Belg. J. Zool., 142 (1) : 39-48 January 2012

Distribution of anticoagulant resistance in the brown rat in Belgium

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ABSTRACT. Anticoagulant resistance is known as one of the major factors interfering with rodent control. Within this context we investigated the distribution of anticoagulant resistance in Flanders, northern Belgium. From 2003 to 2005, we tested 691 rats from different locations with blood clotting response tests for their susceptibility to the anticoagulant compounds warfarin, bromadiolone and difenacoum. Of these, 119 were also screened for a mutation in the VKORC1 gene that is suspected to be responsible for anticoagulant resistance. Warfarin resistant rats were found in the western and eastern parts of Flanders. The same distribution pattern was found for bromadiolone with the exception of the south-eastern area, where this form of resistance was largely absent. We detected difenacoum resistance in only six rats and did not observe any resistant rats in the central part of Flanders. Susceptible rats were found all over Flanders. Genetic analyses showed that anticoagulant resistance in Belgium was related to two different mutations in VKORC1, namely Y139F and L120Q. Our results indicate that rodent control should be regionally tailored to be most effective.

KEY WORDS. blood clotting response, rodent control, warfarin, bromadiolone, difenacoum, VKORC1

INTRODUCTION

Through the ages brown rats have been poisoned because of the damage they cause and the diseases they carry (MEEHAN, 1984; GRATZ, 2006; HEYMAN et al., 2009). Before the discovery of warfarin, rodent control was mostly achieved with acute poisons (BUCKLE, 1994a). With the introduction of warfarin and related anticoagulant compounds, also known as coumarins, a new class of rodenticides became available in the 1940s. The delayed action of anticoagulants does not cause bait-shyness and makes them particularly suitable for the control of a neophobic species such as the brown rat. Furthermore they are relatively safe, due to the existence of the antidote vitamin K1. As a result, rodent control became largely an issue of chemical intervention with less emphasis placed on sanitation and exclusion measures (FRANTZ & PADULA, 1998; PELZ et al., 2005). Since the 1950s, anticoagulants have been the most widely used rodenticides (MEEHAN, 1984).

Coumarins act as a vitamin K antagonist and block the vitamin K cycle in the liver, preventing the reduction of vitamin K epoxide to vitamin K by vitamin K epoxide reductase (VKOR). Vitamin K is an essential co-factor in the activation of several vitamin K-dependant coagulation factors through which it plays an important role in blood coagulation (OLDENBURG et al., 2008). When coumarins bind with VKOR, intoxication with anticoagulants will lead to a deficiency of vitamin K and coagulation factors, causing coagulation disorders such as spontaneous bleeding and eventually death. In resistant rats, VKOR is slightly modified and prevents a proper binding with the rodenticide, which thus fails to work (THIJSSEN, 1995). This mechanism is based on a single nucleotide polymorphism (SNP) in the VKORC1gene, which codes for the VKOR enzyme (ROST et al., 2004). At least eight different SNPs are related to anticoagulant resistance in the brown rat. In Belgium, as in France, the mutation known as TAT-139-TTT or Y139F is present (PELZ et al., 2005; GRANDEMANGE et al., 2010).

After the first discovery of warfarin-resistant rats in Scotland in 1958 (BOYLE, 1960) other foci of resistance arose in Wales and southern England in the 1960s (KERINS et al., 2001). On the European continent the first traces of resistance were found in Denmark in 1962 (LODAL, 2001). About ten years later it also occurred in Germany (PELZ, 1995) and in the meantime this trait developed in North Carolina USA (FRANTZ & PADULA, 1998). Furthermore, resistance to first-generation anticoagulants such as warfarin has also been found in France, Japan, Brazil, Portugal, Italy and Canada (MACNICOLL & GILL, 1987; GREAVES, 1994; PELZ et al., 2005).

