyl	8	20	13 ± 6.86	6	1-59
Difenacoum	4	10	21 ± 11.36	13	4-53
Difethialone	2	5	8 ± 2.96	8	5-11
Flocoumafen	1	3	10		
Liver					
Brodifacoum	32	80	56 ± 8.88	29	2-158
Bromadiolone	24	60	34 ± 9.72	14	2-192
Coumatetralyl	10	25	11 ± 6.37	2	1-62
Difenacoum	9	23	5 ± 2.21	2	1-18
Difethialone	9	23	6 ± 4.11	1	1-38
Flocoumafen	2	5	1 ± 0.03	1	1-1

Occurrence: % of animals with ARs compared to the total number of 40 samples.
Mean ± standard error of the mean (SE), median and range of concentrations are from the cases with detectable concentrations of ARs.

difenacoum, or difethialone. Brodifacoum; however, was detected in significant higher concentration in liver than faeces ($p = 0.003$). Hepatic AR concentrations were >100 ng/g (mean: 178 ng/g) in 28% (11/40) of the foxes, with concentrations >200 ng/g (202–354 ng/g) in four of these animals. Concentrations >100 ng/g were also detected in two faecal samples (113 and 362 ng/g).

Two or more ARs were detected in 28% (11/40) of the faecal samples, with a mean of 1.9 ARs in the positive foxes. In the livers, two or more ARs were found in 68% (27/40) of the samples, with a mean of 2.6 in the positive foxes. Significant differences between number of substances in liver compared to faeces ($p = 0.001$) were detected.

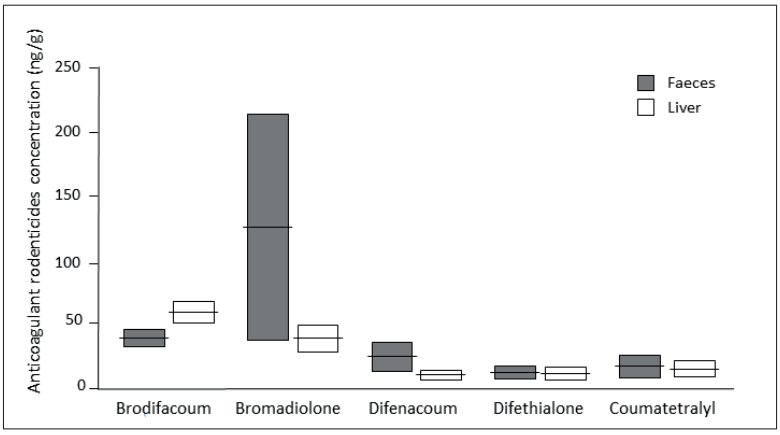


Figure 10. Faecal and hepatic concentrations of anticoagulant rodenticides in faeces and liver samples. The results are given as means ± standard error of the mean (SE; for n, see Table 13).

The total number of positive samples for each AR in faeces and liver, displayed statistically significant differences between samples for brodifacoum ($p = 0.018$) and bromadiolone ($p < 0.0001$; Figure 10). However, no significant differences were found for coumatetralyl ($p = 0.790$), difenacoum ($p = 0.225$) and difethialone ($p = 0.051$).

Paper IV

Determination of anticoagulant rodenticides in faeces of exposed dogs and in a healthy dog population

Blood and faeces were analysed from five accidentally exposed dogs after single ingestions of brodifacoum (n=3), bromadiolone (n=2) and difenacoum (n=1; Table 14). In blood, low concentrations resulted in estimation of distribution half-life from plasma to tissues in one dog only. The α-elimination phase of brodifacoum was calculated to 1 day in this dog, with estimated terminal half-life of 8 days.

Table 14. Overview of the results from studies in dogs included in the PhD project.

Anticoagulant rodenticides	Number of dogs studied	Blood (days)			Faeces (days)				Paper
		$t_{1/2}\alpha$	$t_{1/2}\beta$	Duration of detectable AR residues after ingestion	T max	$t_{1/2}\alpha$	$t_{1/2}\beta$	Duration of detectable AR residues after ingestion	
Coumatetralyl	1	1.8	ND	64	ND	ND	81	204	I
Brodifacoum	3	ND	ND	7	ND	10	330	At least 969*	IV
		1	8	53§	ND	2	200	At least 894§	
		ND	ND	9	2	1	280	At least 700	
Bromadiolone	2	ND	ND	ND	3	2	30	151	IV
		ND	ND	ND	2	1	ND	3	
Difenacoum	1	ND	ND	9	3	10	190	At least 653	IV

T max: Day of maximum measured concentration; ND: Not detected; $t_{1/2}\alpha$: Estimated first phase half-life; $t_{1/2}\beta$: Estimated elimination phase half-life

*Probable new exposure detected day 1032

§After first visit, day of ingestion unknown

Maximum concentrations in faeces were found after day 2-3 for all ARs in the dogs where faeces were collected in the first four days after ingestion (n=4). Concentrations in serially collected samples indicated a biphasic elimination in faeces. The concentrations in faeces declined by 50 % in 1-10 days for brodifacoum, 1-2 days for bromadiolone and 10 days for difenacoum.

Prolonged elimination of ARs was detected with brodifacoum residues detectable for at least 700-969 days after ingestion (Figure 11). Terminal faecal half-lives of brodifacoum were estimated to 200, 300 and 330 days in the three dogs. Difenacoum displayed a terminal faecal half-life of 190 days (n=1). In contrast, estimated terminal half-life of bromadiolone was 30 days (n=1). Despite months with low ARs concentration in the exposed dogs, no clinical signs of poisoning or coagulopathy were observed in this period.

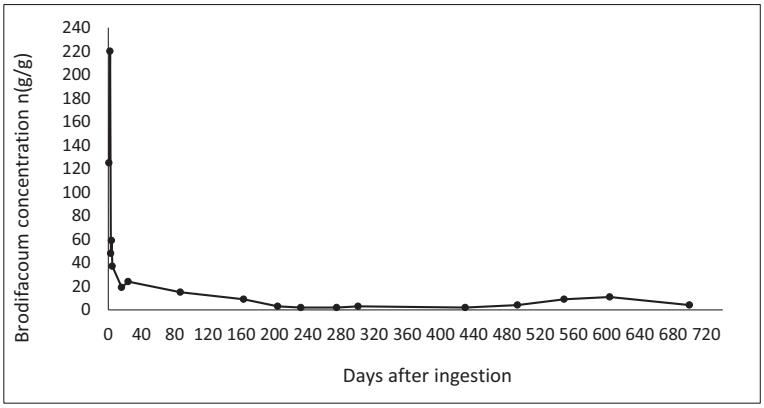


Figure 11. Elimination of brodifacoum in faeces in case 3 (day 1-700 after ingestion).

Four healthy, full-term puppies were born from one of the poisoned dogs 1127 after the first exposure and 95 days after a second suspected exposure. Low concentrations of brodifacoum were detected in all samples from the litter for at least 28 days after parturition.

Blood and faecal samples were collected from 110 healthy domestic dogs, and difenacoum was detected in faeces of one dog.

5. DISCUSSION

5.1 Methodological considerations

5.1.1 Animals and samples

The dog studies were conducted to get a better understanding of the elimination of ARs in this species (Paper I and IV). Inclusion of additional exposed dogs would have strengthened our result of long faecal elimination of ARs. However, despite efforts in including more dogs both from NMBU and other veterinary clinics, this was unsuccessful. Monthly visits to NMBU for 2-4 years turned out to be too time consuming for several of the owners resulting in lack of inclusion of dogs. A higher inclusion rate could have been achieved by limiting the collection of blood and faeces to the first weeks after exposure, but this approach would have limited the result to determination of α-elimination phase only.

In Paper I and IV, we collected blood samples for analysis of ARs at every visit. Measurable concentrations of ARs were not expected in the blood of the poisoned dogs after the first weeks of the studies due to the low concentrations in blood detected in previous studies (Bullard et al., 1976; Huckle et al., 1988). However, blood was collected at each visit to ensure detection of a possible recent reingestion of ARs.

In Paper II we estimated that 54% of the healthy wild red foxes in Norway are exposed to ARs. The samples from wild red foxes represent a population of 21,300 foxes reported hunted in Norway in 2016 (Statistics Norway), hence the sampled animals (n=163) represented only 0.8% of the total population shot. However, we consider the samples an unbiased and representative proportion of the healthy fox population as the foxes were collected from a wide range of different municipalities with varying sizes and density of people, from all but one county in Norway.

Correct collection of samples is important in assessment of AR exposure in wildlife. In the projects presented in Paper II and III, samples were collected by experienced hunters. Nevertheless, there is a possibility of contamination between samples from one fox to another by the use of the same equipment (i.e. contaminated knife) in the field or exchange of labelling between samples.

Sampling faeces from wildlife offers a method for surveillance of ARs. Three different techniques of faecal sampling are conceivable: Direct sampling from dead, hunted animals, direct sampling from living animals and non-invasive collection from the ground. Collection from hunted animals is ethical challenging and represents no advantages over liver sampling once the animal is dead. Direct sampling after chemical immobilisation is beneficial in endangered wildlife, as killing of the animal is avoided. Direct faecal sampling offers an opportunity of repeated sampling from one animal, providing a surveillance method of AR exposure in living wildlife. In addition, sampling faeces directly from animals verifies that only one sample is collected from each individual animal, in contrast to collection of scats from the ground. However, the immobilisation technique is stressful for the animal. Non-invasive sampling of scats from the ground increases the risk of pollution from other sources and environmental degradation. A significant reduction in AR concentration due to washout and natural degradation occurs when scats are exposed to sun and rain over several days, as it is challenging to collect scats from the ground on the same day as the fox defecates (Prat-Mairet et al., 2017). In addition, this technique requires observation of the target species to avoid species misclassification. The species identity of scats is not obvious when sampled from the ground, and studies report 18-25% erroneous identification of presumed fox faeces according to DNA analyses of the faeces (Jacquot et al., 2013b; Fourel et al., 2018). In addition, when each individual animal is not identified, multiple defecations by the same fox cannot be excluded. As defecation rate on

average is eight scats per fox per day, multiple samples from a single fox could influence the result (Webbon et al., 2004). The technique utilised in the present thesis was direct sampling and non-invasive collection of faeces. In the fox studies, samples directly collected for the national *Echinococcus* survey originating from hunted foxes were utilised (Papers II and III). Through this approach we were able to collect samples from animals in most counties in Norway and from areas of different human population densities (rural, suburban and urban), but different numbers of foxes shot in the counties could however present a potential bias of the result. In the dog studies, non-invasive collection was performed as faeces were collected by the owner immediately after the observed defecation from the dog avoiding environmental degradation and misclassification of specimen (Paper I and IV).

As the main objective of our third study was to evaluate the method of analysing ARs in faeces, we chose to compare AR residues in faeces to liver samples from the same animals. In the Norwegian *Echinococcus multilocularis* survey program a piece of the liver and faeces are collected from wild red foxes. Analyses of different liver lobes in red foxes detected a significant difference in brodifacoum concentration between the lobes (Taylor et al., 2020). Distribution in liver of the other ARs has not been examined further, but comparable results are probable. The hunters in our study removed samples from different parts of the livers, hence potential distributional heterogeneity of ARs in the liver could affect our results. This recent finding is not confirmed by others and the biological reason for the different concentration in the lobes needs to be examined further.

Faecal and liver samples were stored appropriately to ensure preservation and subsequent detection of ARs. Previous studies have investigated the stability of ARs in liver and carcass after storage in different environments. Analyses of residues of coumatetralyl, brodifacoum, bromadiolone and difenacoum were within 15% of initial values in stomach content and liver

stored at room temperature for 72 hours (Gallocchio et al., 2014). Flocoumafen residues in liver and carcass were within 20% of the initial values when stored at -18°C for one year (Newton et al., 1994). Brodifacoum, bromadiolone and difethialone residues in liver samples were analysed after storage at benchtop for four days, in refrigerator for one week and freezer for one month resulting in reliable results (Smith et al., 2017). Based on these results, it is doubtful that the storage procedures used in our studies influenced the results.

5.1.2 Analytical method

The faecal samples were freeze dried before further analyses in the laboratory (Papers I, II, III and IV). This process removes liquid from the frozen faeces at very low temperatures, ensuring that stability during storage is increased, while the ARs remain qualitatively and quantitatively unchanged (Boss et al., 2004). By using this method, large visible plant material and hair were less challenging to remove before sample preparation. Freeze drying is time consuming but also reduces odor, which is advantageous during further processing of the faeces in the laboratory. Whether this method influenced the result was not examined, as our aim was not to compare and characterize different analytical procedures. However, based on the stability of ARs, we believe the lyophilized samples are representative for the AR content in faeces.

Faecal samples were analysed by UPLC/MS-MS (Paper I, II, III and IV). A range of different analytical methods have been used to quantify ARs in biological matrices. HPLC, gas chromatography-mass spectrometry (GC-MS), LC-MS and ion chromatography have been utilised in analysis of ARs in blood, urine, liver and bait (Imran et al., 2015). In the current project the primary objective was estimating prevalence and elimination of ARs in C*anidae* by faecal analyses and not comparison of different analytical methods, hence the focus in the thesis is comparison to other analytical

methods in faeces. The first analysis of bromadiolone in fox faeces was done by HPLC-MS (Sage et al., 2010). Both HPLC and UPLC are useful methods in quantifying ARs in biological matrices. However, HPLC operates at lower pressures (<5000 psi) and have a higher column particle size (3-5 μM) compared to UPLC (15,000 psi, <2 μM) (Waters, 2020). Hence, our method utilising UPLC has improved sensitivity and chromatographic separation of the components, shorter run times and lower solvent consumption compared to HPLC, demonstrated by a run time of 6 min in our method compared to Sage and colleagues (2010) of 18 min. A later study analysed brodifacoum, bromadiolone, difenacoum and difethialone in faeces by HPLC-MS/MS based on the method by Sage et al. (Prat-Mairet et al., 2017). Tandem mass spectrometry offers increased sensitivity compared to single MS. However, the LOQs were 10 ng/g for all ARs in the study by Prat-Mairet and colleagues, compared to 2.2-2.7 ng/g in our studies (Paper I, II, III and IV). Compared to the HPLC-MS and HPLC-MS/MS methods described by Sage and colleagues and Prat-Mairet and colleagues, our method provides faster chromatographic runs and better limits of quantitation, however the extraction recoveries are markedly lower.

The main limitation of our UPLC-MS/MS method was inhomogeneous faecal samples with large variability in sample aliquot content. Indigestible hair and plant material are common in fox faeces (Jensen and Sequeira, 1978; Kidawa and Kowalczyk, 2011), and this could influence extraction recovery, although large visible plant material, etc. were removed before sample preparation in our work. Samples were homogenised before analysis, but the size of the tubes limited the amount of faeces prepared (about 3 g). Another option we could have considered was to homogenise several tubes separately, followed by merging, before sample aliquots were taken. This was; however, not practical and homogeneity could still not be guaranteed. Furthermore, ARs are not homogeneously distributed in faeces (Prat-Mairet et al., 2017). As our faecal samples were the remains after previous analysis

of *Echinococcus multilocularis*, complete analysis of the entire sample was in addition not possible.

In Paper II, we examined the prevalence of ARs in wild red foxes. Given the limited number of options in surveillance of AR exposure in living wildlife, faecal analysis was considered a good approach in establishing prevalence in wild red foxes. One limitation in estimating the prevalence was the lack of AR concentrations in the individual foxes (Paper II). Residues in faeces were determined by analysing one parallel for each fox in this study, while comparison of faecal and liver concentrations in Paper III were performed using 3–6 parallels for each faecal sample. We could have determined AR concentrations in the prevalence study and evaluated whether it would have changed the statistical analysis. However, this was not done due to economic limitations. Furthermore, as discussed in Paper III, AR concentrations must be interpreted with caution. AR concentration can be used to verify exposure, but it should not be used as an indicator of toxicosis alone.

5.1.3 Statistical methods

In our main study of prevalence in red foxes, we grouped data according to the density of people in the municipalities (Paper II). As discussed in our paper, a limitation to our results is that the grouping of data did not consider the geographical situation of each sample. As Norway has a territorial distribution with latitude changes that affect fox habitats, this could have added useful information to our results. Rodent population and AR use are influenced by agriculture, building and livestock density, and a more precise landscape analysis of the collection sites would thus have improved the interpretation of our data. However, detailed information of the sampling sites was not available from the hunters. It is possible that increased geographical information would change the results of the

correlation between human population density and AR exposure in the animals, and that future studies might conclude differently.

