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Distribution of anticoagulant rodenticide resistance in *Rattus norvegicus* in the Netherlands according to *Vkorc1* mutations

Bastiaan G Meerburg,* Marga PE van Gent-Pelzer, Bruce Schoelitsz and Theo AJ van der Lee

Abstract

BACKGROUND: Rodenticide resistance to anticoagulants in *Rattus norvegicus* will lead to increased difficulties in combating these pest animals. Here, the authors present the results of a survey in the Netherlands where tissue samples and droppings were tested using a newly developed TaqMan PCR test for genotypic variation at codon 139 in the *Vkorc1* gene associated with anticoagulant rodenticide resistance. Test results are linked to results of a questionnaire that was conducted among pest controllers.

RESULTS: Genetic mutations at codon 139 of the *Vkorc1* gene in *R. norvegicus* can be encountered in many parts of the Netherlands. In 34/61 rat tails, a genotype was found that is linked to anticoagulant rodenticide resistance (56%). In droppings, 42/169 samples (25%) showed a resistance-mediating genotype. In addition, indications of a clear genetic substructure in the Netherlands were found. In some regions, only resistance-mediating genotypes were found, corroborating results from the questionnaire in which pest controllers indicated they suspected resistance to anticoagulant rodenticides.

CONCLUSION: This is the first study to demonstrate the presence of multiple genetic mutations at codon 139 of the *Vkorc1* gene in *R. norvegicus* in the Netherlands. As rodenticides should keep their efficacy because they are a last resort in rodent management, more studies are urgently needed that link specific genetic mutations to the efficacy of active substances.

Supporting information may be found in the online version of this article.

Keywords: rodenticide resistance; rat; genetic mutation; rodent control; anticoagulants, integrated pest management

1 INTRODUCTION

For the last decade, increased attention has been paid to the emergence of rodenticide resistance among *R. norvegicus* in Europe. Rodenticide resistance is defined as the loss of efficacy of rodenticides under practical conditions, even though the rodenticides are properly applied. Anticoagulant rodenticide resistance is associated with mutations in the *Vkorc1* gene,[1–4] causing amino acid substitutions in the VKORC1 protein, a critical factor for blood clotting and the target of anticoagulants. Because of such mutations, rats may (sometimes to a considerable extent) lose their susceptibility to rodenticides (anticoagulants). These mutations are transferred to their offspring, resulting in resistant rat populations.

Although occurrence of resistance to first-generation anticoagulant rodenticides (e.g. warfarin and chlorophacinone) has been known since the 1950s,[4–9] currently there are signs of emerging resistance to second-generation rodenticides.[2,10,11] This is problematic, as *R. norvegicus* are a major pest responsible for food spoilage and transmission of (zoonotic) pathogens and damaging infrastructure.[12] Moreover, it is assumed that in the Netherlands many fires that occur on farms are the result of the gnawing of rodents (often supposed to be *R. norvegicus*) on electric wiring.[13]

In Germany, in 2007 the area in which resistant *R. norvegicus* with the (Tyr139Cys) genotype were encountered stretched from Westphalia into southern Lower Saxony.[10] In the United Kingdom, where virtually all known resistance mutations are found, resistant *R. norvegicus* were found in Cambridge/Essex, Nottinghamshire, Kent, Gloucestershire, Norfolk and Lincolnshire, south-west Scotland, Hampshire and Berkshire, the Anglo-Welsh border, central southern Scotland, Yorkshire and Lancashire.[14,15] Resistance against second-generation rodenticides is also present in a number of other European countries,[2] namely across the whole of Denmark (Bornholm, Fünen, Jutland and Zealand), Belgium (Flanders) and large parts of France (e.g. Yonne, Eure and Loire). Resistance against rodenticides was reported decades ago among *R. norvegicus* and *Mus musculus*.[16,17] Moreover, there have been some indications that resistance against second-generation rodenticides is

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present, based on a small study of a Dutch television programme (Nieuwslicht, 24 September 2008) where researchers of the Julius Kühn Institut (Germany) showed that five out of 12 *R. norvegicus* samples from the Netherlands contained the Tyr139Cys mutation in the *Vkorc1* gene. Here, the authors present the results of comprehensive monitoring in the Netherlands, which involved three parts: a questionnaire for pest controllers, a genetic screening of tissue samples and a countrywide genetic screening using rat droppings.

