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VERTEBRATE PESTS UNIT
UNIVERSITY OF READING

Anticoagulant Resistance in Rats and Mice in the UK – Summary Report with new data for 2019-20

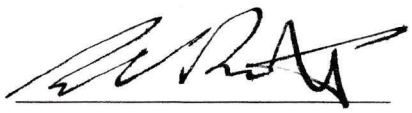
Report from the Campaign for Responsible Rodenticide Use (CRRU) UK for the Government Oversight Group

Test Facility
The Vertebrate Pests Unit
School of Biological Sciences
The University of Reading
Whiteknights
Reading RG6 6AJ, UK

Sponsor
Campaign for Responsible Rodenticide UK
c/o Killgerm Chemicals
Wakefield Road, Ossett
West Yorkshire
WF5 9AJ

Report prepared by:

Alan Buckle, Clare Jones, Montse Talavera and Colin Prescott

Dr C.V. Prescott  Date: 6th October 2020

Honorary Research Fellow

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SUMMARY

1. New resistance data are presented for tissue samples from Norway rats (*Rattus norvegicus*) and house mice (*Mus musculus*) collected in the period September 2019 to February 2020. Coronavirus restrictions at the University of Reading prevented laboratory work after that date. Once again, efforts were made to obtain samples in geographical areas in the UK from which none had been collected in the past.
2. A total of 54 Norway rat tissue samples were analysed, among which 14 were anticoagulant-susceptible and 40 carried one or more of five different resistance mutations (Y139S, Y139C, Y139F, L120Q, L128Q), in either homozygous or heterozygous form. Therefore the prevalence of anticoagulant resistance in this Norway rat sample was 74.1%.
3. For the first time more rats were found to carry the Y139C resistance mutation than the widespread L120Q mutation. This may be because fewer samples were submitted and sequenced from the large and well-known L120Q focus. The observation from previous years was repeated in that resistant rats were again found in places which would not have been expected from prior knowledge of resistance foci. For example, Y139C was found for the first time on the coast of West Sussex. Rats carrying the Y139S mutation (i.e. 'Welsh' resistance) were again recorded from North Yorkshire, far outside the original Welsh focus, at a greater frequency than previously, and the focus had apparently spread into County Durham.
4. These 'break-out' foci, and the increasing geographical spread of existing foci, have resulted in a phenomenon not previously reported for Norway rats in England, that of 'hybrid resistance'. This is where a single individual carries more than one resistance mutation. A surprising 20% of resistant rats carried two different mutations in this limited sample. This is the result of previously distinct resistance foci meeting, merging and interbreeding. The impact of this new phenomenon of hybrid resistance on our ability to manage resistant rodents in the future is discussed.
5. Only six house mouse tissue samples were submitted for analysis. Among these five (83.3%) carried one or more resistance mutations. Although the total number of records for house mouse is small, both for the year reported here and for the accumulated total for all years, these continue to show the wide extent of house mouse resistance to anticoagulants across the UK. Therefore, attention is again drawn to the situation in which permanent anticoagulant baiting is the predominant method for the control of the house mouse among professional pest control practitioners. Yet only the widely resisted difenacoum and bromadiolone active substances are permitted for use in permanent baiting.

1. Introduction

Previous reports produced for the Campaign for Responsible Rodenticide Use (CRRU UK) on the status of anticoagulant resistance among Norway rats (*Rattus norvegicus*) and house mice (*Mus musculus*) in the UK have presented background information on resistance mutations, explained resistance testing methodologies and provided information on the occurrence and geographical distribution of resistance (see Prescott *et al*., 2017 and 2018; Jones *et al*. 2019). This previously-presented information will not be reproduced in this report; rather a summary is provided of new information that has been obtained since the last report was prepared as the result of genomic resistance testing conducted at the University of Reading and funded by the Rodenticide Resistance Action Committee of CropLife International (http://www.rrac.info/).