In Paper III, we compared AR residues in faeces to liver sampled from the same red foxes. Although good concordance in AR concentrations for bromadiolone, coumatetralyl, difenacoum and difethialone between the two matrices were found, a better result would have been achieved by comparison of concentrations in the entire organs. Comparing the total amount in the complete liver and complete faecal volume of the fox could improve the knowledge of AR occurrence in the two compartments. However, the hunters removed a piece of the liver for analysis and the entire liver was not weighed. In addition, we did not have the weight of the intestinal tracts of these foxes. We were thus not able to compare the AR concentrations between the two organs. Another approach could be to determine AR occurrence based on mean weight estimation of these organs; however, such results would be quite uncertain and could result in misleading statistical assumptions.

5.2 General discussion

5.2.1 Prevalence in wild red foxes

It has long been known that AR exposure through predation is a problem for wildlife all over the world (Kaukeinen, 1982; Merson et al., 1984). The Norwegian Environmental Agency has expressed concerns for AR exposure in wildlife in Norway (Hambro, 2012); however, there is a lack of information of AR prevalence in mammals in Norway. The widespread distribution of the red fox makes them good sentinels for mammal-hunting predators in rural, suburban and urban areas, hence we focused on determining AR exposure in this species.

Our study describes for the first time prevalence of ARs in presumed healthy animals across a country, and provides knowledge of AR exposure in the wild red fox population in Norway (Paper II). Despite government restrictions implemented in 2014, ARs are a continuing hazard in nontarget wildlife. One recent study examined the effect of AR exposure in stone martens and polecats after regulatory restrictions in Denmark (Elmeros et al., 2018). No reduction of secondary exposure was detected, and this is reflected in our study with a prevalence of 54% of the wild red foxes. Compared to our findings, previous studies in red foxes have detected a similar exposure with 39-64% in liver samples (Tosh et al., 2011; Sánchez-Barbudo et al., 2012; Geduhn et al., 2015; López-Perea et al., 2019). However, direct comparisons to liver analysis is questionable, as a higher tissue concentration is expected in the liver (Huckle et al., 1988). In addition, ARs are not homogenously dispersed in faeces, lowering the recovery compared to liver analysis. However, as we did not analyse liver samples from the 163 foxes, we do not know how this would have influenced our results. In previous studies, presumed healthy, sick and dead animals have been sampled, whereas in the current study only presumed healthy animals were included. As we discussed in Paper II, AR exposure is a

possible cause of illness and mortality, increasing the likelihood of positive findings in these animals. AR prevalence in the previous studies could thus be overestimated as a value for prevalence in the entire red fox population. Differences between geographic areas and countries in the use of ARs, also play a role in estimation of AR prevalence. The previous studies in red foxes were all multiyear studies in contrast to our single year study; however, results were not compared for annual differences. Rodent cycles influence the quantity of prey consumed by foxes in Norway (Jensen and Sequeira, 1978). Hence variation in rodent population and AR use could potentially result in a different prevalence if we had examined the foxes another year.

Our work indicates a problem with bioaccumulation of ARs in red foxes in Norway. We detected multiple substances in 40% of the positive foxes' faeces, with two (27%), three (7%) and four (7%) compounds in each animal, respectively (Paper II). These findings are consistent with two previous studies with multiple ARs detected in 39-60% of livers in both killed and opportunistically collected dead foxes (Tosh et al., 2011; Geduhn et al., 2015). However, our findings could misestimate exposure to multiple substances as the inhomogeneity and variability in sample aliquot content is more likely to underestimate the prevalence in faeces compared to liver analysis. Different scenarios could explain the high incidence of multiple ARs in the animals. Baits containing several ARs could contribute to multiple residues, but of the 46 government-approved AR products in Norway, only one product consists of a combination of two ARs (bromadiolone and difenacoum). This sole product cannot explain the occurrence of multiple substances detected in the foxes. Furthermore, products with multiple ARs are not commercially sold in other European countries (López-Perea et al., 2015), hence migratory birds and wildlife in Norway will not be exposed to combination products from their country of origin. The more probable explanation of accumulation of multiple

compounds in the foxes, is ingestion of ARs from more than one contaminated prey over time or ingestion of prey with more than one AR.

In our work we detected a significant difference in the occurrence of AR substances in faeces (Paper II). Brodifacoum was most frequent, identified in 46% of the faecal samples, significantly more than coumatetralyl (17%) and bromadiolone (16%). Brodifacoum was also the most frequent substance detected in livers (Paper III). In contrast, bromadiolone and coumatetralyl were the substances most frequently detected in livers from foxes in Sweden and Finland (Tjus, 2014; Koivisto et al., 2018). As we discussed in Paper II, it is possible that the higher prevalence of brodifacoum compared to other ARs is at least partially explained by a difference in sales volume between the Scandinavian countries. Due to lack of data of sales volume and use of ARs in Norway, this cannot be verified (Haraldsen, 2018). Identifying this difference of AR residues in wildlife may provide valuable knowledge on the risk of bioaccumulation of the different substances, although assessments of residues need to be performed in conjunction with the sales of ARs in different countries.

Our work provides novel information of prevalence of AR substances in geographical areas with different population densities in Norway (Paper II). We detected coumatetralyl more frequently in urban compared to rural areas. This difference may not be surprising, as coumatetralyl is more common in rodenticides against mice available to the public (Haraldsen, 2018). In suburban areas; however, there was a significant increase in prevalence of brodifacoum compared to urban areas. Suburban areas comprise a dispersed settlement of both small towns and agricultural land. Farmers are allowed to buy larger quantity of ARs after certification and use ARs against both mice and rats. Whether the higher prevalence of brodifacoum relates to brodifacoum application in livestock areas is not known. Comparable studies in red fox are scarce, but a study in Germany described that livestock density was associated with occurrence of

brodifacoum residues in livers (Geduhn et al., 2015). However, we did not detect any significant difference in brodifacoum prevalence between urban and rural areas, other landscape elements and population factors thus likely play a role in the prevalence of different ARs in red foxes.

In Paper III good concordance between AR residues in faeces and liver was found for coumatetralyl, difenacoum and difethialone. No significant difference was detected for summed number of positive samples or comparisons of AR concentrations between faeces and liver. However, we detected bromadiolone in significantly greater frequency in livers compared to faeces. The chemical structure of bromadiolone with an extra hydroxyl group differs from brodifacoum, difenacoum and difethialone (Table 1). The hydroxyl group facilitates glucuronidation by UDPs (Ge et al., 2016); hence a lower detection in faeces is expected. This finding has also been described in a previous experiment in four foxes after repeated ingestion of poisoned voles (Sage et al., 2010). Sage and colleagues demonstrated a 0.22% detection of bromadiolone in faeces (4.4 ng/g) compared to liver (2000 ng/g) day 28 after two days exposure, and a 1.4% detection (liver 2040-2540 ng/g; faeces 15.0-53.0 ng/g) in three foxes after five days exposure. As the ingested amount and time of exposure in our studies were unknown (Paper II and III), direct comparisons of detected concentrations in liver and faeces to these results are not possible. However, in this respect it is interesting to note similar low detection in faeces in the dogs exposed to bromadiolone (Paper IV). This also raises the question whether diastereomers could impact the excretion and bioaccumulation of bromadiolone. Commercial bromadiolone baits contain a mixture of 70-90% *trans*-isomers and 10-30% *cis*-isomers (Fourel et al., 2017b). The *trans*-isomer is more stable and together with the higher concentration of this isomer in baits result in high accumulation of bromadiolone *trans*-isomers in the liver (Damin-Pernik et al., 2017). However, we do not have the percentages of the two diastereomers in Norwegian bait compared to

France. A previous study examined diastereomers in fox faeces and detected mostly *trans*-bromadiolone (Fourel et al., 2018), but this study was based on faecal analyses of 10 samples from unknown number of foxes. Hence, in order to give an in-depth description of the impact of diastereomers on prevalence and elimination of ARs, more studies of diastereomers in baits and excretion should be applied (Fourel et al., 2017b; Fourel et al., 2018), which was beyond the scope of this work. In our study we found a significant higher concentration and occurrence of brodifacoum in liver compared to faecal samples (Paper III). It is tempting to speculate that this discordance could in part be due to a lower liver elimination rate of brodifacoum. In mice, liver elimination half-life of brodifacoum is 308 days after a single oral dose, compared to 16, 62, and 29 days for coumatetralyl, difenacoum and difethialone, respectively (Vandenbroucke et al., 2008). However, the earlier discussed difference in diastereomers could cause the discrepancy in results for brodifacoum as well (Feinstein et al., 2019). Moreover, the inhomogeneity of the faecal samples could influence our results and probably contributes to the lower detection in faeces compared to liver.

In our liver-faecal comparison study (Paper III), we showed that AR residues can verify exposure in red foxes. Previous studies have determined liver concentrations of 100-200 ng/g SGAR as potentially lethal (Newton et al., 1999; Shore et al., 2005), and a later study suggested a significant risk of acute intoxication with liver concentrations <100 ng/g (Thomas et al., 2011). In our study of 40 red foxes, it is interesting to note SGAR concentrations >100 ng/g in 28% of the hepatic samples. Moreover, in four of these foxes the residues were >200 ng/g. In line with our observations, a study of 48 shot wild red foxes detected liver concentrations >200 ng/g in 38% of the animals and >2000 ng/g in two foxes (Fourel et al., 2018). Hence, the use of a liver threshold toxicity factor for ARs is controversial

and AR concentrations need to be interpreted in conjunction with clinical signs of haemorrhage to determine toxicity.

The occurrence of ARs we detected in red foxes in Norway, indicates a risk for wildlife after human AR use (Paper II). A previous study detected a significant reduction in fox populations after bromadiolone treatment, lasting more than one year (Jacquot et al., 2013a). Subtoxic AR concentrations have previously been associated with increased mortality in different species when subjected to stress or exercise (Jaques, 1962; Carvallo et al., 2015). Moreover, studies have indicated that AR residues in the liver could result in weakened body condition of wildlife and increased risk of accident and predation (Elmeros et al., 2011; Sánchez-Barbudo et al., 2012). However, evidence of a causal association between subtoxic levels of ARs and weakening of the animals together with increased mortality is not strong, due to lack of more extensive examinations of other causes of the animals' deaths and diseases. So far, the consequence of AR residues is disputed, and more large-scale controlled studies are needed to obtain robust results in wildlife.

5.2.2 Prevalence and elimination in dogs

No previous studies have determined the elimination of ARs in dogs after single ingestion. An experiment in four dogs demonstrated however a biphasic depletion curve in plasma after three consecutive days administration of brodifacoum (Woody et al., 1992). Our work indicates for the first time that ARs have biphasic elimination in faeces of dogs after a single ingestion (Papers I and IV). In one of the two dogs exposed to bromadiolone, low concentrations precluded estimation of the elimination phase, but bromadiolone displayed biphasic elimination corresponding to the other ARs in the second case. Hence, the results of bromadiolone must be interpreted with caution with respect to our low number of cases. There are few published reports of elimination of ARs after a single ingestion in

other species, but corresponding biphasic exponential decay of ARs in plasma has been suggested in rodent studies (Bachmann and Sullivan, 1983; Vandenbroucke et al., 2008).

In our work, we calculated the α-elimination phase in plasma in two dogs to be 1.8 days and 1 day for coumatetralyl and brodifacoum, respectively (Paper I and IV). The half-lives were manually calculated using first-order kinetics ($t_{½} = \ln 2/k$, k is the elimination rate constant) (Derendorf and Schmidt, 2020). The results are based on few animals and few analyses, and the findings are thus interpreted with caution. However, these calculations provide important indication of half-lives and detection times of ARs. We did not use computer-based fitting or toxicokinetic software applications. Such models can predict concentration curves and precise estimates of different pharmacokinetic parameters, but due to limited number of animals and concentrations, such results would still be uncertain.

In plasma, ARs display a short distribution half-life to tissues (Huckle et al., 1989). In our work, time of maximum concentration in the exposed dogs could not be estimated due to low detection in blood (Paper IV). The terminal half-life of brodifacoum in plasma was estimated to 8 days in one dog. This is longer than stated in one older study with a half-life of 2.4 days (range 0.9-4.7 days) after an acute ingestion (Robben et al., 1998). The discrepancy from our result may be a consequence of improved quality of analytical methods over the past 20 years. Another study estimated plasma elimination half-life to 6±4 days after three days of ingestion in four dogs (Woody et al., 1992). However, due to few cases both in previous studies and our paper IV, larger pharmacokinetic studies are needed to determine the distribution half-life from blood to tissues in dogs.

In our study, maximum concentration in faeces was found 2-3 days after ingestion for all ARs (Paper IV). However, the ingested dose was unknown in all cases, and this could influence the results. The concentrations in faeces

declined by 50% in 1-10 days for brodifacoum, 1-2 days for bromadiolone and 10 days for difenacoum. Ideally, comparisons of excretion rates should be done by administering the same amount of ARs to the dogs, but ethical considerations preclude this. Low number of cases, which our Paper IV suffered from, reduces the conclusions we can draw from these findings. Although a repeated ingestion experiment, similar results were found in another *Canidae*. Sage and colleagues (2010) detected maximal residues of bromadiolone on day 3 (n=1) and day 5 (n=3) in faeces of red foxes after five days ingestion of poisoned voles.

By our novel method of analysing ARs in faeces, we indicated long elimination of brodifacoum and difenacoum in dogs (Paper IV). Brodifacoum was still detectable in faeces of the three exposed dogs at the termination of the study, 700-969 days after ingestion, with estimated terminal half-lives in faeces of 200, 300 and 330 days. The variation between the individual dogs could be influenced by differences in individual excretion capacities between dogs, as demonstrated in a previous investigation of brodifacoum (Ray et al., 1989). In addition, there were differences in ingested amounts and initial decontamination (gastric emptying and activated charcoal) between the dogs. A study demonstrated that high intake of dietary fibre in rats resulted in increased faecal amount with a lower concentration of bile acids (Reddy, 1981). Furthermore, diets high in fat and beef in humans resulted in higher concentrations of bile acids compared to a normal diet. As ARs undergo enterohepatic circulation, they are affected by bile output. Bile flow is significantly higher after a meal and decreases during the following three hours (Nahrwold and Grossman, 1967). However, the poisoned dogs ingested various diets and we did not estimate AR concentrations in relation to feeding times. In addition, faecal samples were not collected at the same time during the day, therefore we do not know whether or how excretion of bile affected our results.

Other studies of half-life of brodifacoum after a single ingestion are scarce, but a study in mice demonstrated a liver elimination half-life of 307 days after a single ingestion (Vandenbroucke et al., 2008). Direct comparison of half-lives between species is not possible, as there are wide inter-species differences in VKOR inhibition and metabolic activity (Watanabe et al., 2010). However, the long elimination detected in mice indicates similarities to our findings in dogs. We observed a half-life of difenacoum of 190 days in one dog, with still detectable concentration in faeces when the study ended 653 days after ingestion (Paper IV). In mice and rats, a liver elimination half-life of 62 days and 120 days, respectively, was estimated after a single ingestion (Parmar et al., 1987; Vandenbroucke et al., 2008). In contrast to dogs, rats lack gall bladder and continuously secrete bile, hence these comparisons must be interpreted with caution (Cattley and Cullen, 2017).

By comparing the half-lives of the FGAR coumatetralyl (Paper I) to the SGAR bromadiolone (Paper IV) in faeces, we made an interesting observation. Earlier studies have suggested that all SGARs have longer duration of action than FGARs. Although based on few cases, we detected a prolonged half-life of coumatetralyl in dogs (81 days) compared to bromadiolone (30 days). Similarly, liver elimination half-life of coumatetralyl in red deer was estimated to 19 days after a single oral dose, while elimination half-life of diphacinone, a SGAR, was estimated to 6 days (Crowell et al., 2013). However, in this study of red deer the ingested dose of coumatetralyl (8.25 mg/kg) was higher than diphacinone (1.5 mg/kg). Other experiments have similar differences in oral doses between the ARs, as the administered dose is based on LD_{50} for the specific AR in the species studied. In rodents, bromadiolone display a longer elimination half-life compared to coumatetralyl, with liver elimination half-life for coumatetralyl in mice estimated to 15.8 days (8000 μg/mouse) and bromadiolone to 307.4 days (28.2 μg/mouse) (Vandenbroucke et al., 2008). No studies estimate the half-lives of coumatetralyl and bromadiolone after administration of the same

amount of the substances, hence estimation of the impact the different doses have on elimination is not possible based on this data. Furthermore, different metabolism in rodents compared to larger mammals complicates such comparisons. In addition, the difference in stereoisomerism could contribute to the difference, as bromadiolone has two diastereomeric forms and coumatetralyl, on the other hand, is not a diastereomer (European Commission, 2009b; Lefebvre et al., 2017). However, our work indicates that coumatetralyl has a long duration of action in dogs, although it is classified as a FGAR (Watt et al., 2005; King and Tran, 2015). Furthermore, the slow elimination in dogs could contribute to explaining the high occurrence of coumatetralyl in Norwegian foxes (Paper II).