2 EXPERIMENTAL METHODS

2.1 Questionnaire

In order to identify locations with possible rodenticide resistance, 960 questionnaire requests were sent in 2010 to pest controllers throughout the Netherlands. It was possible to fill out the questionnaire on the internet, and pest controllers that received recertification training were also provided with the questionnaire. The questions focused on whether pest controllers have experienced certain regions in the Netherlands where it is difficult to control rodents and what might be possible causes. The questionnaire differentiated among the rodent species *R. norvegicus*, *Rattus rattus* and *Mus musculus*. Farmers were specifically notified by an advertisement in the Dutch farmers' magazine *Nieuwe Oogst* and were also encouraged to fill out the questionnaire for their premises.

2.2 Development of a rapid molecular test and rat tail survey

A rapid molecular test (a TaqMan real-time PCR) for the detection of rodenticide resistance at codon 139 of the *Vkorc1* gene in *R. norvegicus* was developed for this study. Firstly, specific primers and probes were developed by means of CLC computer software (CLC Bio, Aarhus, Denmark) and Primer Express (Applied Biosystems, Carlsbad, CA), and an optimisation/visualisation and calculation step in the program OMP (DNA Software, Ann Arbor, MI). The TaqMan real-time PCR method was validated using DNA reference material kindly provided by the Julius Kühn Institute (JKI). After that, pest controllers were asked to provide animal material, namely the tails of *R. norvegicus*, in the period from November 2010 to June 2011. Samples were shipped in 96% ethanol and labelled with the address. After arrival, tissue samples were stored in 96% ethanol at 4 °C. About 10 mg of freeze-dried tail tissue was used for DNA extraction using the Mag-Bind Tissue DNA kit for KF 96 (Omega Bio-tek, Norcross, GA) protocol on KingFisher™ Flex magnetic particle processors (Thermo Scientific, Waltham, MA). The DNA concentration was determined using Quant-iT™ PicoGreen® (Invitrogen, Carlsbad, CA) in an Infinite® 200 PRO monochromator (Tecan, Männedorf, Switzerland). Approximately 1 ng of DNA was then used in a 25 µL TaqMan reaction with a TaKaRa Premix Ex Taq (Perfect Real Time) master mix (Takara Bio, Otsu, Japan), ROX Reference Dye II, 300 nM of the appropriate primers (*Vkorc1* forward primer 5′-gttctttgtcctgtatgattt-3′ and *Vkorc1* reverse primer 5′-agctaagcaaacatcaggccc-3′) and 100 nM each of three probes (p139Tyr_FAM 5′-FAM-caccaccTATgccatcaa-NFQ-3′, p139Cys_NED 5′-A546-accaccTGTgccatcaatg-NFQ-3′ and p139 Phe_VIC 5′-VIC-caccaccTTTgccatcaatg-NFQ-3′). Nucleotides shown in capitals are locked nucleotide analogue (LNA) modifications integrated to enhance the specificity of the probe.[18] The Alexa fluorophore A456 is a customised fluorophore. The AB7500 instrument can read the fluorescence of this fluorophore in the detection window normally used for the NED fluorophore. The real-time PCR program started with 2 min at 95 °C, then 40 cycles were carried out with temperature steps of 95 °C (15 s) and 60 °C (1 min) using the AB7500 (Applied Biosystems). Afterwards, profiles were manually scored on the basis of the profiles of the reference material (see supporting information Fig. S1).

2.3 Rat droppings and media campaign

The small numbers of tissue samples of *R. norvegicus* that were received from professional pest controllers (66 in total; see Section 3) and their limited geographic spread prompted the authors to change their approach in order to acquire a larger number of samples. Tests were conducted to establish whether the developed TaqMan PCR test also worked on rat droppings (faeces), as this would be easier to acquire from the general public than tissue samples. With some modifications, this was the case, which made it possible to ask the general public to send in rat droppings together with the postal code were these droppings were found. In August 2012, a media campaign was launched, including a public website (www.bruinerat.nl). The news item about this research was covered by television, by national and regional radio stations and by newspapers.