This report has been prepared for CRRU in response to a requirement of the Health and Safety Executive (HSE) and the Government Oversight Group (GOG) to provide resistance monitoring information on an annual basis to support their evaluation of the progress of the UK Rodenticide Stewardship Regime (HSE, 2019) under the heading "Competent Workforce".

2. Materials and Methods

2.1 Origins of samples

The tissue samples analysed for genetical mutations were either submitted by pest control technicians or were collected after trapping by staff of the Vertebrate Pests Unit (VPU) at the University of Reading. Thus, samples were generally received from areas in which technicians had experienced difficulties in obtaining effective control with anticoagulants, possibly because of resistance or, in the case of VPU sampling, were taken from the borders of known resistance areas in an attempt to identify their boundaries.

During 2019 and 2020 additional effort was expended in obtaining samples from areas of the UK from which samples had not previously been received. This was continued in the present sampling period. The maps presented in previous reports had shown that samples have not been obtained, for example, from a large area in the centre of the country, including many counties of the Midlands. This area is of particular interest because, from the very few samples that have been received, there appears to be a low incidence of anticoagulant resistance among Norway rats. Consequently, calls were put out in the magazines serving the UK professional pest control community asking for samples from these areas (see for example Jones and Talavera, 2019; https://www.thinkwildlife.org/free-tests-and-new-guide-tackle-spread-of-resistant-rats/). These efforts have been rewarded with more samples obtained from areas not previously studied.

2.2 Methods of DNA analysis

As in the previous studies described above, genetical material was obtained from the field in the form of either tail tip samples or fresh droppings. Where possible, samples were placed in tubes containing 80% alcohol and then stored at -20°C as quickly as possible. Some unfrozen samples were shipped to the laboratory using a courier service, surface mail or by hand delivery, and were frozen on receipt.

Genomic DNA was extracted using the Qiagen DNeasy tissue extraction kit following the manufacturer's recommendations (Qiagen Ltd., Crawley, West Sussex, UK). Briefly, a small quantity of tissue (approximately 3mm x 2mm x 2mm) was shaved from each tail using a sterile sharp razor blade, and then placed in a 1.5ml microtube. Pre-warmed extraction buffer ATL (180 µl) was added, followed by 20 µl of proteinase K. The mixture was vortexed and incubated at 55˚C on a rocking platform overnight (approx. 17 h). Genomic DNA was then purified and eluted from spin-purification columns in 80 µl of elution buffer and the quality and yield were assessed spectrophotometrically using a nano-drop instrument.

The three exons of the VKORC1 gene, designated 1, 2 and 3, were amplified by PCR following the methodology of Rost et al. (2004). PCR products were purified using the QIAquick PCR purification kit (Qiagen Ltd., Crawley, West Sussex, UK). Product samples (3.5µl) were then sequenced with BigDye version 3.1 terminator chemistry (ABI) on a 9700 ABI thermal cycler, and the terminated products were resolved on an ABI 3130XL capillary sequencer. The sequence trace files were visually analysed and any ambiguous bases were edited using the DNASTAR Lasergene software. The sequence alignments were compiled using ClustalW2.

A list of the VKORC1 mutations found in Norway rats and house mice in the UK is shown in Table 1.

Table 1. VKORC1 mutations in Norway rats (NR) and House mouse (HM) in UK. From: Pelz *et al*., 2005; Rost *et al*., 2009; Prescott *et al*., 2010; Pelz and Prescott, 2015;Clarke and Prescott, 2015 unpublished report. Major resistance mutations with known practical consequences shown in bold.