Due to increasing warfarin resistance, the industry developed stronger second-generation products such as bromadiolone, difenacoum, brodifacoum and difethialone. These rodenticides were also based on the 4-hydroxycoumarin structure, but with increased lipophilicity and thus prolonged half-lives (ATTERBY et al., 2001). Unfortunately, these stronger anticoagulants present a greater risk of primary intoxication of non-target species and secondary intoxication of scavengers and predators (BRAKES & SMITH, 2005; HOARE & HARE, 2006).

Rodent pest management today depends on the anticoagulant rodenticides because of their outstanding efficiency and excellent safety profile. Monitoring for resistance is important if we are to understand the scope of its spread and to manage resistant rodent populations (BUCKLE, 2006). Resistance is defined by the European and Mediterranean Plant Protection Organization as follows; "Rodenticide-resistant rodents should be able to survive doses of rodenticide that would kill 'normal' or 'susceptible' conspecifics" (EPPO, 1995). GREAVES, (1994) describes anticoagulant resistance as a major loss of efficacy in practical conditions where the anticoagulant has been applied correctly, the loss of efficacy being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant. In this study, resistance is based upon a positive blood clotting response (BCR) test result.

In Denmark, but also in Germany and the United Kingdom, scientists monitored the evolution and distribution of resistance (KERINS, 2001; LODAL, 2001; PELZ, 2001). In these countries, they observed that resistance expanded geographically and towards stronger active ingredients e.g. bromadiolone and difenacoum. In the United Kingdom brodifacoum resistance has also been reported (GILL et al., 1992). Resistance to different anticoagulants is known as cross resistance, and evolves often from first- to second-generation anticoagulants (also known as resistance hierarchy) (PELZ, 1995). This means that resistance to anticoagulants of higher potency will always be accompanied by resistance to compounds of lower potency. LUND (1984) mentioned anticoagulant resistance in house mice in Belgium, but no data concerning resistant rats were provided. The work reported here is the first documented study of the distribution of anticoagulant-resistant rats in Belgium. The aim of this study was to assess the presence of any resistant rats in Flanders and to study the extent of this resistance trait, both geographically as well as functionally. More specifically, we wanted to test whether resistance in brown rats was restricted to warfarin, or whether it extended to frequently-used second-generation anticoagulants like bromadiolone and difenacoum. In a later stage, we examined DNA samples to find out if mutations in the VKORC1 gene could explain our positive BCR results.

MATERIALS AND METHODS

Rats

Wild rats (*Rattus norvegicus*) were captured using live traps and caged individually in our laboratory for animal science. Once in the lab they received Carfil Quality maintenance rat food and fresh tap water. No extra menadione or vitamin K3 was administered. The rats were kept in the laboratory for at least three weeks before testing, in order to exclude rats in gestation and diseased or intoxicated rats.

The use of living rats in our study was approved by the local ethics committee of 'The Institute for Agricultural and Fisheries Research of the Flemish Government' and was in agreement with the legislation on laboratory animal science.

Blood clotting response test

To distinguish resistant from susceptible rats we applied a blood clotting response (BCR) test, involving the measurement of changes in coagulation time after the administration of a small dose of anticoagulant. For the detection of warfarin resistance, we used the BCR test described by MARTIN et al. (1979) and MACNICOLL & GILL (1993). BCR tests for bromadiolone and difenacoum resistance were carried out according to GILL et al. (1993, 1994). Briefly, the rats received a solution of anticoagulant (2ml/kg) by oral gavage. The concentration used for warfarin and difenacoum was 0.25% (dose 5mg/kg). For bromadiolone, male and female rats were given a solution of 0.05% (dose 1mg/kg) and 0.12% (dose 2.4mg/kg) respectively. After 24 hours for warfarin and 96 hours for bromadiolone and difenacoum, we took blood by means of a retro-orbital puncture and measured the prothrombin time (PT) (Coadata 501 coagulometer for whole blood, Helena capillary reagent rabbit brain thromboplastin). We converted the PT into the percentage coagulation activity (PCA) by means of a calibration curve based on a dilution series of a mixed blood sample of five Wistar rats for each sex. The cutoff point for warfarin resistance was a PCA of 17%, for bromadiolone and difenacoum this was a PCA of 10%. Warfarin-susceptible rats were euthanized after the experiment. Due to resistance hierarchy, we considered them as also bromadiolone and difenacoum susceptible. Warfarin-resistant rats were subsequently tested with bromadiolone and difenacoum. The minimum interval between warfarin and bromadiolone BCR tests was one week, while between bromadiolone and difenacoum tests it was six weeks. Anticoagulant administration and blood sampling were performed under isoflurane anaesthesia.