In addition, professional pest controllers were asked via telephone to send in rat droppings. Each submitted sample received a unique serial number. DNA was then isolated using the InviMag® Stool DNA Mini kit/KFmL (Stratec Molecular, Berlin, Germany) extraction kit on the KingFisher (Thermo Scientific) extraction robot using the protocol recommended by the suppliers. For each sample, a single, randomly chosen rat dropping was transferred to a reaction vessel. The pellet was shaken twice (30 s at 5000 bpm) with five zirconia beads in a lysis buffer. After centrifugation, the dark-coloured supernatant was transferred to a reaction vessel with adsorbent matrix. Subsequently, the cleaned liquid from the centrifugation was transferred to a 96-deep-well block, and DNA was isolated with a KingFisher robot (Thermo Scientific). The DNA was diluted 10 times before the TaqMan PCR. For the TaqMan PCR, 5 µL of DNA, 300 nM of the appropriate primers (VKORC1 forward and VKORC1 reverse), 100 nM each of the three probes (p139Tyr_FAM, p139Cys_NED and p139Phe_VIC) and ROX Reference Dye II were added to a total volume of 25 µL of TaKaRa Premix Ex Taq (Perfect Real Time) mix (Takara Bio). The real-time PCR program started with 2 min at 95 °C, then 45 cycles were carried out with temperature steps of 95 °C (15 s) and 60 °C (1 min), using the AB7500 (Applied Biosystems). Afterwards, profiles were manually scored. To prevent cross-contamination, all samples of rat droppings were packed in seal-locked plastic bags per sending, special UV cabinets were used for the pipetting and filter tips were employed to prevent carryover. In addition, care was taken never to open amplification plates after the amplification step.

3 RESULTS

3.1 Outcome of the questionnaire

In total, 158 questionnaire responses were returned (117 online and 41 as hard copy), and these covered most of the Netherlands. Unfortunately, no farmers participated. The majority of respondents (59%) worked for local governments, while 30% worked for private pest control companies, water boards (6%) or other employers such as recreational parks or golf centres (5%). The major part (82%) did not experience problems with rodent control, while 18% did. However, only 8% of the respondents attributed these problems to emerging rodenticide resistance,

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while others mentioned other reasons: the availability of other feed sources during the rodent control phase, limited attention for rodent presence or absence of rodent proofing. Eight respondents suspected rodenticide resistance among *R. norvegicus* in 16 municipalities (Fig. 1A).

3.2 Outcome of the rat tail survey

Of 66 rat tail samples, five were not analysed because the quality or quantity of the DNA was insufficient. Twenty-seven of the remaining samples (44%) were wild type. In ten samples (16%), an abnormal FAM profile was found that could have contained an unknown mutation affecting the probe binding. In 14 samples (23%), the Tyr139Cys mutation (German type) was found heterozygously, and in eight samples (13%) the Tyr139Cys mutation was found to be in the homozygous state. Finally, one (2%) heterozygous Tyr139Phe genotype (French/Belgian type) and one (2%) heterozygous genotype (Tyr139CysTyr139Phe) were found. An overview of the different samples is given in Fig. 1B.

3.3 Results from the *R. norvegicus* droppings survey

The campaign to send in droppings started on 8 August 2012 and lasted until 25 January 2013. In total, 361 samples with droppings were sent in, covering almost all parts of the Netherlands and one location in Belgium. The number of submissions originating from parts of Drenthe, Zeeuws-Vlaanderen, Noord-Oost Groningen and Limburg was small. Out of 361 samples, 172 (48%) originated from pest controllers and 189 (52%) were sent in by the general public. DNA extraction was performed on 290 samples that showed visual characteristics of droppings of *R. norvegicus* (although a visual inspection is not 100% reliable) and were in appropriate condition for further analysis. All samples were then tested in the TaqMan PCR assay. Of these, 121 samples (41%) showed no PCR amplification. This could include samples in which insufficient DNA was isolated or samples that could have originated from *R. norvegicus* by visual characteristics but came from other species. Samples submitted by pest controllers more often produced a reliable test result (63%) than those from the general public (37%), potentially because pest controllers better recognised droppings of *R. norvegicus*. In 169/290 samples (59%) there was a reliable test result. Of these, 127 (75%) showed the wild-type genotype, while 42 (25%) showed either the Tyr139Cys mutation or the Tyr139Phe mutation. Both mutations were found in the heterozygous and homozygous state. If submissions by the general public and professional pest controllers are compared, there are only small differences in the frequency distribution of the various genotypes (Table 1).