Species	**Mutation**	**Abbreviations**	**Where present**
NR	**Leucine128Glutamine**	**L128Q**†	**Central Southern Scotland, Yorkshire, Lancashire**
NR	**Tyrosine139Serine**	**Y139S**†	**Anglo-Welsh border**
NR	**Leucine120Glutamine**	**L120Q**†	**Hampshire, Berkshire, Essex, Norfolk and elsewhere**
NR	**Tyrosine139Cysteine**	**Y139C**†	**Gloucestershire, Norfolk, Lincolnshire, Yorkshire, SW Scotland and elsewhere**
NR	**Tyrosine139Phenylalanine**	**Y139F**†	**Kent, Sussex, Norfolk, Suffolk**
NR	Argenine33Proline	R33P‡	Nottinghamshire
NR	Phenylalanin63Cysteine	F63C*	Cambridge/Essex
NR	Tyrosine39Asparagine	Y39N*	Cambridge/Essex
NR	Alanine26Threonine	A26T#	Cambridge/Essex
HM	**Tyrosine139Cysteine**	**Y139C**†	**Reading**
HM	**Leucine128Serine**	**L128S**†	**Cambridge**

† Known either from field experiments and/or field experience to have a significant practical effect on anticoagulant efficacy

‡ Known from laboratory experiments to confer warfarin resistance

* Shown in laboratory experiments to have a significant impact on protein function

Unlikely to confer any significant degree of resistance

2.3 The Rodenticide Resistance Action Committee (RRAC) interactive global resistance map

The results from this study were provided to the funding body, the Brussels-based RRAC of CropLife International (http://www.rrac.info/). The results are collated with those obtained from other global studies and presented in an interactive form on the RRAC web-site. The maps available (see example for the UK at: http://guide.rrac.info/resistance-maps/united-kingdom/) use Google 'heatmap' technology to ascribe different weightings to records depending on the numbers of positive samples and the frequencies of their closest neighbours. Users of the maps are able to scroll in to find their own location, that of the nearest confirmed incidence of anticoagulant resistance, the mutation of that record and to obtain advice about the correct use of anticoagulants in the area. It is anticipated that this scheme will help pest control practitioners to make informed choices about which anticoagulant active substance to use and will support a 'competent workforce'.

3. Results

3.1 Norway rats

During the period September 2019 and February 2020 a total of 54 Norway rat tissue samples was received that were capable of analysis using the gene sequencing technique. Six samples were incapable of being sequenced. This number of samples was regrettably fewer than in previous years because restrictions implemented by the University of Reading to protect staff and students during the coronavirus outbreak prevented any laboratory work being done after February 2020.

Among these 54 samples, 40 were found to possess one or more of the five known Norway rat resistance single nucleotide polymorphisms (SNPs) and 14 were found to be susceptible animals (Table 2). Hence, 74.1% of the samples received possessed one of the resistance mutations, in either their homozygous or heterozygous form.

Table 2. The numbers of Norway rats tissue samples received and analysed and their status of resistance or susceptibility. A total of six samples could not be sequenced. (See Table 1 for further explanations of the different resistance mutations.)

Resistance Mutation	Homozygous	Heterozygous	Total
L120Q	2	5	7
L128Q	7	1	8
Y139C	2	10	12
Y139F	2	0	2
Y139S	1	2	3
L128Q and Y139C	0	4*	4
L128Q and Y139S	0	1*	1
L120Q and Y139C	0	3*	3
Susceptible	-	-	14
Total	**28**	**26**	**54**

*These eight animals were heterozygous for each of two the resistance mutations.

The geographical origins of these new samples are shown in Figure 1. The discovery of several new resistance foci and the further apparent spread of others are revealed when a comparison is made of these findings and those published in the previous report (Jones *et al*., 2019). Of course, as before, it is impossible to determine whether these are newly-developed resistance foci or have been present undetected for some time.

The proliferation of foci of the Y139C focus continues with a new occurrence in West Sussex near Shoreham. The nearest previous record of this mutation was in south-east Surrey. Once again, a focus of the Y139C SNP has been discovered in association with a maritime/harbour setting, indicating a possible link with shipping and transport from the continent where this mutation predominates.