Genetic analysis

Rat DNA was extracted from tissue samples (tail tip) with the Qiagen tissue kit (Qiagen). PCR amplification of part of exon 3 of the VKORC1 gene was performed using the primers and conditions described in PELZ et al. (2005). The presence of a mutation in individual samples was analysed by temperature gradient capillary electrophoresis (TGCE) (CHOU et al., 2005) on a SCE9610 Genetic Analyzer (Spectrumedix Inc.) applying a 45-55 °C gradient with a ramp period of 24 minutes. Electropherograms were analysed with the Revelation 2.41 software (Spectrumedix). Samples showing a heteroduplex were considered as heterozygous mutants. Samples showing a homoduplex were further analyzed by mixing the test sample with a known homozygous wild type (WT) reference sample and repeating the TGCE analysis to distinguish between homozygous mutants and WT. In this way, homozygous WT animals could be discriminated from homozygous and heterozygous mutants.

Samples from animals carrying a mutation as revealed by TGCE were further analysed using an allele-specific amplification refractory mutation system (ARMS) PCR (YE et al., 2001) for the presence of the Y139F mutation, which has been previously detected in Flanders (PELZ et al., 2005). As the mutation Y139F is different from the one published (Y139C), the original inner ARMS primers were slightly modified: F-primer: 5'-TGATTTCTGCATTGTTTGCATCACCAC GTT-3' and R-primer: 5'-CAACATCAGGCCC GCATTGATGGAAT-3'. Amplification products were analyzed by agarose gel electrophoresis.

Some of the samples that were negative in the ARMS PCR were sequenced and revealed the presence of the L120Q mutation (PELZ et al., 2005). For this mutation, a new ARMS PCR was developed with inner primers F: 5'-TGGTGTCTGTCGCTGGTTCTCTGTAGC A-3' and R: 5'-ATACAGGACAAAGAACAGG ATCCAGGGCA-3'.

For routine analysis, the forward inner primers of the mutations Y139F and L120Q were labelled with an FAM-dye and a NED-dye, respectively, and analysed simultaneously on the SCE9610 sequencer. Results were analysed with the Genospectrum v3.0.0 software (Spectrumedix).

Statistical analysis

We used a Log-Linear analysis to test, per river catchment, whether more rats than expected were resistant to warfarin and subsequently to bromadiolone. The analysis was based on the Fisher exact test and the level of significance was corrected for multiple testing (Table 1).

To measure the level of agreement between the BCR test and the genetic analysis we calculated kappa. Kappa expresses the proportion of agreement beyond chance and is only valuable when the results of both tests are not significantly different and the prevalence is between 0.2 and 0.8 (Dohoo et al., 2003).

RESULTS

From 2003 to 2005 we tested 691 rats from different locations for warfarin resistance. Of these, 550 had a PCA less than 17% and 141 rats had a PCA above 17% and were respectively warfarin susceptible and resistant. Between the BCR tests for warfarin and bromadiolone resistance, 19 rats died. Consequently only 122 warfarin-resistant rats were tested with bromadiolone. Of these, 88 were also bromadiolone resistant. Between the bromadiolone and difenacoum BCR tests we lost 28 rats. Six bromadiolone resistant rats were also difenacoum resistant.

Because advances in genetic research on resistance took place after the onset of this study we were not able to test all our rats for mutations in VKORC1. We screened 26 susceptible and 93 resistant rats for the presence of a mutation in VKORC1. None of the 26 susceptibles carried a mutation in VKORC1 but 87 resistant rats did. Six rats that had a positive BCR result for warfarin resistance did not carry a mutation in VKORC1; their PCA varied between 17 and 27 %. All the bromadiolone and difenacoum resistant rats tested genetically carried a mutation. Two different mutations were found in exon 3. Mutation one showed an SNP in codon 139 where adenine was replaced by thymine (TAT-139-TTT), which resulted in a replacement of the amino acid tyrosine by phenylalanine in VKOR (Y139F). The second mutation was found in codon 120 where thymine was replaced by adenine (CTG-120-CAG) and the amino acid leucine was substituted by glutamine (L120Q).