Our work indicates a potentially shorter faecal elimination half-life of difenacoum (Paper IV). Interestingly, mice displayed similar differences in liver elimination half-lives after single ingestion in one study (Vandenbroucke et al., 2008). However, there are large differences between studies in the same species, with liver elimination half-life estimated from 130 to 350 days in rats (Table 3). Differences in ingested amount, experimental conditions and analytical methodology complicate comparisons between studies; furthermore, these details are not accessible in some studies. Individual differences in metabolism may also affect estimations (Ray et al., 1989), and our findings must be interpreted with caution due to the low number of cases.

5.2.3 Clinical aspect of anticoagulant rodenticides in dogs

In our work, low AR concentrations were detected for months to years in faeces of the exposed dogs (Paper I and IV). Full biochemistry and haematology profiles obtained several times during these months did not display any significant values outside the reference ranges, nor did we detect any infections or other signs of impact on the immune system in the dogs. In the healthy dog population investigated in Paper IV (n=110), we

detected one dog exposed to difenacoum. Neither of the exposed dogs displayed any clinical signs of poisoning or illness. Only one exposed dog in the population precluded meaningful analyses of possible predisposing factors to AR exposure such as region, housing, food, type of exercise (unleashed, leashed) in our study. One must be cautious when attempting to associate findings in observational studies with biological causation, as correlation does not imply causality and correlation between effects on the immune system and AR exposure need to be distinguished from mere coincidence.

Several studies have examined ARs' impact on the immune system in *Felidae* (Fraser et al., 2018; Kopanke et al., 2018; Serieys et al., 2018). However, there are inter-species differences in pharmacokinetics between *Canidae* and *Felidae* affecting the glucuronidation of ARs (Toutain et al., 2010; Court, 2013), and there is no information whether AR residues in dogs or foxes have similar effect. Further studies should aim to detect causal relationship between AR residues and clinical effect on the immune system in *Canidae*, with exclusion of other possible explanatory variables.

Traditional vitamin K_1 administration in AR poisonings is a costly and lengthy treatment in dogs, and there is a need for new treatment options. The enterohepatic circulation in dogs and foxes is a contributing factor to the extensive accumulation in liver and prolonged elimination (Watt et al., 2005), as detected in our work (Paper I and IV). Herein lies the most promising area of AR poisoning therapy. Single dose of activated charcoal (AC) is recommended in decontamination of AR poisoning in dogs (DeClementi and Sobczak, 2018). AC binds to substances in the gastrointestinal tract, decreases absorption and reduces systemic toxicity. Repeated doses of AC increase the elimination of substances already absorbed and may be beneficial in substances undergoing enterohepatic recirculation (American Academy of Clinical Toxicology and European Association of Poisons Centres and Clinical Toxicologists, 1999). Enhanced

elimination has been confirmed after repeated AC administration in several drugs undergoing enterohepatic recirculation (Wakabayashi et al., 1994; Bradberry and Vale, 1995). Consequences of repeated doses of AC has not yet been investigated in AR poisonings in dogs, but a human study with two patients is frequently used as verification of the lack of clinical effect (Donovan et al., 1990). Both patients received AC the first day (25 g every 4 hours) and a later administration at 3 days and 33 days post ingestion, respectively. Plasma elimination half-life was reduced during treatment, but an increased plasma concentration was seen after discontinuation of AC. This abstract offers thus a weak scientific basis for discarding the therapy. For the canine population, repeated AC administration is a simple, cheap and safe therapy which may prevent serious poisoning and reduce the need for long-term vitamin K_1 treatment. However, to evaluate the efficacy of this therapy, a case-control study with concentration measurements and outcome evaluation needs to be assessed.

Our work provided knowledge of perinatal transmission of brodifacoum (Paper IV). Earlier studies have detected teratogenic effects, abortion and postpartum death after brodifacoum exposure in pregnant dogs. Eight out of 13 puppies died (liver concentration 230 and 630 ng/g in two puppies, not detected in one) after exposure during gestation, but the bitch displayed no clinical signs of poisoning (Munday and Thompson, 2003). Four puppies out of nine died of haemothorax in utero in a bitch with normal coagulation parameters (PT and aPTT), and the bitch died after caesarean section (liver concentration 24 ng/g) (Fitzgerald et al., 2018). However, birth complications could have contributed to the outcome of the bitch in this case. In our work, a dog with detectable faecal brodifacoum residues gave birth to four full-term, healthy puppies. Low concentrations of brodifacoum were detected in all faecal samples from the litter of puppies from day 1 (63 ng/g) to 28 (6.0 ng/g) after parturition, despite absence of clinical signs in the bitch (day 1: 219 ng/g). Corresponding to our findings, a dog poisoned

by brodifacoum 10 days after mating with coagulopathy gave birth to clinically normal puppies after vitamin K_1 treatment throughout gestation (Hornfeldt and Phearman, 1996). Although our results confirm transmission of brodifacoum from the bitch to puppies, the concentrations cannot be used in comparison with liver concentrations in the other published cases.

5.2.4 Anticoagulant rodenticides in faeces

In our work we provided a novel method of monitoring AR exposure in exposed dogs (Paper I and IV). In addition, this technique offers an opportunity of repeated sampling from animals, providing a surveillance method of AR exposure in living wildlife. Government radio tagging under sedation is performed in free-ranging gray wolves, wolverines, brown bears (*Ursus arctos*) and Eurasian lynx (*Lynx lynx*) in Norway (Arnemo and Evans, 2017). During these procedures, faeces can be sampled from the animals, enabling authorities to monitor the occurrence of ARs in living endangered wildlife.

A previous study demonstrated excretion of up to 20% of ingested ARs in faeces of rats before death at day 4–6 (Fisher et al., 2017). Brodifacoum poisoning by faecal–oral route has been suggested in chronic accidental exposure in a child (Watts et al., 1990). In addition, poisoning by AR contaminated potatoes due to inadequate hygienic conditions has been described (Huić et al., 2002). In our work we detected a prolonged excretion of difenacoum and brodifacoum in faeces for more than 653-969 days in dogs (Paper IV). Hence, it is tempting to speculate that faecal excretion of ARs potentially can lead to exposure in other animals. In a population of 110 healthy dogs, we identified one dog with faecal AR residues unknown to the owner (Paper IV), but we were not able to detect the source of AR exposure in this dog.

5.2.5 Ethical considerations

The study protocol of the suspected poisoned dogs was approved by the Ethics Committee at the Faculty of Veterinary Medicine, NMBU (14-04723-44). Collection of blood and faeces of the healthy dogs were approved by the Norwegian Food Safety Authority (FOTS ID 13821). All dog owners gave written, informed consent before participation. Wild red foxes were shot during the licensed hunting season (January to mid-April and mid-July to late December) commissioned by the Norwegian Food Safety Authority as part of the pathogen-specific surveillance program of *Echinococcus multilocularis* in Norway in response to EU/EØS regulation 998/2003/EC.

6. CONCLUSIONS

- More than half of the presumed healthy wild red foxes in Norway are exposed to ARs.
- A high proportion (40%) of the foxes with AR residues are exposed to more than one AR, up to four different ARs were detected in a single animal.
- There is no statistically significant seasonal, age or sex difference in exposure to ARs in red foxes in Norway.
- There is good concordance between AR residues in faeces and liver for coumatetralyl, difenacoum and difethialone in red foxes.
- Analysis of faeces from carnivores is a non-invasive and valuable method to estimate exposure to ARs.
- ARs have a biphasic elimination in faeces of dogs.
- Coumatetralyl, difenacoum and brodifacoum display long terminal half-lives in faeces of dogs estimated to 81, 190, 200-330 days, respectively.
- Bromadiolone display a shorter terminal half-life (30 days) in faeces compared to the other ARs in dogs.
- Brodifacoum is detectable in puppies for at least a month after perinatal transmission of low concentrations.
- ARs have low prevalence in the healthy dog population in Norway.

7. FUTURE PERSPECTIVES

- Rodenticides are needed to control the increasing rodent population in urban and rural environment. However, benefits of ARs must be balanced against the risk of poisoning of domestic animals and exposure in wildlife. Although the papers presented here give information regarding residues of ARs in dogs and red foxes, they have not provided data on how ARs impact these animals. This is the natural next step and can be achieved by large-scale studies designed to investigate physiological health parameters such as general haematological parameters and immunological parameters, as well as overall clinical parameters like condition factors, concurrent diseases, lifespan etc. in animals exposed to ARs compared with a relevant group of unexposed animals.

- It is unclear how low levels of ARs impact reproduction in *Canidae* as current knowledge is based on a few published cases in dogs and occasional findings in wildlife. More work is needed to understand the effect of subtoxic levels of ARs on reproducing animals and their offspring.

- We detected a significant difference in the occurrence of the different AR substances in faeces of foxes. This raises the question how diastereomers impact the excretion and bioaccumulation of each AR. Future studies should thus focus on the bioaccumulation of specific substances and their diastereomers, in conjunction with the sales of ARs in the region, in assessment of residues in wildlife.

- Another important question generated by this thesis, is whether ARs are the best rodenticides when looking at both the need for effective rodenticides, as well as risk of poisoning in domestic animals and wildlife health. There is a continuing need for eco-friendly and effective

rodenticides and further investigation of reproductive inhibitors, contraceptives and combination products with cholecalciferol should be elucidated in further studies to determine how commercial products can reduce the risk of secondary poisoning of predators, while retaining primary toxicity for commensal rodents.

- The analyses of AR prevalence in foxes from geographical regions with different human population density revealed no statistically significant differences between the animals. Study of AR prevalence combined with more precise landscape analysis of the geographical situation of each sample would increase the knowledge of different AR use in varying human population density areas and would be interesting to investigate in the future. Faecal analyses make it possible to address this issue in further studies of living wildlife.

- We applied the method of analysing ARs in faeces from exposed dogs, producing results that indicated very long half-life of ARs. However, these studies are based on limited number of cases and inclusion of more animals is necessary for full evaluation of the excretion of ARs in this species.

- The optimal acute therapy of AR poisoning is not known. Activated charcoal is recommended in the acute phase of the poisoning, but whether repeated doses of activated charcoal reduce absorption of ARs due to the enterohepatic circulation and hence Vitamin K_1 treatment time, need to be investigated in further studies.

- We detected one AR exposed dog in a population of 110 healthy dogs. Future studies should follow up this finding with a larger prevalence study in healthy dogs to increase the understanding of AR exposure in this species.

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9. Errata

Page 18, 21: “agent” is replaced by “agents”

Page 23, 23: “are” is replaced by “is”

Page 28, 9: “its” is replaced by “their”

Page 34, 19: “cause” is replaced by “causes”

Page 41, 23: “…haemostasis is the…” is replaced by “…haemostasis is a disturbance of the…”

Page 82, 8: “number” is replaced by “numbers”

Page 84, 6: “have” is replaced by “has”

Page 90, 4: “plays” is replaced by “play”

Page 98, 15: “need” is replaced by “needs”

Page 102, 15: “casual” is replaced by “occasional”

10. Papers I – IV

Paper I

Seljetun *et al. Acta Vet Scand (2018) 60:3*
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Acta Veterinaria Scandinavica

RESEARCH

Open Access

Quantitative method for analysis of six anticoagulant rodenticides in faeces, applied in a case with repeated samples from a dog

Kristin Opdal Seljetun[1,2*], Elin Eliassen[3], Ritva Karinen[3], Lars Moe[1] and Vigdis Vindenes[3,4]

Abstract

Background: Accidental poisoning with anticoagulant rodenticides is not uncommon in dogs, but few reports of the elimination kinetics and half-lives in this species have been published. Our objectives were to develop and validate a new method for the quantification of anticoagulant rodenticides in canine blood and faeces using reversed phase ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) and apply the method on a case of anticoagulant rodenticide intoxication.

Results: Sample preparation was liquid–liquid extraction. Six anticoagulant rodenticides were separated using a UPLC® BEH C_{18}-column with a mobile phase consisting of 5 mM ammonium formate buffer pH 10.2 and methanol. MS/MS detection was performed with positive electrospray ionization and two multiple reaction monitoring transitions. The limits of quantification were set at the levels of the lowest calibrator (1.5–2.7 ng/mL or ng/g). The method was successfully applied to a case from a dog accidentally poisoned with anticoagulant rodenticide. Coumatetralyl and brodifacoum concentrations were determined from serial blood and faecal samples. A terminal half-life of at least 81 days for coumatetralyl in blood was estimated, which is longer than previous reported in other species. A slow elimination of brodifacoum from the faeces was found, with traces still detectable in the faeces at day 513.

Conclusions: This study offers a new method of detection and quantification of six frequently used anticoagulant rodenticides in canine faeces. Such drugs might cause serious health effects and it is important to be able to detect these drugs, to initiate proper treatment. The very long elimination half-lives detected in our study is important to be aware of in assessment of anticoagulant rodenticide burden to the environment.

Keywords: Brodifacoum, Bromadiolone, Coumatetralyl, Difenacoum, Difethialone, Flocoumafen, Half-life, Pharmacokinetics, Rodenticide poisoning, Serum

Background

Anticoagulant rodenticides (AR) are used worldwide in pest control. The first generation AR includes warfarin, chlorophacinone, diphacinone and coumatetralyl that were developed in the 1950s. Increasing resistance in rodents led to the development of second generation compounds [1, 2]. These long-acting anticoagulant rodenticides include brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen, which are far more toxic and lethal for strains of rodents resistant to the first generation rodenticides [2].

The AR produce their anticoagulant effect by inhibition of vitamin K_1 epoxide reductase. This prevents regeneration of active vitamin K_1 and thus impairs formation of vitamin K_1 dependent clotting factors II, VII, IX and X, and proteins C and S in the liver [3]. The anticoagulant effect is mainly due to depletion of factors II and X [4]. In the dog, the plasma half-lives of factors II and X are 41 and 17 h, respectively [5]. After the depletion of the already circulating clotting factors, spontaneous coagulopathy develops. Clinical signs after ingestion of AR are

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expected to develop after about 3.5 days, which represents minimum two half-lives of clotting factor II [4].

Ingestion of AR is not uncommon in dogs and other non-target animals and has been documented over several years [6–9]. We do not know, however, how many dogs in a population are exposed to these rodenticides during their lives. In 2014, a survey of prevalence of previous exposure to AR in diseased dogs was undertaken at the Norwegian University of Life Sciences (NMBU) [10]. Liver samples were taken from all the dogs that were necropsied during 6 months' time, irrespective of the cause of death, illness or clinical signs. Rodenticides were detected in the liver in one in five dogs (20%) of the 63 dogs included in the study. In 8% of the necropsied dogs more than one type of AR were present. The source of the rodenticide in these dogs could not be determined.

The liver is the organ with the most significant accumulation of AR, and the major route of elimination is through bile to the faeces [11–13]. The long duration of action is explained by their enterohepatic circulation and high lipid solubility [14, 15]. In an experiment done in foxes with multiple doses of bromadiolone, residues persisted in the liver even when bromadiolone was no longer detectable in plasma [16]. The excretion in faeces continued throughout the study period of 31 days and was still present at the end of the study.

Detection of AR requires rapid, sensitive and specific methods. Warfarin and its metabolites are regularly analysed by gas chromatography or high-performance liquid chromatography, but owing to larger mass and lower volatility of some of the AR, liquid chromatography–mass spectrometry has been considered a more suitable method [17]. Several analytical methods for detection of AR have been published [18]. There are no published methods for determining concentration of AR in faeces from dogs. In addition, there is sparse information describing the toxicokinetics of coumatetralyl in blood in the canine species.

The main objective of this study was to develop an analytical method for analysis of six AR in faeces. We used the method to determine the elimination time of coumatetralyl in blood and faeces after an acute poisoning of AR in a dog; a case history is presented.

Case history

A 7.2 kg, 6-month-old intact female Dachshund presented to the University Animal Hospital at NMBU after an ingestion of 1.5 block of AR nicked from the owners pocket. The information of the product or AR dose were not available. Within one and a half hour following ingestion, the dog was given apomorphine to induce vomiting, which revealed some large pieces of rodenticide. The dog was given activated charcoal and referred to a veterinary clinic for measurement of prothrombin time (PT) and activated partial thromboplastin time (aPTT) at 48 and 72 h after exposure. Due to a misunderstanding, the blood samples were not examined at the clinic, but sent to an external laboratory and the prolonged coagulation was not discovered.