The spread of Welsh resistant Y139S rats from their original focus on the Anglo-Welsh border was reported for the first time in the 2019 report (Jones *et al*., 2019). More Y139S rats were

again found in North Yorkshire in the 2019-20 sample and another heterozygous individual was located over the border in County Durham. If the findings in North Yorkshire and County Durham indicate a contiguous focus, it suggests a much larger area infested with Y139S rats than previously thought and the likelihood that this resistance focus was present for some time before it was discovered.

When the boundaries of previously isolated resistance foci expand and eventually merge, because of the continued use of ineffective rodenticides, it is to be expected that rats carrying different resistance SNPs will meet and interbreed. To date, this survey had identified only one individual Norway rat which possessed two different resistance SNPs, an animal found near Edinburgh carrying the L128Q and L120Q mutations. We use the new term "hybrid resistance" to describe this apparently rare phenomenon. However, in the relatively small 2019-20 sample we have found no fewer than eight rats with hybrid resistance. This is a surprising and troubling increase over the period of just one year.

Three different hybrids were found (L120Q/Y139C, L128Q/Y139C, L128Q/Y139S) in many widely separate locations, although all explicable by nearby documented foci of the separate SNPs. L120Q and Y139C are perhaps the most severe of the resistance SNPs found anywhere and animals carrying this hybrid combination were found in Greater Manchester, East Sussex and Dorset. Individuals with both L128Q and Y139C were found widely across the north of England, in Greater Manchester, East Yorkshire, West Yorkshire and County Durham. Finally, an animal carrying the L128Q and Y139S mutations was found in Merseyside. This brings to four the number of different resistance hybrids now found among Norway rats in the UK. The consequences for rodent pest management of the widespread emergence of hybrid resistance will be discussed later in this report.

Once again efforts were made better to delineate the contiguous area of putative anticoagulant susceptibility that appears to exist in the Midlands. Consequently, susceptible animals were found in Northamptonshire, Lincolnshire and Bedfordshire. These counties can now be added to others in the Midlands and central southern England, namely West Midlands, Leicestershire, Nottinghamshire and Hertfordshire where, to date, no resistant Norway rats have been found. However, it must be emphasised that only very small sample sizes are involved (in some cases only a single animal), and further confirmatory sampling will be conducted if possible.

The map shown in Figure 2 gives all accumulated data on the distribution of anticoagulant resistance for Norway rats in the UK and includes the 2019-2020 data.

Figure 1. Map showing the geographical locations of Norway rat tissue samples submitted to the Vertebrate Pests Unit in the period September 2019 to February 2020 and their resistance status.