The results of the BCR test and the mutation screening were not significantly different and the prevalence was between 0.2 and 0.8. With kappa=0.864, CI 95%: 0.758-0.969 higher than 0.8 both tests agreed almost perfectly (Dohoo et al., 2003).

The geographical distribution of resistant brown rats in Flanders was significantly different from random (Table 1, Fig. 1). We used the existing river catchments as geographical units. Anticoagulant resistance was found in three different regions. In the southeast region, which corresponds to the Demer river catchment, 26 rats were resistant to warfarin. Only four of these rats were bromadiolone resistant, resulting in significantly less bromadiolone resistance than expected (Table 1). One rat was also difenacoum resistant. Between bromadiolone and difenacoum BCR tests there was a significantly higher loss of rats; 14 out of 25 rats compared to 14 out of 97 rats for the other regions. All 26 warfarin-resistant rats were tested genetically and carried mutation L120Q.

In the west and in the east of Flanders, we saw that the majority of the warfarin resistant rats (91%) were also resistant to bromadiolone. Five of these bromadiolone resistant rats were resistant to difenacoum. The rats in this region carried mutation Y139F. In the central part the resistance trait was absent.

TABLE 1

Warfarin- and bromadiolone-resistant rats (by BCR test) per river catchment. 691 rats were tested with warfarin, of which 141 were warfarin resistant. Of these rats 122 animals were also tested with bromadiolone. The geographical distribution of warfarin- and bromadiolone-resistant brown rats in Flanders was significantly different from random. WS: warfarin susceptible, WR: warfarin resistant, BS: bromadiolone susceptible, BR: bromadiolone resistant, level: level of significance based on Fisher exact and corrected for multiple testing.

RIVER CATCHMENT	WS	WR	level	BS	BR	level
1 Yser	30	18	ns	5	11	ns
2 Bruges Polder	20	13	ns	0	11	ns
3 Ghent Canals	30	4	ns	2	2	ns
4 Lower Scheldt	145	6	<0.001	1	3	ns
5 Leie	47	32	<0.001	4	24	ns
6 Upper Scheldt	0	2	ns	0	1	ns
7 Dender	81	1	<0.001	0	1	ns
8 Dijle	38	0	<0.01	0	0	ns
9 Demer	61	26	ns	21	4	<0.001
10 Nete	49	0	<0.001	0	0	ns
11 Meuse Antwerp	7	0	ns	0	0	ns
12 Meuse Limburg	42	39	<0.001	1	31	<0.01
total	550	141		34	88	