Five days after exposure, the dog returned to the University Animal Hospital. Clinical signs included lethargy, weakness, tachycardia, weak pulse, pale mucous membranes, tachypnea and dyspnea. The initial coagulation profile showed a markedly prolonged PT of 51 s and aPTT of 131 s. Vitamin K_1 was administered orally and symptomatic treatment was initiated. The clinical condition improved gradually over the next 2 days and the dog's PT and aPTT levels returned to normal. The dog was discharged to her owners' care on day 9, and the vitamin K_1 antidote treatment continued for 50 days after ingestion. This improved the clinical condition, but is not expected to affect the kinetic curve of AR [19]. The dog remained healthy with a complete resolution of clinical signs throughout the study period.

Methods

Sample collection and storage

Faecal samples were collected from the poisoned dog in dark plastic bags or plastic containers after natural defecation on the same day as the blood collection. Samples were maintained at − 20 °C. Within a few weeks, the samples were lyophilized to dryness. The sample residues were analyzed at the laboratory at the Department of Forensic Sciences, NMBU.

Blood for analyses of AR was collected into vacuum tubes containing sodium fluoride as preservative and potassium oxalate as anticoagulant. Blood samples were frozen (− 20 °C) shortly after collection and stored until analyses.

Blood for analyses of aPTT and PT was obtained in a vacutainer tube containing sodium-citrate (3.2%). The blood was analyzed within 2 h of collection at NMBU by a Coag Dx Analyzer (IDEXX Laboratories Europe B.V., The Netherlands).

Blood and faecal samples for determination of AR in our dog were obtained 6, 7, 11, 18, 22, 24, 32, 39, 50, 64, 93, 121, 204, 422, 470 and 513 days after ingestion. Corresponding measurements of PT and aPTT were made in the acute phase of the poisoning.

Reference substances and chemicals

Brodifacoum, bromadiolone, difenacoum, flocoumafen were supplied by Fluka Chemika (Sigma-Aldrich Norway AS, Oslo, Norway), difethialone and coumatetralyl by Dr. Ehrenstorfer (Dr. Ehrenstorfer GmbH, Augsburg, Germany). Figure 1 presents the compounds' molecular

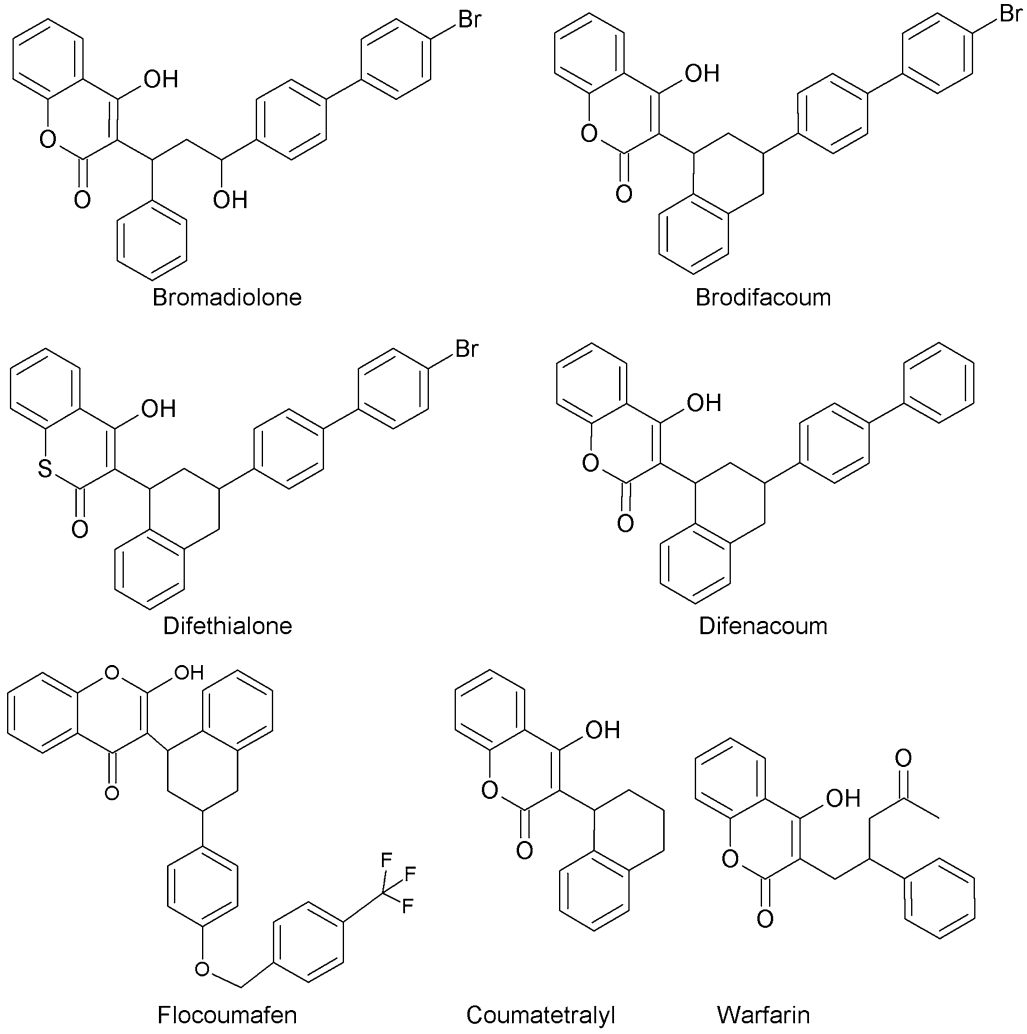

Fig. 1 Molecular structures of the compounds and warfarin

structures. Warfarin-d5 (internal standard) was purchased from Chiron AS (Chiron AS, Trondheim, Norway). Ethyl acetate and dichloromethane were obtained from Chemi-Teknik as (Oslo, Norway). Methanol (LC–MS Chromasolv®), acetonitrile (ACN), ammonium formate and heptane (99%) were purchased from SIGMA (Sigma-Aldrich Norway AS, Oslo, Norway). Type 1-water (18 MΩ-cm) was obtained from a Milli-Q A10 water purification system (Millipore, Bedford, MA, USA). Human whole blood was supplied by Blood Bank at Ullevål University Hospital, Oslo, Norway, and the blank dog faeces samples were collected from other healthy dogs by the authors.

Stock solutions of the analytes were prepared separately in ACN, and working standard solutions for brodifacoum, bromadiolone, difenacoum, flocoumafen, and coumatetralyl were prepared in ACN from the stock solutions at seven concentration levels. Working

standard solutions for difethialone was prepared separately because of the lower concentration of the reference substance solution. Calibration samples were prepared from whole blood or faeces spiked with working standard solutions. The concentration ranges are shown in Table 1. Quality control (QC) samples were prepared independently at three concentration levels.

Blood sample preparation

Sample preparation for calibrators and controls was performed by adding 50 µL of each working standard solutions in ACN to an aliquot of 100 µL whole blood. 100 µL ACN was added to the unknown samples (100 µL). 50 µL of the internal standard (0.078 mg/L in Type 1 water) was added to all samples followed by immediate agitation on a Multitube vortexer. 100 µL borate buffer pH 11 and 1.2 mL ethyl acetate/heptane mixture (4:1 *v/v*) were added and the samples were agitated for 10 min followed by centrifugation at 4500 rpm (3900×*g*) at 4 °C for 10 min. The organic layer was transferred to a clean 5 mL glass tube, dried under N_2 (nitrogen gas) at 40 °C, reconstituted with 100 µL of methanol/Type 1 water mixture (20:80 *v/v*) and shaken well before transferring into auto sampler vials.

Faecal sample preparation

The fecal samples homogenized and exact aliquots of 100 mg were weighed in using a precision weight (XS-precision weight, ©Mettler-Toledo International Inc., UK). Preparation of the calibrators and QC-samples were performed by adding 50 µL of each working solutions to the blank faeces samples. To the case samples, 100 µL ACN was added. To all samples, 50 µL internal standard and 400 µL borate buffer pH 11 were added followed by immediate agitation on a Multitube vortexer. 1.0 mL ACN was added followed by agitation. 1.0 mL dichloromethane was added and the samples were mixed for 10 min using a blood mixer followed by centrifugation at 4500 rpm (3900×*g*) at 4 °C for 10 min. The thin, upper messy layer was carefully removed; and the dichloromethane phase was transferred to a clean glass tube, dried under N_2 at 40 °C, and reconstituted with 100 µL of methanol/Type 1 water mixture (20:80 v/v), shaken, and centrifuged before transferring into auto sampler vials.

Analysis

The samples were analyzed in on a Waters ACQUITY UPLC-system (Waters Corporation, Milford, MA, USA), applying an Acquity UPLC® BEH C_{18}-column (2.1 mm × 50 mm, 1.7 µm particles, Waters Corporation, Milford, MA, USA) using gradient elution with a mobile phase consisting of 5 mM ammonium formate buffer pH 10.2 (A) and methanol (B). The column temperature was held at 65 °C and the mobile phase flow rate was 0.5 mL/min. The gradient profile was: 10% B in 0.00–1.50 min, 30% B in 1.50–1.80 min, 58% B in 1.80–1.81 min, 60% B in 1.81–3.50 min, 60% B in 3.50–3.52 min, 100% B in 3.52–4.00 min, 100% B in 4.00–4.50 min, and 10% B in 4.50–4.51 min. A linear curve profile for the change in mobile phase composition was used. Run time was 6.00 min and the injection volume 3 µL.

Positive electrospray ionization (ESI+) MS/MS detection was performed on a Xevo TQS triple quadrupole mass spectrometer from Waters (Milford, MA, USA), using two multiple reaction monitoring (MRM) transitions for each analyte and the internal standard. Data acquisition, peak integration, and calculation were interfaced to a computer workstation running MassLynx 4.1 software. The MRM transitions monitored, along with the respective cone voltage and collision energy values, and retention times for the analytes, are listed in Table 2. The chromatograms of the lowest QC sample and the blank sample with the internal standards are shown in Fig. 2.

Method validation

Quantitative results were obtained by integrating the peak height of the specific MRM chromatogram in reference to the integrated height of the internal standard. A 2nd order calibration curve ($y = ax^2 + bx + c$) was used for quantification because of the wide concentration range (Table 1). Origin was excluded and a weighing factor 1/x was used. Limits of quantification (LOQ) were set at the level of the lowest calibrators, signal-to-noise ratios were above 10. Within-day ($n = 6$) and between-day variations ($n = 6$) were determined by analyses of spiked human whole blood and blank faeces samples at three different concentration levels for all compounds. Faecal analyses were performed using 3–6 parallels for each sample. Extraction recovery and matrix effect were studied using the method developed by Matuszewski et al. [20]. For this study, five dog blood samples and faeces samples were spiked at two concentration levels for all compounds. Extraction recoveries for blood samples were studied at two concentration levels and at one level for faeces samples.

Results

The calibration curves were evaluated and mean values of R^2 were above 0.995 for all compounds in both blood and faeces (Table 1). The levels of the lowest calibrator (1.5–2.7 ng/mL blood or ng/g faeces) fulfilled the criteria for LOQ for all compounds. Precision and accuracy, determined as bias, are shown in Table 1, and was within ± 20% for all compounds. For blood, no pronounced matrix effects were seen, while for faeces ion suppression

Table 1 Validation parameters of six anticoagulant rodenticides

Compound	Calibration range (ng/mL or ng/g)	Blood		Faeces		QC-sample conc.	Blood					Faeces				
		Mean R^2	RSD (%)	Mean R^2	RSD (%)		Within-day precision RSD (%)	Between-day precision RSD (%)	Bias (%)	ME%	RE%	Within-day precision RSD (%)	Between-day precision RSD (%)	Bias (%)	ME%	RE%
Coumatetralyl	1.5–731 (0.0050–2.5 µM)	0.999	0.061	0.998	0.24	2.3	4.0	7.9	− 3.5	90	10	2.8	15	− 3.6	109	69
						88	3.6	8.7	5.2	93	15	5.7	13	1.6	96	
						585	6.0	11	− 1.8			6.5	15	8.4		
Bromadiolone	2.6–1319 (0.0050–2.5 µM)	0.998	0.21	0.998	1	4.2	5.8	17	16	100	44	15	16	− 7.7	63	32
						158	5.7	14	13	97	52	17	18	− 3.1	56	
						1055	7.5	17	− 4.1			16	13	− 11		
Difenacoum	2.2–1111 (0.0050–2.5 µM)	0.997	0.33	0.999	0.10	3.6	9.1	13	5.5	101	43	13	18	− 5.3	62	26
						133	2.2	15	7.8	97	87	14	17	− 6.1	60	
						889	8.9	15	6.1			15	15	− 11		
Flocoumafen	2.7–1356 (0.0050–2.5 µM)	0.997	0.41	0.995	1.1	4.3	8.0	14	2.7	95	80	11	19	− 5.5	45	18
						163	3.0	14	6.6	92	90	9.0	17	− 2.8	32	
						1085	6.4	15	− 4.6			6.3	19	− 14		
Brodifacoum	2.6–1309 (0.0050–2.5 µM)	0.998	0.37	0.999	0.11	4.2	8.6	15	8.4	93	61	9.2	19	− 1.7	32	25
						157	3.1	14	6.2	90	78	9.3	18	− 2.1	32	
						1047	9.1	11	− 3.1			6.4	19	− 18		
Difethialone	2.7–1349 (0.0050–2.5 µM)	0.997	0.37	0.998	0.17	4.3	8.9	14	16	86	69	10	17	1.3	31	22
						164	6.4	12	12	81	84	11	11	0.8	27	
						1079	13	17	3.1			5.1	16	− 9.4		

Table 2 Multiple reaction monitoring transition ions

Compound	RT (min)	MRM transitions (m/z)	Cone voltage (V)	Collision energy (eV)
Coumatetralyl	1.86	*239.1 > 107.1*/91.0	40/30	32/28
Bromadiolone	2.73	*511.1 > 251.2*/173.0	26	24/42
Difenacoum	2.83	*445.3 > 179.1*/257.2	30	32/22
Flocoumafen	3.17	*543.2 > 159.1*/335.2	28	42/24
Brodifacoum	3.27	*525.2 > 337.1*/178.2	34/55	34/55
Difethialone	3.33	*539.1 > 178.1*/335.1	36	32/22
Warfarin-d_5	1.62	*314.2 > 163.1*/256.0	24	14/22

Transitions in italics font were used for quantification

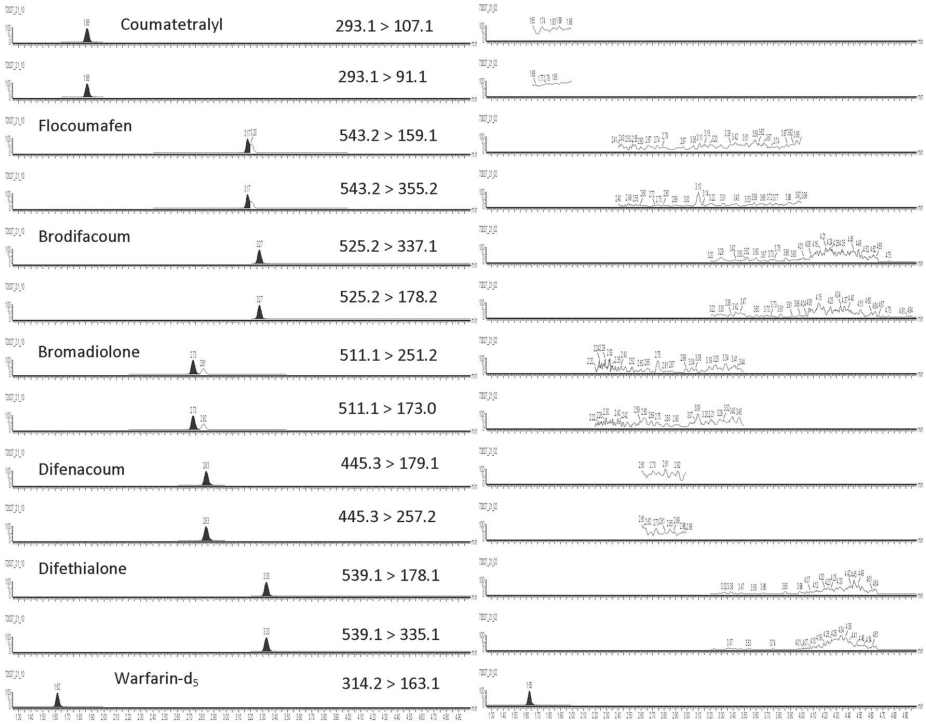

Fig. 2 The chromatograms of the lowest quality control sample and the blank sample with the internal standards

was observed for all compounds except for coumatetralyl. Extraction recovery was likewise lower from faeces than from blood.