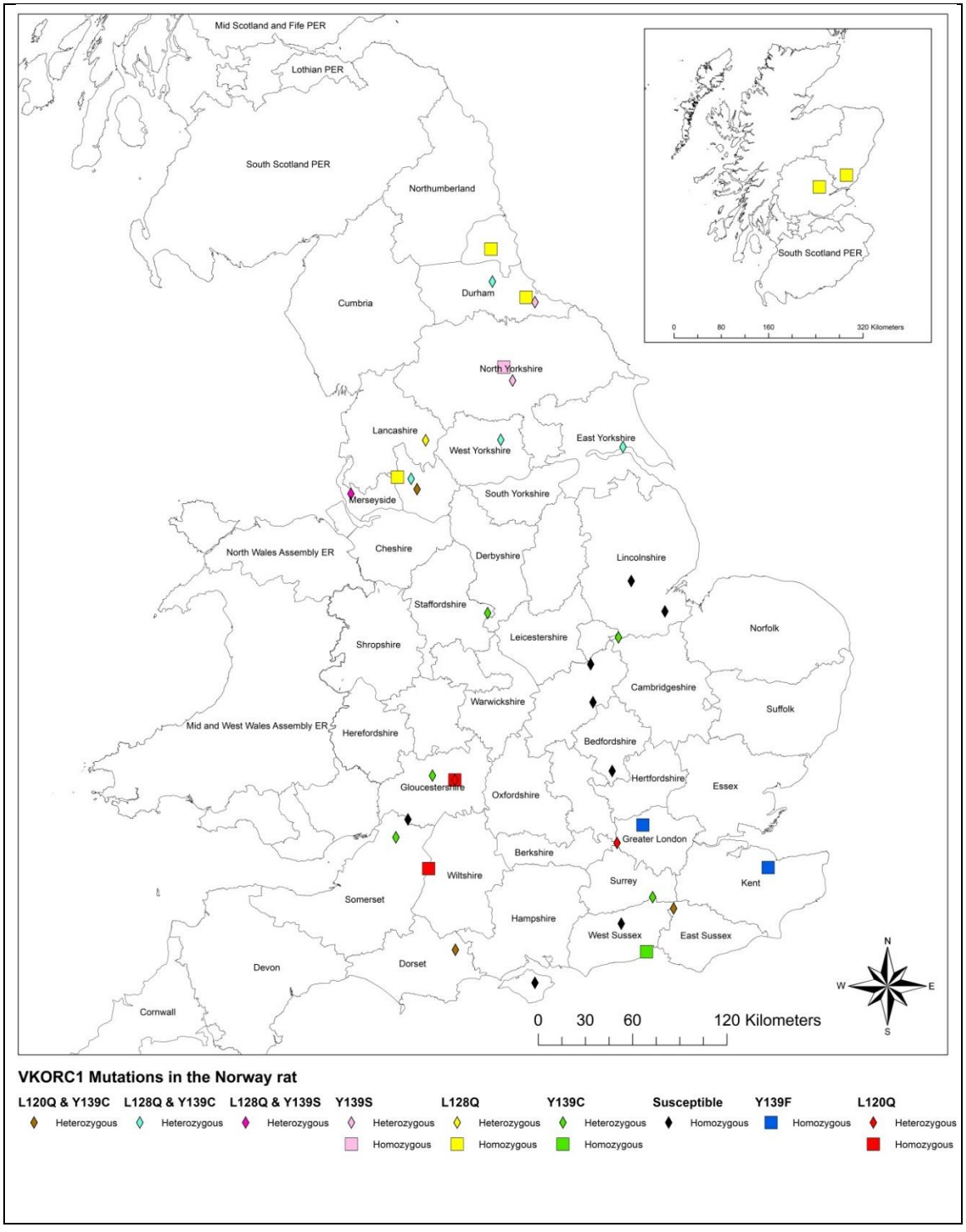
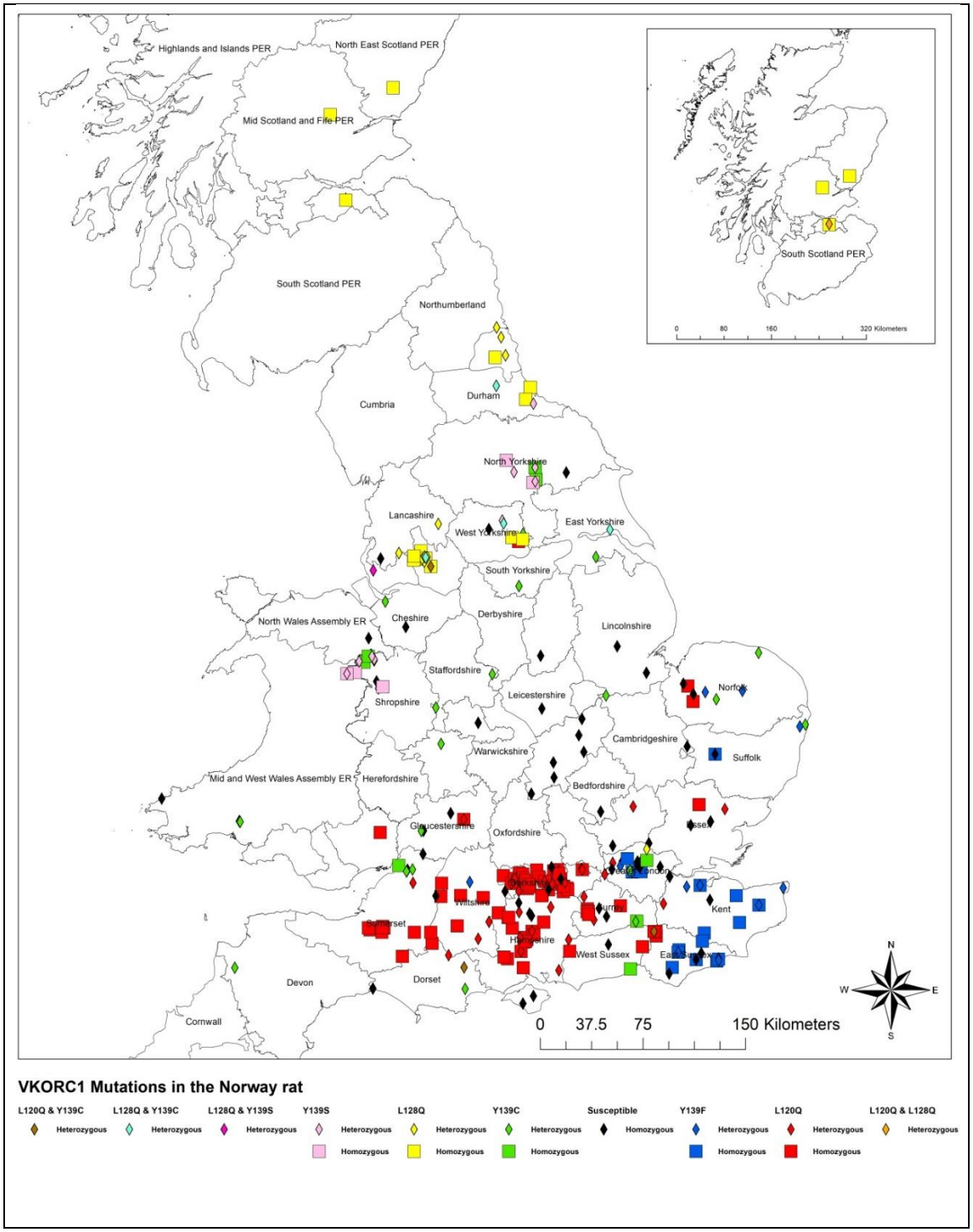