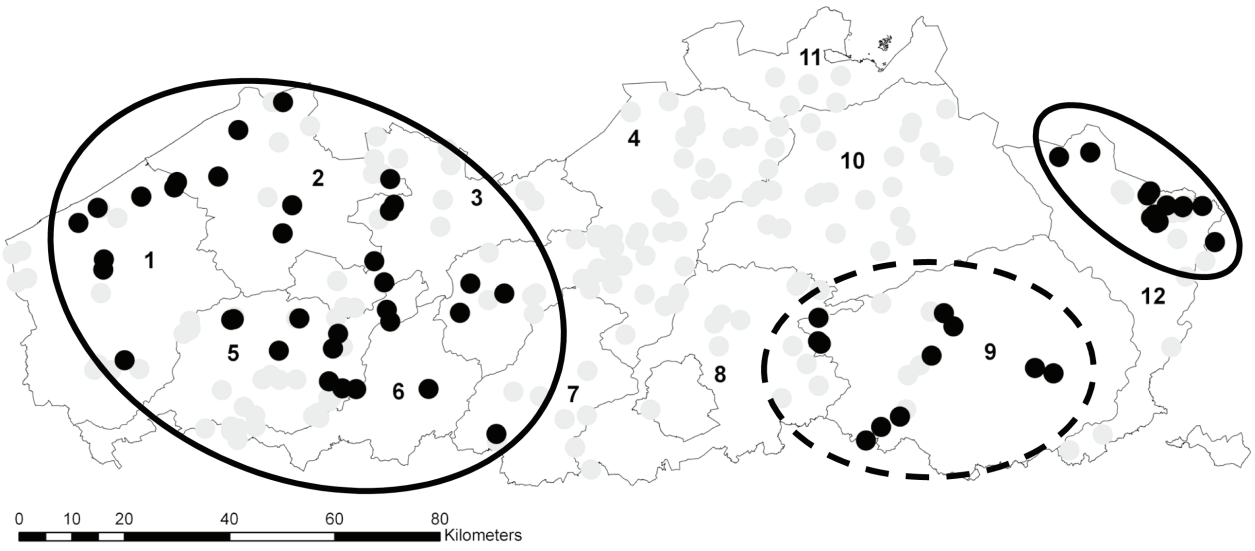

Fig. 1. − Three different areas with resistance were found in Flanders. In the west and east these areas were characterised by bromadiolone resistance (full line) and linked to mutation Y139F in VKORC1. In the south-east the area was characterised by warfarin resistance (broken line) and the presence of mutation L120Q. The grey dots represent locations where only susceptible rats were found. The black dots stand for locations where at least one resistant rat was caught. The numbers correspond with the river catchments in Table 1.

DISCUSSION

This study shows that resistance to anticoagulants also occurs in Flanders, with a clear distribution pattern caused by a different genetic background and resistance to different coumarins. In a small region such as Flanders, which is one of the most densely populated and urbanised areas in Europe (EEA 2007) and is characterized by a large amount of traffic along roads and watercourses enhancing rat migration, we expected less regional variation in anticoagulant resistance. We know that brown rats are widespread (STUYCK, 2003) and the availability and use of different types of anticoagulant rodenticides does not differ much between localities. Therefore we believe that neither increased rodent control intensity, due to higher rat densities, nor different use of poisons, is responsible for the different levels of resistance. Similarly, we can not explain the lack of resistance in the central part of Flanders; in Upper-Scheldt, Dender, Dijle and Nete river catchments, significantly fewer than expected resistant rats were recorded (Table 1). Data from other countries such as Denmark, Germany and the United Kingdom has shown that anticoagulant resistance expands (KERINS, 2001; LODAL, 2001; PELZ, 2001), both geographically and functionally, from first to second generation rodenticides. The situation observed in Flanders provides a unique opportunity to follow the trend of resistance in a resistance-free area, surrounded by areas with resistant rats.

In the west and east of Flanders, we found warfarin-resistant rats, most of which were also bromadiolone resistant. Only a few of these bromadiolone resistant rats were difenacoum resistant and this to a lesser degree than to bromadiolone. Therefore we believe that the resistance hierarchy in both areas was as follows: warfarin < bromadiolone < difenacoum. The same hierarchy pattern was previously found in Denmark and Germany (PELZ et al., 1995). The rats in these regions of Flanders carried mutation Y139F. This mutation is common in France where it also confers resistance to bromadiolone (GRANDEMANGE et al., 2009). More recently it was also found in Korea and in the UK (ROST et al., 2009; PRESCOTT et al., 2011), the latter in a place where applications of the anticoagulant rodenticide bromadiolone had been unsuccessful. The situation in France as well as in the UK is consistent with our findings.