The elimination curves for coumatetralyl in blood and faeces were estimated (Fig. 3). The initial distribution phase could not be established in this case, as the analysis of blood was first performed 6 days after ingestion. However, the elimination from day 6 to day 11 indicates a first phase with an estimated half-life of 1.8 days, which indicates an initial α-elimination phase. At 18 days after ingestion, the blood concentration was below LOQ but continued to vary around and below this concentration for 4 months after ingestion. The last positive blood sample was seen 64 days after ingestion.

The corresponding faecal analyses of coumatetralyl were performed using 3–6 parallels for each sample. Relative standard deviations (RSD) were between 4 and 41%, with an average of 25%, for all the samples from our case. Large visible plant material, etc. were removed before sample preparation, but the variability in sample aliquot

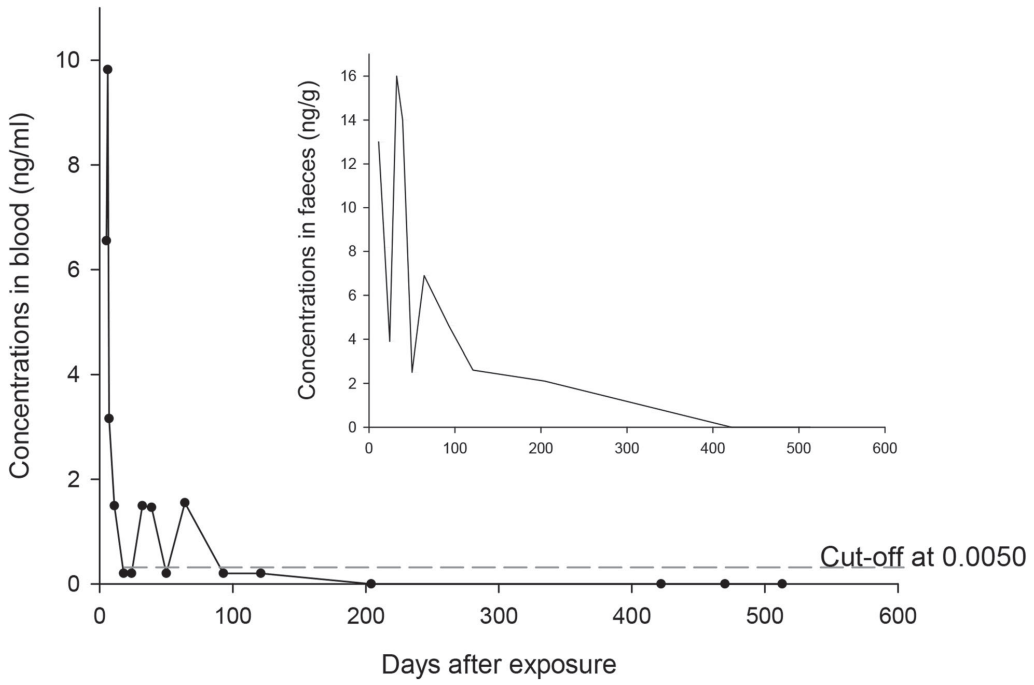

Fig. 3 Concentrations of coumatetralyl in blood (ng/mL) and faeces (ng/g)

content will always be large in this type of samples. This partly explains the relatively large relative standard deviations of the analysis between the concentrations found for the sample aliquots. The first elimination phase in faeces could not be accurately determined as the first samples were taken 11 days after ingestion. The second elimination phase from day 64 to 422 gives an estimated eliminations half-life of at least 81 days. Coumatetralyl was still detectable in faeces 204 days after ingestion, which demonstrates a considerably longer presence in the faeces compared to blood.

Detectable levels of brodifacoum were found in blood throughout the study period. Since only one of the concentrations where above LOQ, these results are not presented in Fig. 4. Corresponding analyses of brodifacoum in faeces demonstrated relatively high levels throughout the study (Fig. 4).

Discussion

We developed a novel method for analysis of six AR in faeces from dogs, and detected coumatetralyl and brodifacoum in blood and faeces and a very long elimination in the faeces. Accidental poisoning with AR is not uncommon in dogs, but few reports of the elimination kinetics and half-lives are published.

Elimination of coumatetralyl in faeces

The enterohepatic circulation and major route of elimination through the faeces support analyzing AR in faeces as a measurement of the residues in the body. Previous studies have found the highest concentration of AR in the liver, followed by kidney, muscle and fat [13, 21]. The lowest concentration was detected in blood [13]. In our case concentrations of coumatetralyl in faeces increased from day 24 to day 32 (Fig. 3). One explanation for this second peak could be a new exposure to AR, but we consider this unlikely since the corresponding concentrations in blood displayed only trace amounts of coumatetralyl and no clinical signs of exposure. Analytical error has been explored; and six parallels of the sample from day 32 were run, and the concentration in the following sample continued to be elevated. An explanation is biological variabilities between samples from the same animal [22–25]. A more probable explanation of the second peak is enterohepatic recirculation. Bile is released from the gallbladder shortly after ingestion [26]. Our samples

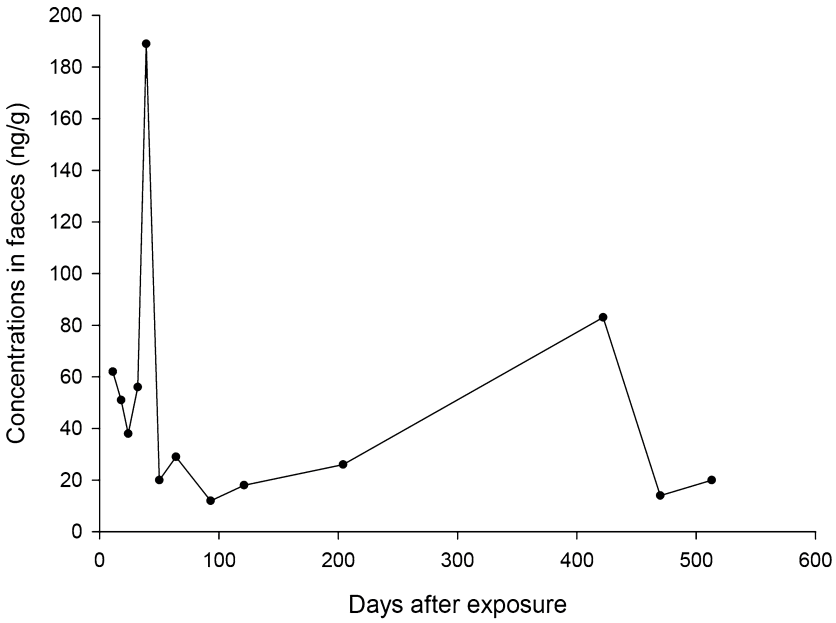

Fig. 4 Concentrations of brodifacoum in faeces (ng/g)

were not collected at the same time after meals. The dog was in her first estrus at day 32 and 39. Both estrogen and progesterone have extensive enterohepatic recirculation and are shown to decrease bile flow [27, 28], but the impact on the excretion of anticoagulant rodenticides is not known.

The faecal coumatetralyl concentration of 2.1 ng/g at day 204 suggests a substantial storage in the liver. Our analyses give an estimated terminal half-life of at least 81 days in this dog. A previous study in rats determined the elimination half-life of coumatetralyl in the liver to 55 days [29]. A stipulation of the elimination half-life in the liver of mice is 15.8 days [30] and 18.9 days in red deer (*Cervus elaphus scoticus*) [31]. Our results indicate that coumatetralyl might be present in the liver for more than 7 months after a single ingestion in dogs, depending on the amount ingested. As no samples were taken between days 204 and 422, we could not verify the elimination more precisely.

Detection of coumatetralyl in blood

There is limited toxicokinetic data available for coumatetralyl. Our case demonstrates a rapid initial α-phase in whole blood with concentrations decreasing from 9.8 to 1.5 ng/mL from day 6 to day 11, representing a half-life of 1.8 days. A terminal phase with slower elimination followed, until coumatetralyl was not detectable at day 204. This indicates a biphasic elimination, suggesting a two-compartment model, in accordance with studies from mice [30]. Compared to studies of other first-generation AR, our results indicate that coumatetralyl is detectable in the blood of dogs for a longer period compared to other investigated species. In rats, a single dose of chlorophacinone was completely excreted within 4 days [32]. A potential interaction on coumatetralyl elimination from brodifacoum cannot be excluded, but only trace amounts of brodifacoum was detectable in the blood throughout our study. The coumatetralyl dose ingested will affect the detection time.

Sources of brodifacoum

The source of brodifacoum in our case could not be determined. The dog had not showed any signs of illness prior to this ingestion and the owners were unaware of previous AR exposure. The trace amounts in blood indicated no recent, large ingestion. There are no AR products legally available in Norway, which contains both coumatetralyl and brodifacoum [33]. A previous exposure of small amounts of brodifacoum could have taken place. Another explanation of the small amounts of brodifacoum found in this young dog may be through

exposure to a resistant or sublethally poisoned rodent. Resistance to second-generation AR is observed in the brown rat (*Rattus norvegicus*) and house mouse (*Mus musculus*) in several European countries [34, 35]. In Germany, sublethally contaminated mice are detected in large areas around baiting stations [36]. Fisher et al. demonstrated an excretion of up to 19.4% of the ingested AR in the faeces of rats before death at day 4–6 [37]. Exposure to faeces from poisoned animals may be another origination of brodifacoum. Brodifacoum poisoning by a fecal–oral route has been suggested in one human case after a chronic accidental exposure [38]. The extent of this impact requires further investigation.

Faecal elimination of brodifacoum

We demonstrated high brodifacoum residues in the faeces throughout the study (Fig. 4). Extraction recovery for brodifacoum was 25%, which increases the risk of false negative results in our analyses. Our LOQs were set at the levels of the lowest calibrators (1.5–2.7 ng/g), which is below 3 µg/kg dry matter faeces in a previous study in foxes [16]. Our validation procedures yielded a satisfactory result for blank faeces samples, and precision and accuracy was within ± 20%. We believe this substantiates our method as precise, in spite of the low extraction recovery. After an initial reduction, brodifacoum concentration increased from day 24 to day 39, which corresponds to similar increase in concentration of coumatetralyl. Equivalent explanations as for coumatetralyl is probable for this peak. A second peak in faecal concentration of brodifacoum was seen at day 422, with corresponding concentration in blood displaying trace amounts. The owners were unaware of any new exposure and had removed all rodenticides from their property after the initial poisoning. The dog had not displayed any clinical signs of poisoning during these 7 months, but as no samples were collected between day 204 and 422, re-exposure to AR cannot be excluded.

No canine studies of hepatic half-life of brodifacoum could be found, and we propose to use of serial faecal levels to determine AR liver residues. Brodifacoum was still detectable at the conclusion of the study at day 513. Studies of the second-generation AR brodifacoum in rats after a single oral dose indicate biphasic elimination from the liver, with an estimated half-life of 150–350 days [32]. A single dose of brodifacoum in possums produced high liver concentrations at the time of sacrifice at 254 days [39]. An experiment with a single oral dose of brodifacoum in sheep demonstrated detectable levels in the liver at the end of the trial at day 128, but below the limit of detection in the faeces at day 32 [40]. This comparatively short elimination time could be explained by the limit of detection in faeces of 0.05 mg/kg (equivalent to 50 ng/g), compared to our study with a LOQ of 1.5 ng/g. A species difference between the ruminants and the monogastric dog may also be a contributing factor.

Detection of brodifacoum in blood

Few studies have reported half-life of brodifacoum in blood from dogs. A study with four dogs and administration of brodifacoum for 3 consecutive days, suggested a terminal half-life of 6 ± 4 days, revealing a two-compartment model and biphasic elimination [19]. A non-compartment model is suggested in one report, with a median plasma half-life of 2.4 days in seven poisoned dogs [41]. As the source and time of ingestion of brodifacoum were unknown in our case and only trace amounts were detectable in blood during the 513 days, we were not able to establish the elimination half-life. Our data suggests, however, a more prolonged half-life compared to previous studies.

Coumatetralyl poisoning

Coumatetralyl is classified as a first generation anticoagulant that requires multiple ingestions in order to exert its effect [42]. In our case, a single ingestion produced a severe poisoning. However, the trace amounts of brodifacoum detected in the blood and faeces at the time of ingestion may be a contributing factor to the severe effect of coumatetralyl in this case. The correlation between residues in the liver and sublethal effects in the animal is poorly described [43]. Riley et al. [44] showed a significant association between death in mange-infested bobcats and secondary anticoagulant exposure, suggesting that small exposures to AR lead to increased susceptibility to other diseases. Another study [45] did not find association between exposure to AR and immune suppression in cats. Other studies in rats have demonstrated severe poisoning after a single exposure of coumatetralyl [46, 47]. Different susceptibility to coumatetralyl between species has been suggested in several previous studies [42, 48]. Chopra et al. [47] described 50.5 mg/kg body weight of coumatetralyl in rats to be lethal to all the Indian mole rat (*Bandicota bengalensis*), but ingestion of 176.5 mg/kg was necessary to achieve equivalent effect in the common house rat (*Rattus rattus*). Species variation in susceptibility to coumatetralyl has also been demonstrated between Malaysian house rat (*Rattus rattus diardii*) and ricefield rat (*Rattus argentiventer*) [49]. There is a lack of data of the toxicity of coumatetralyl in dogs, but this species is suggested to be the most sensitive of the non-target mammals to coumatetralyl after a single ingestion [50]. One report suggested the lowest dose with effect on the coagulation to be 1 mg/kg in dogs, while similar effect is achieved after 5 mg/kg in cats [51]. The ingested dose of coumatetralyl in our case was unknown,

as the product was not identified and the dog vomited some of the AR a short time after ingestion.

Limitations

Faeces contain varying concentrations of AR, due to the inhomogeneity of the sample aliquots. This will further affect extraction recovery and the concentration of AR. Due to ethical considerations, studies of AR in non-target animals such as dogs are unacceptable in many countries. Naturalistic studies like ours will thus provide valuable contribution to this field.

Conclusions

We have developed a new method for the quantitative determination of six anticoagulant rodenticides in blood and faeces from dogs, by using UPLC–MS/MS. This analysis of AR in faeces offers a rapid, precise and non-invasive technique to monitor rodenticide exposures and adds value to diagnosing intoxication. The assay was successfully applied to a case of accidental rodenticide poisoning in a dog with analyses of faeces and blood. The faecal analyses of coumatetralyl revealed an estimated terminal half-life of at least 81 days in dogs. Brodifacoum was still detectable at the conclusion of the study at day 513, and displayed a prolonged half-life compared to previous studies. To our knowledge, this is the first report of a method for analysis of anticoagulant rodenticides in the faeces from dogs.

Abbreviations

ACN: acetonitrile; aPTT: activated partial thromboplastin time; AR: anticoagulant rodenticides; LOQ: limits of quantification; MRM: multiple reaction monitoring; N2: nitrogen gas; NMBU: Norwegian University of Life Sciences; PT: prothrombin time; QC: quality control; RSD: relative standard deviations; UHPLC–MS/MS: ultra-high performance liquid chromatography–tandem mass spectrometry.

Authors' contributions

KOS and LM designed the study and collected the samples. VV gave input on the study design and data collections. EE performed analyses. EE and RK drafted statistical analyses. The manuscript was drafted by KOS and RK and revised with assistance of EE, LM and VV. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The owner signed an informed consent form. NMBU adhere to a high standard (best practice) of veterinary care.

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Paper II

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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 (ARs) 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 single 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 analyzed for six ARs: brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, and flocoumafen. We detected ARs 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 ARs. 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 ARs in live endangered wildlife in connection with radio tagging or collaring operations.

Key words: Carnivores, fecal analyses, nontarget animal, predators, rat poison, secondary exposure, wildlife.

INTRODUCTION

Use of anticoagulant rodenticides (ARs) 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 (FGARs), including warfarin, diphacinone, coumatetralyl, and chlorophacinone, were developed in the 1950s. Extensive use of FGARs led to resistance against these rodenticides in both 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 (SGARs), such as brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen. Compared to FGARs, SGARs have higher toxicity and prolonged liver half-life and are effective after a single exposure (Watt et al. 2005). The SGARs can cause mortality after several days, allowing animals to ingest multiple doses and accumulate high concentrations in their body (Daniels 2013).