The distribution of resistance-mediating genotypes and the distribution of heterozygous and homozygous mutants seem to be uneven in the Netherlands (Fig. 2).

4 DISCUSSION AND CONCLUSIONS

The results of this study demonstrate that several genetic mutations at codon 139 of the *Vkorc1* gene that are linked to anticoagulant rodenticide resistance in *R. norvegicus* occur in the Netherlands. The rapid molecular test that was developed can be used for molecular detection of such genetic mutations via either rat tails or rat droppings.

Only 18% of the pest controllers experienced problems, and of these a mere 44% attributed these problems to emerging rodenticide resistance. Others mentioned other reasons for the problems, such as the availability of other feed sources during the rodent control phase, limited attention for rodent presence or absence of rodent countermeasures. This once again underlines the need for effective rodent management procedures [based on integrated pest management (IPM) with a proper working rodenticide as the final tool]. Although there was no 100% overlap, nearly all regions where professional pest controllers suspected rodenticide resistance in *R. norvegicus* were confirmed by the genetic screening of both the rat tails and the rat droppings.

In the rat tail survey, 24/61 (39%) of the rats carried genetic mutations that were associated with rodenticide resistance.[19] However, the number of screened rat tail samples was low, and the majority of samples came from areas where it was later confirmed by the rat dropping survey that mutants were present. The authors attribute this clearly biased sampling of the rat tails to the fact that pest controllers in those areas may have been more cooperative in submitting their samples as they were interested to know why their control methods did not work. In the rat droppings survey, national media attention and the involvement of the general public generated a large geographic spread of samples. Therefore, 25% is thought to be a good estimate for the whole country. During a recent study in France,[20] a comparable number was found (28%).

However, in the Netherlands there are specific regions where this average number is far exceeded. In the eastern part of the Netherlands, only droppings from resistant specimens were found. The presence of homozygote genotypes with the Tyr139Cys and Tyr139Phe mutation in such areas further indicates that the percentage of rodenticide-resistant rats there is high.

Specimens with the homozygous Tyr139Cys genotype (German) were found not only in the eastern part of the Netherlands (Twente, Achterhoek) but also in the region around Rotterdam and the Noord-Oostpolder. Also, rats with the homozygous Tyr139Phe (French) mutation were found in a number of locations throughout the country (Gelderse Vallei, South-Eastern Brabant and Northern-Limburg). Perhaps this is linked to migration along the main rivers that connect these regions with France and Belgium where this genotype occurs frequently.[20,21] The rat droppings derived from Belgium had the Tyr139Phe genotype, which was consistent with a study from Belgium that was conducted in 2003–2005 where the presence of Tyr139Phe genotype was reported from that region and directly linked to bromadiolone resistance.[21]

Moreover, it is assumed that the regions in which resistant *R. norvegicus* are found are expanding in the Netherlands, as has already happened in Germany.[9,10,22] Figure 2 shows that rats with either the Tyr139Cys or the Tyr139Phe genotype can be encountered in a significant part of the Netherlands. The occurrence of the Tyr139Cys genotype (German) seems to be more widespread, probably because this genotype was present earlier in the country compared with the Tyr139Phe genotype (French/Belgian). This seems to be similar to the situation in Germany, where the Tyr139Phe genotype has been encountered in one location, whereas the Tyr139Cys mutation seems to be widely distributed throughout north-western Germany.[10,22]

Substances such as bromadiolone (the only toxic rodenticide in the Netherlands that was allowed to be used outdoors until June 2014) and difenacoum may lose or may have lost their efficacy partly or even completely.[23] A recent study in Germany[14] demonstrated that the resistance factor for difenacoum in German *R. norvegicus* carrying the Tyr139Cys mutation was about 2.5. Although this resistance factor is quite low, difenacoum has been unable to create an appropriate level of control for