In the Demer river catchment, the majority of the resistant rats were resistant only to warfarin, with only a few rats also testing bromadiolone resistant. Compared to other river catchments this difference was significant (Table 1). Additionally, the bromadiolone-resistant rats showed a BCR test result for bromadiolone close to the cutoff point, indicating a very low degree of resistance. We also noticed a major loss of rats between the bromadiolone and difenacoum BCR tests. Such a high mortality after a BCR test is exceptional as the doses used in these tests are considered as non-lethal (GILL et al., 1993). This observed mortality confirmed the presence of fewer bromadiolone resistant rats and resulted in a possible underestimation of difenacoum resistance. Moreover, the only difenacoum-resistant rat caught in the Demer river catchment showed a higher level of difenacoum resistance than bromadiolone resistance. The much lower degree of bromadiolone resistance and a possibly different resistance hierarchy, can be explained by the presence of another resistant strain in the Demer river catchment. All the resistant rats in the Demer carried mutation L120Q, a mutation initially found in the Berkshire and Hampshire strain in the UK (PELZ et al., 2005), and later, also in France (GRANDEMANGE et al., 2010). These strains in the UK are known for their difenacoum resistance (GILL et al., 1993), but in our study this mutation's contribution to anticoagulant resistance is mainly restricted to the first generation rodenticide, warfarin.

At the beginning of this study, we chose to work with BCR tests to evaluate anticoagulant resistance in Flanders. At that time, it was probably the best solution as the BCR tests then had replaced feeding tests for reasons of accuracy and animal welfare (KERINS et al.,

2001). Nowadays, often only genetic tests are used to evaluate the presence of resistance. It is not clear how closely positive BCR test results correlate with the definition of GREAVES (1994), which emphasises major loss of efficacy of the rodenticide in practical conditions. Although BCR tests provide no direct indication of the practical impact of the resistance observed (BUCKLE, 1994b), we now know that a positive BCR result has a high, positive predictive value for the presence of an SNP in the VKORC1 gene (PELZ et al., 2005), which certainly contributes to resistance. To assess the practical implications of anticoagulant resistance on rodent control using the BCR test, it is possible to work with a resistance factor based on the multiple of the discriminating dose as suggested by PRESCOTT et al. (2007). In our opinion, the major advantage of a BCR test remains the fact that it measures the effect caused by the rodenticide itself apart from the resistance mechanisms behind it. This means that changes in pharmacodynamics or pharmacokinetics (MARKUSSEN et al., 2008) that differ from changes in VKORC1 will also be detected. A disadvantage of the BCR test is that rats trapped in the wild could bias the BCR test result because of an earlier bait uptake in the field. Indeed, the half-life of second-generation anticoagulants in the liver can extend to 300 days (EPA 2007). The same bias also plays a role with subsequent testing of different compounds in the lab. At the beginning of our study, we found that an interval of three weeks between the bromadiolone and difenacoum BCR tests was not enough to normalise the PT within its normal range. As a result, we extended the interval up to six weeks for all the following tests to normalize the PT. However, this still did not guarantee the absence of any negative effects on the difenacoum BCR result. For these reasons, we believe that it is better not to test rats with more than one second generation anticoagulant, as this can lead to an underestimation of the resistance level. But more importantly, BCR tests should be part of each resistance screening as they indicate resistance independent of the mechanism behind it.

The BCR tests we used were based on the methods described by MARTIN et al. (1979), MACNICOLL & GILL (1993) and GILL et al. (1993, 1994). It was PRESCOTT et al. (2007) who re-evaluated these BCR tests and designed the standardised BCR (SBCR) test. This is a sensitive method designed to detect the slightest form of anticoagulant resistance. The discriminating dose was determined by using a group of susceptible rats. To predict the likely impact on field control, a resistance factor based on the multiple of the discriminating dose is used. The SBCR is based on the International Normalised Ratio (INR) and allows comparison of blood clotting data obtained by different thromboplastin reagents used in different labs. This is not possible with PCA values. As a consequence, the thresholds of PCA we used –17% for warfarin and PCA, 10% for bromadiolon and difenacoum, corresponding with INR values of about 3.5 and 7 respectively – probably do not match the thresholds used in the original BCR tests. The usual threshold for the SBCR test is an INR value of 5, and the time between administration of the anticoagulant and the blood sampling is reduced for all anticoagulants to 24h.