Predators can accumulate ARs through ingesting bait (primary exposure), by consuming poisoned prey (secondary exposure), or by

ingesting prey secondarily exposed to ARs (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 SGARs have been detected in raptors found dead in the wild, such as the golden eagle (*Aquila chrysaetos*) and eagle owl (*Bubo bubo*; Langford et al. 2013). To our knowledge, no publications have investigated AR occurrence in wild mammals in Norway.

Large amounts of ARs may cause bleeding 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, and 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 bacterium *Francisella tularensis*. 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 ARs have an enterohepatic circulation and accumulate in the liver (Huckle et al. 1988; Watt et al. 2005). Nontarget animal exposure to ARs 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 ARs, we suggest fecal analysis as a suitable method to investigate this unintended exposure.

The aim of our study was to estimate the occurrence of ARs 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 to 310,600 ha), representing 18 out of 19 counties in Norway and including areas surrounding three major cities in Norway (Oslo, Bergen, and Trondheim). The municipalities were divided in groups based on human population density. Population density per square kilometer for each municipality in 2016 was obtained from Statistics Norway (Statistics Norway 2018).

Sample collection

The hunter removed feces directly from the rectum immediately after death and submitted fresh samples to the Norwegian Veterinary Institute 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 to February and December, spring (n=30) from March to May, summer (n=20) from June to August, and autumn (n=23) from September to 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) and 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 analyzed, 65 were male, 64 female, and the sex of 10 was not determined. The samples were immediately frozen at –80 C upon arrival at the Norwegian Veterinary Institute and kept frozen at this temperature for 3 d, before being stored at –20 C until preparation. One sample per fox was analyzed.

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 AR analysis (Seljetun et al. 2018). In brief, ARs 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, Massachusetts, USA) with a mobile phase consisting of 5 mM ammonium formate buffer (pH 10.2) and methanol. Positive electrospray ionization tandem mass spectrometry detection was performed on a triple quadrupole mass spectrometer (Waters), using two multiple reaction monitoring transitions. Limits of quantification 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 ARs above limits of quantification were classified as positive, while detectable AR concentrations below quantitation limits were labeled as trace concentrations. The ARs analyzed 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. To test the sensitivity of the specific categorization of rural, suburban, and urban from human population density, we included variants of population measures. Municipalities with fewer 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 categorized 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 ARs was calculated for the total of all samples (n=139) and within groups. Differences between prevalence of AR substances were tested using the McNemar χ^2 test, whereas significant differences in AR exposure between groups were tested using the Pearson χ^2 tests. P values of the Pearson χ^2 test were obtained with 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 the Akaike information criterion value. 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 2016). Results were considered significant when P values were below 0.05.

RESULTS

Prevalence of ARs

At least one AR compound analyzed 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

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

Fox classifications	No.	% 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

(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 ARs 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 ARs, 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).

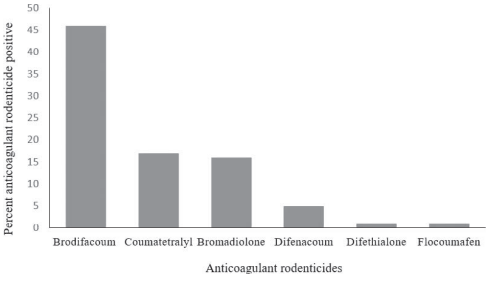

FIGURE 1. Occurrence of different anticoagulant rodenticide compounds in 139 fecal samples collected from presumed healthy wild red foxes (*Vulpes vulpes*) in Norway in 2016.

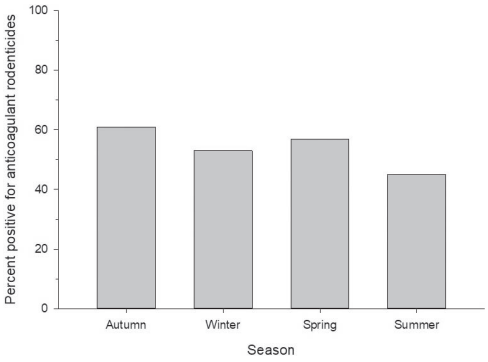

FIGURE 2. Seasonal occurrence of anticoagulant rodenticide compounds in 139 fecal samples from red foxes (*Vulpes vulpes*) in Norway in 2016. Exposure varied by season with 61% (14/23) foxes positive for anticoagulant rodenticides in the autumn (September–November), 53% (35/66) in the winter (January–February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

Sex and age differences

Of the 139 foxes analyzed, 65 were male, 64 female, and the sex of 10 was not determined (Table 1). Fecal residues of at least one AR were detected in 49% (32/65) males, 59% (38/64) females, and 50% (5/10) of those of unknown sex. There was no significantly different in AR exposure between sexes (χ^2=1.34, P=0.299). Exposure to ARs 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 or multiple AR exposure (P>0.437). Logistic regression indicated a tendency of positively association between sex and exposure to ARs when combined with age. In adult female foxes, 68% (23/34) were positive to ARs, compared to 49% (46/93) in a combined group of juveniles and adult male foxes (P=0.066).

TABLE 2. The percent (number) of fecal samples from wild red foxes (*Vulpes vulpes*) containing different anticoagulant rodenticides (ARs) 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	Fecal samples with ARs						
		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)

Prevalence of ARs in foxes correlated to human population densities

Foxes in suburban areas had an AR occurrence 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). 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 ARs 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 that foxes 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 exposure to ARs 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 ARs 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 ARs 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 ARs in the liver that results in its being the organ with highest tissue concentration (Huckle et al. 1988; WHO 1995). In addition, ARs 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 ARs may also be a factor. Furthermore, these previous studies were multiyear studies, compared to our single-year study. This could affect the results, because rodent population and AR use can vary between years. Last, 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, ARs can decrease fitness and cause abnormal behavior 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 ARs, 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 analyzed in that study. In Sweden and Finland, bromadiolone and coumatetralyl were the most common residues found in foxes (Tjus 2014; Koivisto et al. 2016). We suspect the difference between the countries in occurrence of these ARs 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 ARs 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 FGARs and SGARs, and the public is restricted to indoor use only. However, our results demonstrated continued exposure to nontarget wildlife despite these legislative measures.

More than one AR were detected in 40% of the positive foxes. Only one commercial product contains a combination of two ARs (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 ARs are not commercially sold in other European countries (López-Perea et al. 2015). We believe that accumulation of ARs 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, SGARs 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 necessarily 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 nontarget animals due to varying rodenticide use and differences in the foxes' diets. Wildlife in urban areas is considered to be at greater risk of exposure to ARs, 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 ARs 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 such as 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, 2019; Lohr 2018), 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 transference 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 ARs. 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 ARs 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 ARs. Despite government restrictions implemented in 2014, our results demonstrated that ARs are a continuing hazard in nontarget 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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Paper III

Brief Communication

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Comparison of anticoagulant rodenticide concentrations in liver and feces from apparently healthy red foxes

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Abstract. Exposure of wildlife and domestic animals to anticoagulant rodenticides (ARs) is a worldwide concern, but few methods exist to determine residue levels in live animals. Traditional liver detection methods preclude determining exposure in live wildlife. To determine the value of assessing AR exposure by fecal analysis, we compared fecal and liver residues of ARs in the same animals. We collected liver and fecal samples from 40 apparently healthy red foxes (*Vulpes vulpes*) potentially exposed to ARs, and quantified brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, and flocoumafen residues by liquid chromatography–tandem mass spectrometry. Residues of ARs were detected in 53% of the fecal samples and 83% of the liver samples. We found good concordance between AR residues in feces and liver for coumatetralyl, difenacoum, and difethialone. Bromadiolone occurred in significantly greater frequency in livers compared to feces, but no significant difference in concentration between feces and liver in individual foxes could be detected. Brodifacoum displayed a significant difference in concentration and occurrence of positive samples between liver and feces. Our findings demonstrate that fecal analysis of ARs provides a feasible and valuable non-lethal means of determine AR exposure in live wildlife.

Key words: anticoagulant rodenticides; non-target animals; secondary exposure; wildlife.

Anticoagulant rodenticides (ARs) have been used worldwide in pest control since the 1950s. ARs include first-generation ARs (FGARs), such as warfarin, diphacinone, coumatetralyl, and chlorophacinone, and second-generation ARs (SGARs), such as brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen.

Secondary exposure (ingestion of poisoned prey) in wildlife is a worldwide problem, and AR residues have been verified in 84–99% of livers from predators such as the red fox (*Vulpes vulpes*), stone marten (syn. beach marten; *Martes foina*), and European polecat (*Mustela putorius*).[4,21] Subtoxic levels of ARs may induce behavioral changes and reduced body condition in predators, impairing hunting ability and predisposing them to accidents and injury.[2] The threat of secondary poisoning in the critically endangered arctic fox (*Vulpes lagopus*) is of particular concern. The red fox may act as a sentinel for this species because of its widespread distribution and similar feeding resources.

ARs accumulate in the liver, and the major route of elimination is through feces.[5] Exposure in wildlife is normally assessed by residue analyses in liver, restricting examination to potentially biased opportunistically sampled dead animals. ARs have been analyzed in plasma or assessed by coagulation test to verify AR exposure in animals,[1,12] but this is inadequate in verifying sublethal exposure because residues can be detected in feces even when ARs are no longer detectable in plasma of either foxes or dogs.[17,18] During chemical immobilization and radio-tagging procedures of endangered species, there is an opportunity to use noninvasive techniques to sample feces from sedated animals. However, to be able to interpret such results, studies are needed to compare concentrations of ARs in feces with corresponding liver concentrations. We measured concentrations of ARs in liver and fecal samples collected from the same animal to evaluate the value of assessing AR exposure by analyzing AR concentrations in feces from live wildlife.

The 40 wild red foxes included in our study were apparently healthy animals shot in Norway by experienced hunters during the winter and spring of 2016. Feces and a piece of the liver were removed immediately after death and sub-

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Table 1. Number of red foxes from which anticoagulant rodenticides were detected.

			Residues in positive individuals (ng/g)		
	n	Occurrence (%)	Mean ± SE	Median	Min.–max.
Feces					
Brodifacoum	21	53	35 ± 6	28	4–103
Bromadiolone	3	8	122 ± 89	44	23–299
Coumatetralyl	8	20	13 ± 7	6	1–59
Difenacoum	4	10	21 ± 11	13	4–53
Difethialone	2	5	8 ± 3	8	5–11
Flocoumafen	1	3	10		
Liver					
Brodifacoum	32	80	56 ± 9	29	2–158
Bromadiolone	24	60	34 ± 10	14	2–192
Coumatetralyl	10	25	11 ± 6	2	1–62
Difenacoum	9	23	5 ± 2	2	1–18
Difethialone	9	23	6 ± 4	1	1–38
Flocoumafen	2	5	1 ± 0.03	1	1–1

Occurrence = % of animals with anticoagulant rodenticides (ARs), compared to the total of 40 samples; SE = standard error of the mean. Mean, median, and range of concentrations (ng/g) are from the cases with detectable concentrations of ARs.

mitted to the Norwegian Veterinary Institute (NVI) within 2 d. The submitted samples were frozen at −80°C and kept frozen at this temperature for 3 d, before being stored at −20°C until preparation and analysis. In our study, we analyzed the ARs used most commonly in Norway: brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, and flocoumafen.

Fecal samples were lyophilized to dryness and analyzed at the laboratory of the Department of Forensic Sciences, Oslo University Hospital. Procedures for fecal extraction and analysis of ARs have been validated in our laboratory and applied in our previous study in foxes.[18,19] Briefly, fecal samples were homogenized and aliquots of 100 mg removed. ARs were extracted with acetonitrile and dichloromethane from the aliquots and separated (Acquity ultra performance liquid chromatography BEH C18 column; Waters) with a mobile phase consisting of ammonium formate buffer and methanol. Positive electrospray ionization (ESI) tandem mass spectrometry (MS/MS) detection was performed on a triple quadrupole mass spectrometer (Waters), using 2 multiple reaction monitoring transitions. Signal-to-noise ratios were >10; precision and accuracy were within ±20%. In feces, limits of quantification (LOQs) were 1.5 ng/g for coumatetralyl, 2.2 ng/g for difenacoum, 2.6 ng/g for brodifacoum and bromadiolone, and 2.7 ng/g for difethialone and flocoumafen.

Liver samples were analyzed at NVI by a previously validated method.[9] Liver extracts (0.5 ± 0.1 g) were homogenized twice with acetone, before evaporating the liquid fraction to dryness. Residues were re-dissolved in acetonitrile and washed twice with hexane (Fluka Chemika; Millipore Sigma). ARs were separated (1200 series high performance liquid chromatography, Agilent Technologies; Xbridge C18 column, Waters). The column was equilibrated with ammonium acetate (Fluka Chemika) in water and acetonitrile at a ratio of 20:80 (v/v). The ARs were detected (negative ESI; G6470A triple quadrupole LC-MS; Agilent Technologies). Fragment ion spectra were recorded using 2 multiple reaction monitoring transitions. The recovery rates of ARs from liver tissue were 87–95%. Wet liver tissue LOQs were 0.5 ng/g for coumatetralyl, 0.8 ng/g for difenacoum, 1.8 ng/g for brodifacoum and bromadiolone, and 0.3 ng/g for difethialone and flocoumafen.

Comparisons between frequencies of AR occurrence between compounds in feces and liver were assessed by the Fisher exact test, and statistical comparisons were conducted using statistical software (Epi Info v7.2.3.1; Center for Disease Control and Prevention, Division of Health Informatics & Surveillance, Atlanta, GA). Statistical computations of AR concentrations between feces and liver were assessed by Wilcoxon signed rank test and conducted by JMP Pro (v14.2.0; SAS Institute). Nonparametric tests were used when data were not normally distributed; $p \leq 0.05$ was considered statistically significant.

Of the 40 wild red foxes examined, 35 of 40 (88%) contained detectable residues of 1 or more ARs. Residues of ARs were detected in 21 of 40 (53%) fecal samples and 33 of 40 (83%) liver samples. The number of detected ARs differed between feces and liver, but brodifacoum was most prevalent in both (Table 1). Given the low number of samples positive for flocoumafen, we excluded this substance from further statistical comparisons.

Comparing summed number of positive samples for each substance between feces and liver, there was a statistically significant difference between specimens for brodifacoum ($p = 0.018$) and bromadiolone ($p < 0.0001$; Fig. 1). No

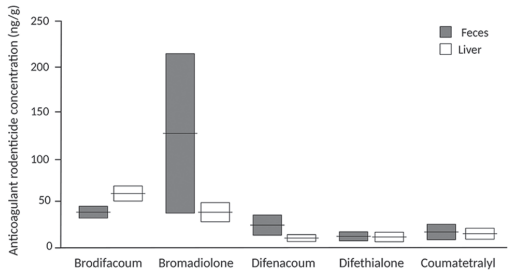

Figure 1. Fecal and hepatic concentrations of anticoagulant rodenticides from 40 wild red foxes. The results are given as means ± SE. For *n*, see Table 1.

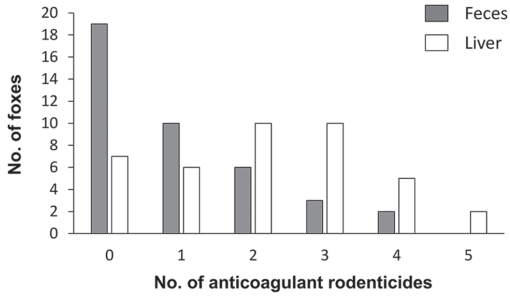

Figure 2. Number of anticoagulant rodenticides detected in samples of feces and liver collected from 40 wild red foxes. The samples were analyzed for brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, and flocoumafen.

significant differences were found for coumatetralyl (p = 0.790), difenacoum (p = 0.225), and difethialone (p = 0.051).

Comparisons of AR concentrations between feces and liver demonstrated no statistically significant difference for bromadiolone, coumatetralyl, difenacoum, or difethialone. Brodifacoum, however, was detected in significant higher concentration in liver than feces (p = 0.003).

In 11 of 40 (28%) foxes, the hepatic AR concentrations were >100 ng/g (mean: 178 ng/g). In 4 of these animals, the concentrations were >200 ng/g (202–354 ng/g). Concentrations >100 ng/g were also detected in 2 of the fecal samples (113 and 362 ng/g).