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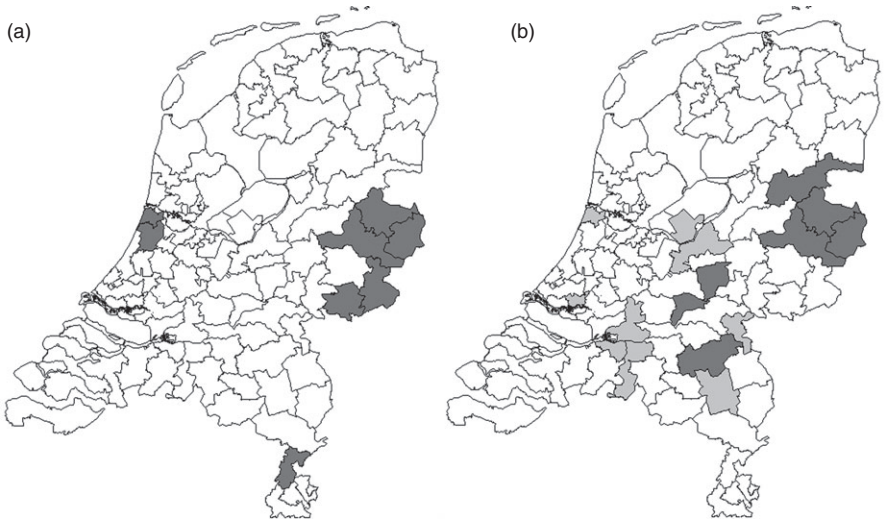

Figure 1. Overview of the questionnaire outcome among pest controllers (A) and the outcome of the analysis of tissue samples of *R. norvegicus* (B) presented on the map of Dutch postal codes (two digits). On map A, the dark-grey areas represent regions where the presence of resistant rats was suspected. On map B, in the dark-grey areas at least one mutation was discovered, based on tail tissue samples, while in the light-grey areas only the wild type (susceptible to anticoagulant rodenticides) was encountered.

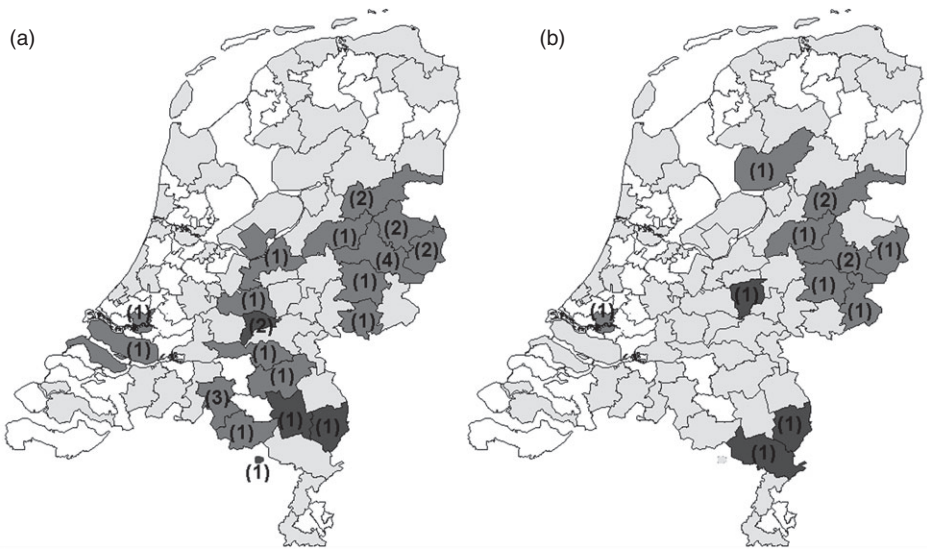

Figure 2. Spatial distribution of mutations at codon 139 of the *Vkorc1* gene in *R. norvegicus* in the Netherlands, based on dropping samples. The grey areas are regions with reliable genotyping results. A: the light-grey areas are regions with heterozygous Tyr-Cys genotypes, and the dark-grey areas are regions with heterozygous Tyr-Phe genotypes. B: the light-grey areas are regions where homozygous Tyr-Cys genotypes were encountered, and the dark-grey areas are regions where homozygous Tyr-Phe genotypes were found. Numbers in parentheses show the number of positive specimens in that area.

rat populations carrying this mutation and should not be used. Moreover, Buckle *et al.*[14] also mention that a successful rodent control effort may be influenced by homo- or heterozygosity, whereby individuals that carry the Tyr139Cys mutation in the homozygous state are more resistant than individuals that carry this mutation in the heterozygous state. Given the large number of *R. norvegicus* in the Netherlands that have the Tyr139Cys genotype in the homozygous state, this could have serious implications. In another study in Münsterland, Germany, it was reported that 0.005% brodifacoum is still effective against rodents with the Tyr139Cys (when heterozygous) mutation.[24] An overview of the current status of the efficacy of active substances in the Netherlands is presented in Table 2. More studies that link the efficacy of active substances to specific genetic mutations are urgently needed.