For the detection of warfarin resistance we used a discriminating dose of 5mg/kg versus 3.02 mg/kg for male and 4.26 mg/kg for female rats used in the SBCR test. A higher discriminating dose together with a lower threshold means that we probably underestimated warfarin resistance compared to the SBCR test. A correction for the different threshold alone means that about 12% of the rats that we regarded as susceptible should have been resistant under the SBCR test. This rather small difference in result between the BCR and the SBRC tests can be explained by the variation in our results. About 75% of the warfarin resistant rats have a PCA>30% or INR<2 and about 88% of the susceptible rats have a PCA<12% or INR>5. This means that about 15% of the rats that we tested were in the range of INR 2 – 5. For this minority of rats, it is not always possible to tell whether they are resistant or susceptible, an uncertainty which also exists when using the SBCR test, since the cut off for that test was arbitrarily defined (PRESCOTT

et al., 2007). This again shows that resistance to anticoagulants is not clear-cut. Based on our and previous results (PELZ et al., 2005), it seems that mutations in VKORC1, resulting in changes in pharmacodynamics, contribute more clearly to anticoagulant resistance as illustrated by the high kappa value we found. Since the mutations in VKORC1 are probably responsible for the variation in blood clotting between the groups of resistant and susceptible rats, variation within the groups and overlap between the groups could then be explained by changes in metabolism or pharmacokinetics (MARKUSSEN et al., 2008).

Comparison of our results for bromadiolone and difenacoum resistance in the light of the SBCR is complex. The major advantage of second-generation anticoagulants in rodent control is their prolonged half-life, which is dependent on changes in clearance and apparent volume of distribution (BRECKENRIDGE et al., 1985). As a result, the plasma concentration and, therefore, also the effect of the rodenticide, remain higher for a longer period. In the SBCR, the interval between administration and blood sampling is reduced from 96h to 24h, to exclude changes in clotting time by pharmacokinetically-based effects (PRESCOTT et al., 2007). By shortening the interval, however, the beneficial effect of the prolonged half-life of second-generation anticoagulants is untested, as is its benefit in the context of anticoagulant resistance. Furthermore, there are indications that changes in pharmacokinetics affect anticoagulant resistance in brown rats (MARKUSSEN et al., 2008). For the same reason HEIBERG (2009) did not shorten the time interval of her BCR tests.

CONCLUSIONS

In Flanders, the degree of resistance to different anticoagulants used in rodent control showed a clear geographical distribution and was linked to the presence of two different mutations in VKORC1. One strain of rats in the west and the east of Flanders was characterised by its resistance to warfarin and bromadiolone apparently related to mutation Y139F in VKORC1. Another strain located in the southeast, a region corresponding with the Demer river basin, was mainly warfarin-resistant. Here, resistance was linked to mutation L120Q. In both strains, the first signs of difenacoum resistance had appeared. The central part of Flanders did not reveal any resistant rats and no mutations in VKORC1 were found.

Our results show that resistance monitoring should be an essential part of adaptive rodent management when confronted with rodent control failure caused by anticoagulant resistance.

We suggest that future research should focus on resistance monitoring not only in the central area of Flanders, where this trait is currently lacking but also in the resistant areas where we could determine possible changes in resistance prevalence and hierarchy. The BCR test and the detection of mutation in VKORC1 resulted in a similar outcome for warfarin resistance. Despite their close agreement, a combination of genetic and (S)BRC tests should be used, especially for second generation anticoagulants and as long as some of the mechanisms causing anticoagulant resistance remain unclear.

ACKNOWLEDGEMENTS

We are grateful to the department Operational Water Management of The Flemish Environment Agency (VMM) for providing the rats and their support. We also want to thank our INBO colleagues Tim Adriaens, Frank Husentruyt, Thomas Scheppers and Koen Van Den Berge for their valuable contribution to this manuscript, Sabrina Neyrinck and David Halfmaerten for the genetic analysis and Sebastien Pieters, Filip Berlengee and Bram Lens for their technical assistance in our laboratory. Special thanks go to Andrea Tongue for proofreading the manuscript.

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Received: March 2nd, 2011

Accepted: November 30th, 2011

Branch editor: Huybrechts Roger