Two or more ARs were detected in 11 of 40 (28%) fecal samples, with a mean of 1.9 ARs in the positive foxes. In the liver samples, 2 or more ARs were found in 27 of 40 (68%), with a mean of 2.6 in the positive foxes (Fig. 2). There was a significant difference between number of substances in liver compared to feces (p = 0.001).

Overall, our results revealed good concordance between residues in feces and liver for coumatetralyl, the only FGAR analyzed. We detected the compound in 20% of the fecal and 25% of the liver samples; this is a high number considering previous suggestions of more rapid elimination of FGAR than SGAR.[16] Earlier studies have estimated the half-life of coumatetralyl of 15.8 d in mice and 55 d in rats.[16,23] The prevalence detected in our study suggests that coumatetralyl has a longer half-life in red foxes than previously estimated in rodents, which is in accordance with previous findings of estimated terminal half-life of at least 81 d after a single ingestion in a dog.[18]

We found good concordance of difenacoum and difethialone residues between feces and liver, both in concentration and frequency of positive foxes. The consistency between similar concentrations of difethialone found in liver and feces is probably a result of its exclusive fecal elimination as unchanged parent material.[8] Difenacoum displays similar elimination in feces with <2% excretion in urine.[22] On the other hand, 5% of bromadiolone is eliminated through urine, and similar excretion is seen with brodifacoum.[6] Although this difference in urinary elimination is small, a contribution to the difference in the concentrations between liver and feces of bromadiolone compared to difethialone and difenacoum is possible.

Bromadiolone was identified in a significantly higher number of livers compared to fecal samples. However, no significant difference in concentration of bromadiolone between feces and liver in the individual foxes was detected. This discrepancy is probably a result of the low number of positive fecal samples compared to liver. We detected bromadiolone in feces in only 3 animals, but in high concentrations. In one of these foxes, fecal concentration was 299 ng/g, with corresponding liver concentration of 35 ng/g. The high fecal concentration could indicate recent ingestion of either bait or rodent containing a high amount of bromadiolone. Another reason for the discordance in results may be low sensitivity in detection of bromadiolone in feces. A comparatively low detection in feces was identified in a previous experiment in 4 foxes, with a mean of only 1.1% bromadiolone in feces compared to liver 26 d after exposure.[17] On the other hand, given the low number of foxes in that experiment, direct comparison to our results is specious.

We detected brodifacoum significantly more often and in higher concentration in liver than in feces. We examined whether the significance in our results was influenced by the different LOQs in feces (2.60 ng/g) and liver (1.80 ng/g), but no such effect was found. One reason for this discrepancy in test results could be the result of variation in metabolism. Rats resistant to bromadiolone are suggested to have different metabolism of the compound compared to susceptible rat breeds or strains.[14] Whether this is valid for other ARs or affects the animals' metabolism after secondary exposure is not known. Furthermore, the discordance could in part be the result of a longer liver elimination half-life of brodifacoum (350 d detected in rats).[5] Bromadiolone has an equivalent half-life of 318 d.[6] In comparison, difenacoum and difethialone have an estimated liver elimination half-life of 118 and

126 d, respectively.[7,8] On the other hand, as bromadiolone was detected in only 3 fecal samples, extended comparisons are inconclusive. Furthermore, feces from foxes contain plant material and hair influencing extraction recovery and AR concentration, which is likely to contribute to the lower detection in feces compared to liver.[19]

Thresholds of toxicity for liver residues of ARs have not been established. In barn owls (*Tyto alba*), hepatic concentrations >200 ng/g SGAR were previously determined as potentially lethal[15]; a later study indicated a significant risk of acute intoxication with levels <100 ng/g.[20] However, one study demonstrated no signs of ill health in barn owls with liver residues up to 690 ng/g brodifacoum, 140 ng/g difenacoum, and 520 ng/g flocoumafen.[11] This discrepancy could be the result of large variation in individual susceptibility to ARs within species. Furthermore, tolerance to ARs is highly variable between species. Liver concentrations of 39 ng/g and 160 ng/g bromadiolone were lethal in poisoned dogs.[3] In contrast, liver residues of up to 2,060 ng/g bromadiolone were detected in randomly shot wild red foxes.[10] This difference could be because of a large variation in metabolism and vitamin K epoxide reductase activity between species.[24] In our study of presumed healthy foxes, 28% of the hepatic samples of SGAR were >100 ng/g, the previously stated threshold of acute toxicity. In 4 of these foxes, the residues were >200 ng/g, previously indicated as potentially lethal concentrations. This confirms that residue levels can verify exposure, but AR concentrations alone cannot be used to determine effect on animal health or serve as an indicator of toxicosis.

We collected feces directly from the rectum after death. Other studies have suggested analyzing ARs in scats sampled from the ground, but DNA analyses have detected 18–25% misclassification of presumed fox feces in these studies.[10,13] In addition, repeated fecal samples from one individual could skew the results. We therefore suggest collecting feces directly from the animals, also avoiding natural degradation of scats in the environment.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Paper IV

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RESEARCH

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Determination of anticoagulant rodenticides in faeces of exposed dogs and in a healthy dog population

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Abstract

Background: Exposure to anticoagulant rodenticides (ARs) in dogs is among the most common causes of poisoning in small animal practice, but information about toxicokinetic of these rodenticides in dogs is lacking. We analysed blood and faeces from five accidentally exposed dogs and 110 healthy dogs by reversed phase ultra-high performance liquid chromatography-tandem mass spectrometry. The aim of the study was to estimate elimination of brodifacoum, bromadiolone and difenacoum after acute exposure, calculate the half-lives of these rodenticides in dogs, estimate faecal elimination in a litter of puppies born, and further to identify the extent of AR exposure in a healthy dog population.

Results: Three dogs were included after single ingestions of brodifacoum; two dogs ingested bromadiolone and one dog ingested difenacoum. Maximum concentrations in faeces were found after day 2–3 for all ARs. The distribution half-lives were 1–10 days for brodifacoum, 1–2 days for bromadiolone and 10 days for difenacoum. Brodifacoum and difenacoum had estimated terminal half-lives of 200–330 days and 190 days, respectively. In contrast, bromadiolone had an estimated terminal half-life of 30 days. No clinical signs of poisoning or coagulopathy were observed in terminal elimination period. In blood, the terminal half-life of brodifacoum was estimated to 8 days. Faeces from a litter of puppies born from one of the poisoned dogs were examined, and measurable concentrations of brodifacoum were detected in all samples for at least 28 days after parturition. A cross-sectional study of 110 healthy domestic dogs was performed to estimate ARs exposure in a dog population. Difenacoum was detected in faeces of one dog. Blood and faecal samples from the remaining dogs were negative for all ARs.

Conclusions: Based on the limited pharmacokinetic data from these dogs, our results suggest that ARs have a biphasic elimination in faeces using a two-compartment elimination kinetics model. We have shown that faecal analysis is suitable and reliable for the assessment of ARs exposure in dogs and a tool for estimating the AR half-lives. Half-lives of ARs could be a valuable indicator in the exposed dogs and provides important information for veterinarians monitoring AR exposure and assessment of treatment length in dogs.

Keywords: Brodifacoum, Bromadiolone, Canine, Difenacoum, Intoxication, Non-target animal, Rodenticide

Background

Ingestion of anticoagulant rodenticides (ARs) is among the most common causes of poisoning in dogs worldwide [1, 2]. After ingestion, the ARs exert their effect by inhibiting vitamin K_1 epoxide reductase. Consequently, regeneration of active vitamin K_1 and formation of vitamin K_1

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dependent clotting factors II, VII, IX and X are disrupted [3]. The anticoagulant effect is mostly due to reduction of factors II and X, with plasma half-lives of 41 and 16.5 h, respectively [4]. After about 3–5 days, representing minimum two half-lives of factor II, circulating clotting factors are depleted and coagulopathy occur. The clinical signs and findings of ARs poisoning are unspecific and dogs may present with lethargy, pallor, dyspnoea, tachycardia and inappetence [5]. Prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT) confirms coagulopathy after ARs exposure.

The ARs are highly lipid soluble and accumulate in the liver [6]. In rat studies, liver elimination is suggested to be biphasic with a rapid distribution phase followed by a prolonged terminal phase [7, 8]. Information about liver elimination of ARs in dogs is lacking. ARs have an enterohepatic circulation which result in long half-lives and prolonged duration of anticoagulant effect [9]. Vitamin K_1 is used as antidote in these poisonings, and treatment up to 4 weeks may be necessary [10]. The main elimination is through faeces, and faecal residues appears to be equivalent to hepatic residues for most ARs [8, 11]. Studies have demonstrated that ARs are detectable for a longer period in faeces compared to blood, indicating faecal analysis as a feasible method to determine half-lives of ARs in poisoned dogs [12–14].

Despite the frequency of ARs poisoning in dogs, reports with serial measurements of ARs concentrations, toxicokinetic data and estimated half-lives are scarce [14–16]. The primary aim of this study was to estimate the faecal elimination phase after a single ingestion and calculate the half-lives of ARs in acutely exposed dogs. The second aim was to estimate faecal elimination in a litter of puppies born from a previous poisoned dog. Finally, we aimed to estimate the occurrence of ARs exposure in a healthy dog population.

Methods

Animals

Exposed dogs

Six privately owned dogs shortly after a witnessed ingestion of ARs or with clinical signs of ARs poisoning brought to the University Small Animal Hospital at the Norwegian University of Life Sciences (NMBU) were included in the study (Table 1). Two dogs (cases 1 and 2) arrived at the NMBU displaying clinical signs of ARs poisoning. Cases 3–6 were examined 0.5–1.5 h after a witnessed rodenticide ingestion before occurrence of clinical signs. The amount ingested ARs were unknown in all cases. Patient demographics (including age, breed, sex, weight), information on residence, concurrent medications and previous possible exposures were recorded for all dogs.

Case 1 gave birth to four healthy, full-term puppies 1127 days after the first exposure and 95 days after a second suspected exposure. The puppies were included in the study for 12 weeks.

Healthy dogs

In this cross-sectional study of non-randomly selected 110 privately owned dogs were enrolled, selected at routine visits to veterinary clinics and national dog shows. Dogs were included from all 18 counties in Norway with a variety of living conditions (rural, suburban and urban) between November 2017 and October 2018. The dogs were of 59 different breeds, average age 5.2 years (range 1.5–13 years), average body weight 21 kg (range 2.9–70 kg) and both sexes (46 males and 64 females).

Table 1 Characteristics and clinical presentation for six dogs exposed to anticoagulant rodenticides (ARs)

Case number	Age (years)	Weight (kg)	Clinical signs	Coagulation status	Detected ARs	Treatment	Duration of detectable AR concentrations after ingestion	
							Blood	Faeces
1	0.5	7	L, PMM, TP, DP, T	Prolonged	Brodifacoum	Vitamin K_1, oxygen, fluids	7	At least 969[a]
2	2	6	L, PMM, TP, DP, T, I, LT	Prolonged	Brodifacoum	Blood transfusion, Vitamin K_1, oxygen, fluids	53[b]	At least 894[b]
3	8	16	None	Normal	Brodifacoum	Emeticum, activated charcoal	9	At least 700
4	0.6	11	None	Normal	Bromadiolone	Emeticum, activated charcoal	n.d.	151
5	0.8	22	None	Normal	Bromadiolone	Emeticum, activated charcoal, fluids	n.d.	3
6	9.5	26	None	Prolonged (day 3)	Difenacoum	Emeticum, Vitamin K_1	9	At least 653

n.d. Not detected, *L* lethargy, *PMM* pale mucous membranes, *TP* tachypnea, *DP* dyspnea, *T* tachycardia, *I* inappetence, *LT* low temperature

[a] Probable new exposure detected day 1032

[b] After first visit, day of ingestion unknown

According to owner's signed declaration and information obtained in a comprehensive questionnaire, the dogs were healthy with no previous known exposure to ARs.

Coagulation analyses

Blood samples for coagulation analyses (PT and aPTT) were obtained from the exposed dogs at each visit. Blood was collected into vacutainer tubes containing sodium-citrate (3.2%) and analysed by a Coag Dx Analyzer (IDEXX Laboratories Europe B.V., The Netherlands) within 2 h of collection. Elevated values day 3–5 after ingestion corresponded with the clinical signs in case 1. Coagulation was longer than the range of the method in case 2 at arrival. Both dogs received vitamin K_1 and symptomatic treatment, and coagulation normalised. Vitamin K_1 was administered per os for 50 days and 26 days in cases 1 and 2, respectively. Case 6 displayed increased PT (24 s; reference value 11–17 s) with normal aPTT and no clinical signs of poisoning at day 2 after ingestion. The dog was started on vitamin K_1 treatment and coagulation normalised. Vitamin K_1 therapy was continued for 26 days. The remaining dogs (cases 3, 4 and 5) displayed normal coagulation throughout the study.

Sample collection

Exposed dogs

Blood and faecal samples were collected daily to weekly in the first month after exposure, followed by once a month until ARs were no longer detectable or the study ended. Faecal samples were collected by the owners after spontaneous defecation and brought to NMBU on the same day as blood was sampled. Faeces was collected in dark plastic bags, maintained at − 20 °C and within a few weeks lyophilized to dryness. Blood was collected in vacuum tubes containing sodium fluoride as preservative and potassium oxalate as anticoagulant. Blood samples were frozen (− 20 °C) shortly after collection and maintained frozen until analyses.

The litter of puppies was included from birth, and faecal samples were collected at day 1, 19, 23, 24, 27, 28 and 86 after parturition. Faeces was collected by the owner in dark plastic bags and maintained at − 20 °C. Within a few weeks the samples were brought to NMBU and lyophilized to dryness.

Healthy dogs

Blood and faeces were sampled once from each dog on the same day. Faeces was collected after spontaneous defaecation in dark plastic bags by the owner and brought to the veterinarian. Faeces was maintained at − 20 °C and were lyophilized to dryness within a few weeks after collection. Blood was collected in vacuum tubes containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Blood samples were frozen (− 20 °C) shortly after collection and maintained frozen until analyses.

Sample analyses

Blood and faeces were analysed for ARs at the Department of Forensic Sciences at Oslo University Hospital. Brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, and flocoumafen were analysed in this study, as all were previously registered for use in Norway. Sample preparation and faecal extraction were conducted as previously described [14]. Briefly, faecal samples were subjected to a liquid–liquid extraction with acetonitrile and dichloromethane, blood samples with ethyl acetate/heptane mixture. Separation followed by Waters Acquity reversed phase ultra-high performance liquid chromatography BEH C18 column (Waters Corporation, Milford, MA, USA) with a mobile phase consisting of 5 mM ammonium formate buffer (pH 10.2) and methanol. Positive electrospray ionization MS/MS detection was performed on a triple quadrupole mass spectrometer (Waters Corporation), using two multiple reaction monitoring transitions. An internal standard, warfarin d5, was added and analysed for all samples.

The extraction recoveries have previously been reported [14], and were 48% and 26% for difenacoum, 65% and 32% for bromadiolone, and 70% and 2% for brodifacoum, from blood and faeces respectively. Dog faeces is inhomogeneous with large variability in sample aliquot content. Even though large visible plant material, etc. were removed before sample preparation, this can influence both inter- and intra-individual extraction recovery. Good linearity and as well as precision and accuracy within ± 20% for all compounds were however found. In cases with more than one faecal sample per day, a mean of the ARs concentrations was calculated. Limits of quantification were 2.2 ng/g for difenacoum and 2.6 ng/g for brodifacoum and bromadiolone.

Calculation of half-lives

The concentration versus time profile in the post-peak phase for drugs with first-order kinetics is an exponential function ($dc/dt = -kC$), where k is the elimination rate constant [17]. The elimination half-life ($t_{½}$) can be calculate directly from the rate constant; $t_{½} = \ln 2/k$ or $t_{½} = 0.693/k$.

Results

Characteristics, clinical presentation and treatment for the six dogs exposed to anticoagulant rodenticides are given in Table 1.

Anticoagulant rodenticides analyses

In the dogs where faeces were collected during the first four days after ingestion (n=4), analyses displayed maximum ARs concentration at day 2–3 for bromadiolone, brodifacoum and difenacoum.

Brodifacoum was identified in samples from three dogs (cases 1–3). In blood, brodifacoum was quantifiable for 7, 9 and 53 days (Table 1). For the remainder of the study, brodifacoum was identified in only trace amounts or not detectable as previously reported [14]. We identified low residues of brodifacoum in faeces of one dog for 969 days (Fig. 1). At day 1032 our results displayed a second peak in both blood and faecal levels, and a recent minor ingestion unknown to the owner was suspected. As the dog remained healthy with no recurrence of clinical signs, no vitamin K_1 treatment was initiated. In case 2, brodifacoum was detected in faeces throughout the study and still detectable at the conclusion of the study (894 days; Fig. 2). Case 3 was lost to follow up after 700 days with still detectable levels of brodifacoum in faeces (Fig. 3).