Recently, a nationwide survey was conducted in France. In total, 268 rats were analysed, and 100 of these demonstrated at least one single nucleotide polymorphism on the VKORC1 gene.[20] Resistance was conferred in 37% of the rats across the country.[20] It is known that Tyr139Phe confers resistance to first-generation anticoagulants.[25] However, during the survey in France, other known mutations were also found, namely Tyr139Cys and Leu120Gln, and some unknown mutations without information on their phenotypic expression occurred.[20] In Germany, different mutations in the *Vkorc1* gene can be encountered (e.g. Ala26Thr, Ser79Phe, Tyr139Phe, Tyr139Cys), with known resistance effects for Tyr139Cys and Tyr139Phe.[26] Strain Tyr139Cys is resistant to warfarin, and the majority of rats are resistant to bromadiolone as well as to coumatetralyl: neither bromadiolone[27] nor difenacoum provides a sufficient level of control.[14] On the

Table 1. An overview of the expected and observed frequency distribution among tested *R. norvegicus* droppings

Genotype	Number expected	Number encountered (% of total)	Number encountered by general public (%)	Number encountered by pest controllers (%)
Wild type	118	127 (75)	45 (71)	82 (77)
Tyr139Cys heterozygous	37	24 (14)	10 (16)	14 (13)
Tyr139Cys homozygous	2	10 (6)	2 (3)	8 (8)
Tyr139Phe heterozygous	9	5 (3)	4 (6)	1 (1)
Tyr139Phe homozygous	0	3 (2)	2 (2)	1 (1)
Total		169	63	106

Table 2. An overview of the efficacy of the different active substances. The table is partly based on the work of the Rodenticide Resistance Action Group[23] (× = active substance has lost its efficacy for this resistance mutation; +/− = active substance can be effective, but is not suitable for complete pest control; √ = active substance is expected to be effective for this resistance mutation; ? = efficacy is unknown)

	Resistance mutation							
Active substance	Tyr139Phe[a]	Tyr139Cys[a]	Tyr139Ser	Leu128Gln	Leu120Gln	References	Available on the Dutch market?	Reduced efficacy proven in the Netherlands
Warfarin	×	×	×	×	×	2, 4, 8, 17, 21, 22, 23	No	Yes
Chlorophacinone	×	×	×	×	×	23	No, recently retracted	Yes
Coumatetralyl	×	×	×	×	×	20, 23, 27	No	No
Difenacoum	+/−	+/−	√	√	+/−/×[b]	23	Yes	Yes
Bromadiolone	×	×	√	√	+/−/×[b]	21, 23, 27	Yes	Yes
Brodifacoum	√	√	√	√	√	23, 24	Yes	No
Flocoumafen	√	√	√	√	√	23	Yes	No
Difethialone	?	?	?	?	?		Yes	No

[a] Resistance mutation encountered in *R. norvegicus* in the Netherlands.
[b] Differs by region in the United Kingdom.

other hand, brodifacoum has been found to be fully effective against *R. norvegicus* with the Tyr139Cys mutation in Germany.[24]

Resistance among *R. norvegicus* may not only lead to more problems concerning the transfer of (zoonotic) pathogens, infrastructural damage and food spoilage but also be a threat to non-target species (especially predators) through secondary exposure. A recent study showed that the accumulation of chlorophacinone is the same in resistant rats as in susceptible rats, but, because survival times differ, non-target species (especially predators) may be more at risk.[28]

A good knowledge of the occurrence and distribution of rodenticide resistance is a prerequisite for proper application of IPM. The use of rodenticides is a final step in rodent management, if all other options fail. It is important that their efficacy remains or that new and better active substances are developed to control rodent damage.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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