In the litter of puppies born from case 1, low faecal concentrations of brodifacoum were detected in all samples up to 28 days after parturition, but not detected at day 86.

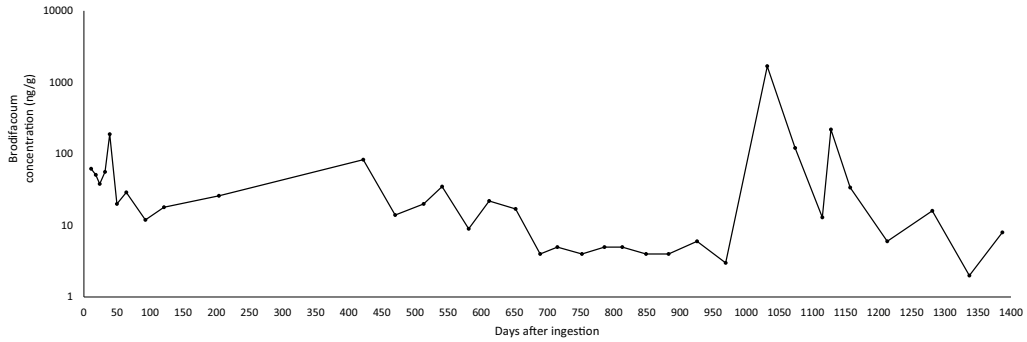

Fig. 1 Logarithmic elimination kinetics of brodifacoum in faeces in case 1 (day 11–1387 after ingestion). The dog gave birth to four puppies day 1127 days after the first poisoning. Brodifacoum was detected for at least 969 days after first exposure, with an estimated terminal half-life of 330 days

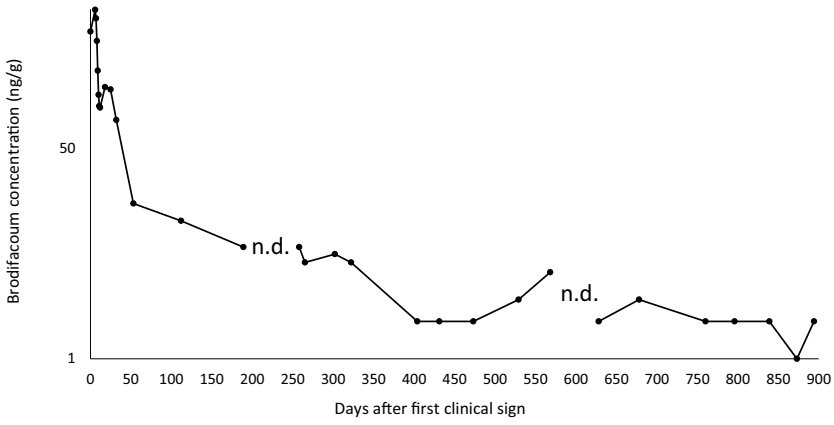

Fig. 2 Logarithmic elimination kinetics of brodifacoum in faeces in case 2 (day 0–894 after first visit). Terminal elimination half-life was calculated to 200 days

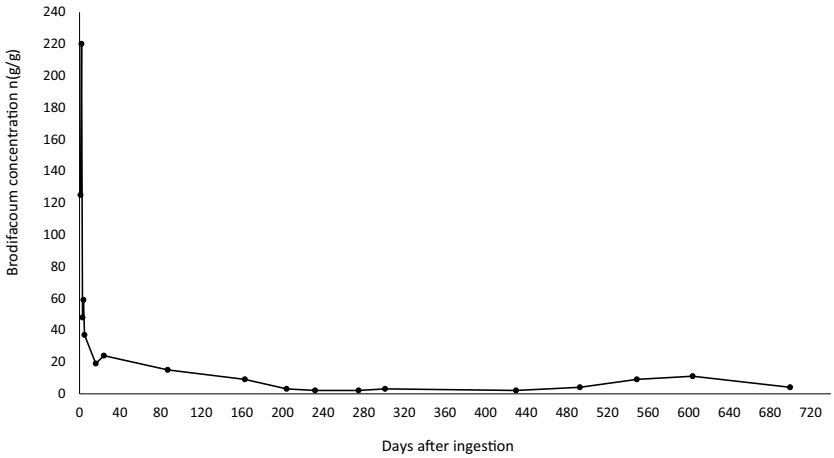

Fig. 3 Elimination kinetics of brodifacoum in faeces in case 3 (day 1–700 after ingestion). Terminal elimination half-life was calculated to 300 days

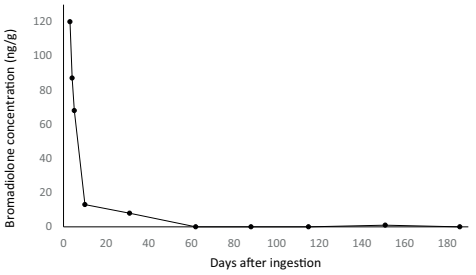

Fig. 4 Elimination kinetics of bromadiolone in faeces in case 4 (day 3–186 after ingestion) Terminal elimination half-life was calculated to 30 days

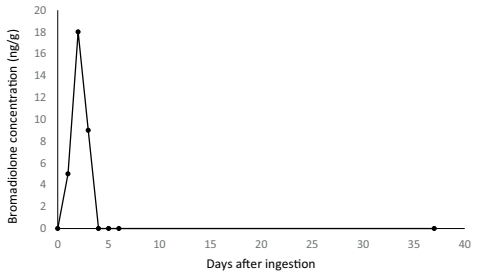

Fig. 5 Elimination kinetics of bromadiolone in faeces in case 5 (day 0–37 after ingestion)

Bromadiolone was detected in two dogs in the study (cases 4–5). Both dogs remained healthy throughout the study period with no clinical signs of ARs exposure. Coagulation remained normal and bromadiolone was not detected in blood. In faeces, bromadiolone was identified for 151 days in case 4, however samples were negative from day 62 to 115 (Fig. 4). In case 5, bromadiolone was detected for 3 days (Fig. 5).

Difenacoum was only identified in case 6. In blood, difenacoum was detected in trace amounts until day 9. Throughout the remainder of the study, difenacoum was identified in trace amounts in blood or not detected. In faeces, difenacoum was detected throughout the study and still detectable at the conclusion of the study (653 days; Fig. 6).

Estimated half-lives

In faeces, the serial levels indicate biphasic elimination and a two-compartment model for all ARs in dogs. The distribution half-life varied between the substances with 1–10 days in brodifacoum (case 1–3), 1–2 days in bromadiolone (case 4–5) and 10 days in difenacoum (case 6). The terminal half-lives were prolonged in brodifacoum with 200–330 days and difenacoum with 190 days. Elimination phase of bromadiolone was estimated in case 4 only, due to low concentration in case 5, and terminal half-life was calculated to 30 days.

In blood, as a result of low concentrations, distribution and elimination half-lives were only possible to estimate in case 2. Serum distribution half-life of brodifacoum was calculated to 1 day and the terminal half-life to 8 days.

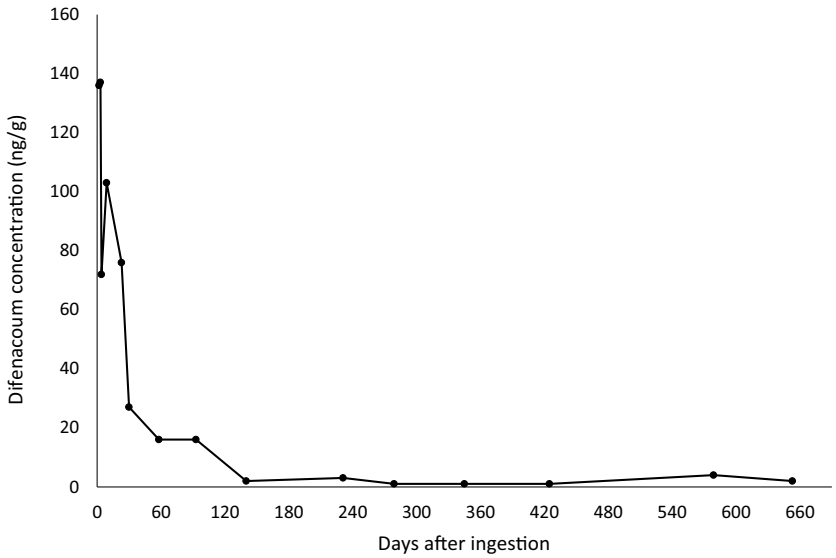

Fig. 6 Elimination kinetics of difenacoum in faeces in case 6 (day 2–653 after ingestion) Terminal elimination half-life was calculated to 190 days

The healthy dog population

Faeces and blood from 110 healthy domestic dogs assumed previously unexposed to ARs were sampled to establish prevalence of AR in a healthy dog population. The analyses revealed difenacoum in faeces (2 ng/g) of only one dog. All blood samples were negative for brodifacoum, bromadiolone, coumatetralyl, difenacoum, difethialone, and flocoumafen.

Discussion

This study confirms a biphasic elimination of brodifacoum, difenacoum and bromadiolone in faeces of dogs. Our findings suggest that ARs in dogs are stored for months in different tissues in the body after a single ingestion.

We estimated plasma terminal half-life of brodifacoum to 8 days in one case. A median plasma half-life of brodifacoum was estimated to 2.4 days (range 0.9–4.7 days) in analyses of seven poisoned dogs by high performance liquid chromatography (HPLC) in 1997 [16]. An experiment in four dogs in 1992 with repeated ingestions for three consecutive days estimated the serum half-life by HPLC to 6 ± 4 days [15]. As our result is based on only one case, comparisons are uncertain, but the prolonged half-life in our case could be due to improved analytical methods over the past 20 years.

Our study demonstrates that faecal sampling is a feasible method of monitoring ARs exposure in dogs, although faeces display a large variability in extraction recovery due to inhomogeneous samples and presence of plant material. We detected a substantially longer persistence of ARs in faeces compared to blood, corresponding to similar differences between liver and blood in quail and possums [18, 19].

Brodifacoum displayed a distribution phase of 1–10 days in faeces. The variation between the cases is probably partly due to exposure to different amounts and more frequent sampling might have improved the estimated distribution phase. However, due to poor clinical condition in two of the dogs, defecation was sparse during the first days after presentation. In addition, the amount distributed is influenced by initial decontamination of gastric emptying and activated charcoal which differed between the dogs. Although case 3 displayed no clinical signs of exposure, coagulation remained normal and no treatment was administered after initial decontamination, brodifacoum was still detectable for more than 700 days in faeces. Brodifacoum was detected in faeces of cases 1 and 2 for 894–969 days until the end of the study. Faecal terminal half-lives in the three dogs were estimated to 200–330 days. A previous investigation demonstrated a variation in individual susceptibility to brodifacoum [20], and this could contribute to the differences between the dogs detected in our study. In addition, as brodifacoum was present in faeces in all dogs at drop-out or conclusion of the study, different estimated

terminal half-lives of these dogs are conceivable. As no studies have examined half-lives of brodifacoum in dogs, comparisons to other species have been done. A half-life of 307 days in liver was observed in mice after a single ingestion of brodifacoum, which corresponds with our findings [21]. In sheep, brodifacoum was below the limit of detection in faeces at day 32 after a single ingestion [13]. The discrepancy from our study could be due to different analytical methods. Secondly, difference in level of detection is a contributing factor, with 0.05 mg/kg (equivalent to 50 ng/g) in the sheep compared to our limit of quantification of 2.6 ng/g. In addition, difference in species could contribute to the variance as metabolism and inhibition of vitamin K 2,3-epoxide reductase of ARs vary between species [22].

Bromadiolone was less persistent in faeces compared to brodifacoum in our study, correlating to previous studies with a longer hepatic persistence of brodifacoum compared to bromadiolone both in mice and rats [20, 21]. Bromadiolone displayed a biphasic elimination in faeces with an initial distribution phase of 1–2 days, equivalent to an experiment in pigs [23]. We calculated faecal terminal half-life of bromadiolone to 30 days in case 4. This correlates to an estimated liver half-life of 28 days and 24 days after a single ingestion in mice and humans, respectively [21, 24]. Our estimate is however based on only one case.

There are few reports estimating half-lives of difenacoum. A half-life of 62 days in liver was detected after a single ingestion in mice [21]. Several experiments have been conducted in rats, and a half-life of 120–128 days in liver has been suggested [7, 25]. However, in contrast to dogs, rats lack gall bladder and continuously secrete bile, problematizing direct comparisons between these species [26]. Difenacoum was detected in faeces in case 6 when the study ended 653 days after ingestion, and the estimated half-life was 190 days. Our estimate is however only based on the findings from one dog exposed to difenacoum. The prolonged elimination in this dog differs from our findings of shorter terminal elimination of bromadiolone but corresponds to the detected elimination of brodifacoum in cases 1–3. Corresponding pharmacokinetics of difenacoum and brodifacoum were detected in an experiment with analyses of plasma concentrations in rabbits [27].

In the 110 healthy dogs assumed unexposed to ARs, we detected one dog with low faecal concentrations of difenacoum. A previous investigation of 115 domestic pets revealed two dogs with trace amounts of diphacinone in the liver [28]. However, clinical status of the dogs was not specified in the study. Due to the limited number of dogs, where ARs were found, detection of predisposing factors contributing to ARs exposure was not possible. More research is needed to detect the cause and occurrence of ARs exposure in the healthy dog population.

The dogs did not display any clinical effects of their subtoxic AR concentrations in the months after exposure. Previous studies have detected reduced body condition of wildlife with sublethal ARs concentrations [29–31]. In addition, subtoxic ARs levels are suspected to increase susceptibility to pathogens, while others have not found such association [32–34]. Several studies have examined AR-related effects on the immune system in different species, and ARs changed the expression of immune-related genes in bobcats (*Lynx rufus*), increased levels of immature red blood cells in red-tailed hawks (*Buteo jamaicensis*), and decreased production of cytokines in domestic cats [34–36]. Further studies are required to assess the relevance in dogs with sublethal ARs concentrations.

Earlier studies have detected teratogenic effects, abortion and postpartum death after ARs exposure in pregnant animals [37, 38]. Stillbirth and neonatal death were seen in one report at least 4 weeks after a possible brodifacoum exposure of an unaffected bitch not displaying clinical signs of coagulopathy [39]. However, in the present study, case 1 gave birth to four full-term, healthy puppies after exposure. Brodifacoum was detected in faeces in both puppies and bitch for 1 month after birth, but all remained asymptomatic.

Conclusions

Faecal analysis has shown to be suitable and reliable for the assessment of ARs exposure in dogs and a valuable tool in estimating ARs half-lives in dogs. This study suggests that brodifacoum and difenacoum might be present in dogs' faeces after a single ingestion for more than 700 days and 650 days, respectively, and were still detectable at the conclusion of the study. Bromadiolone showed a comparatively shorter half-life in dogs. In a litter of puppies born from a poisoned dog, low faecal concentrations of ARs were detected for at least 28 days after parturition. The results may indicate a rather low prevalence of AR exposure among healthy dogs in Norway, but due to the limited number of dogs in this study, detection of prevalence and predisposing factors contributing to ARs exposure were not possible.

Abbreviations

aPTT: Activated partial thromboplastin time; ARs: Anticoagulant rodenticides; HPLC: High performance liquid chromatography; NMBU: Norwegian University of Life Sciences; PT: Prothrombin time.

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Prior publication
Our method for the detection and quantitative determination of anticoagulant rodenticides in faeces have previously been published with the early data from case 1 [14]. An abstract describing the findings in the litter of puppies was approved for presentation at the EAPCCT congress in Tallinn, Estonia in May 2020, but this was cancelled due to Covid-19 [40].

Authors' contributions
KOS designed and coordinated the study, collected and prepared the samples and drafted the preliminary manuscript. VV and LM contributed to study execution and data interpretation, and preparation of the manuscript. GB performed toxicologic analyses of blood. EE performed toxicologic analyses of faeces. ELØ analysed faeces and approved the analytical results. All authors participated in critical revision of the manuscript and have read and approved the final version.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study was approved by the Ethical Committee at NMBU (14-04723-44). All dog owners gave written informed consent before participation. Collection of blood and faeces from healthy client-owned dogs was approved by the National Ethics Committee for animal care and use, which falls under the Norwegian Food Safety Authority (FOTS ID 13821).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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