Figure 2. Map showing the geographical locations of all Norway rat tissue samples submitted to the Vertebrate Pests Unit to date and their resistance status.



3.2 House mice

The results from the analysis of a total of 6 house mouse tissue samples submitted in the period September 2019 to September 2020 are shown in Table 3. Among six samples examined, one carried the fully susceptible genotype. Table 1 shows that one or other of the two resistance mutations commonly found among house mice in the UK were present in five out of the six animals. L128S was found in homozygous form in 3 animals and Y139C in homozygous form in another. One individual carried both mutations, each heterozygous.

Table 3. The numbers of house mouse tissue samples received and analysed and their status of resistance or susceptibility. (See Table 1 for further explanations of the different resistance mutations.)

Mutation	**Homozygous**	**Heterozygous**	**Total**
L128S	3	0	3
Y139C	1	0	1
L128S and Y139C	0	1*	1
Susceptible	1	0	1
Total samples	**5**	**1**	**6**

*This animals was heterozygous for each of two the resistance mutations.

The geographical distribution of the samples analysed during September 2019 to February 2020 and reported here is shown in Figure 3. The combined data for all years is shown in Figure 4. Resistance distribution data for house mice recorded in the previous reports (Prescott *et al*., 2017 and 2018; Jones *et al*., 2019) were mainly from Greater London and the south-east of England. The samples now reported were again much more widely dispersed and demonstrate conclusively the extent of anticoagulant resistance in UK house mice.

Figure 3. Map showing the geographical locations of house mouse tissue samples submitted to the Vertebrate Pests Unit in the period September 2019 to February 2020 and their resistance status.

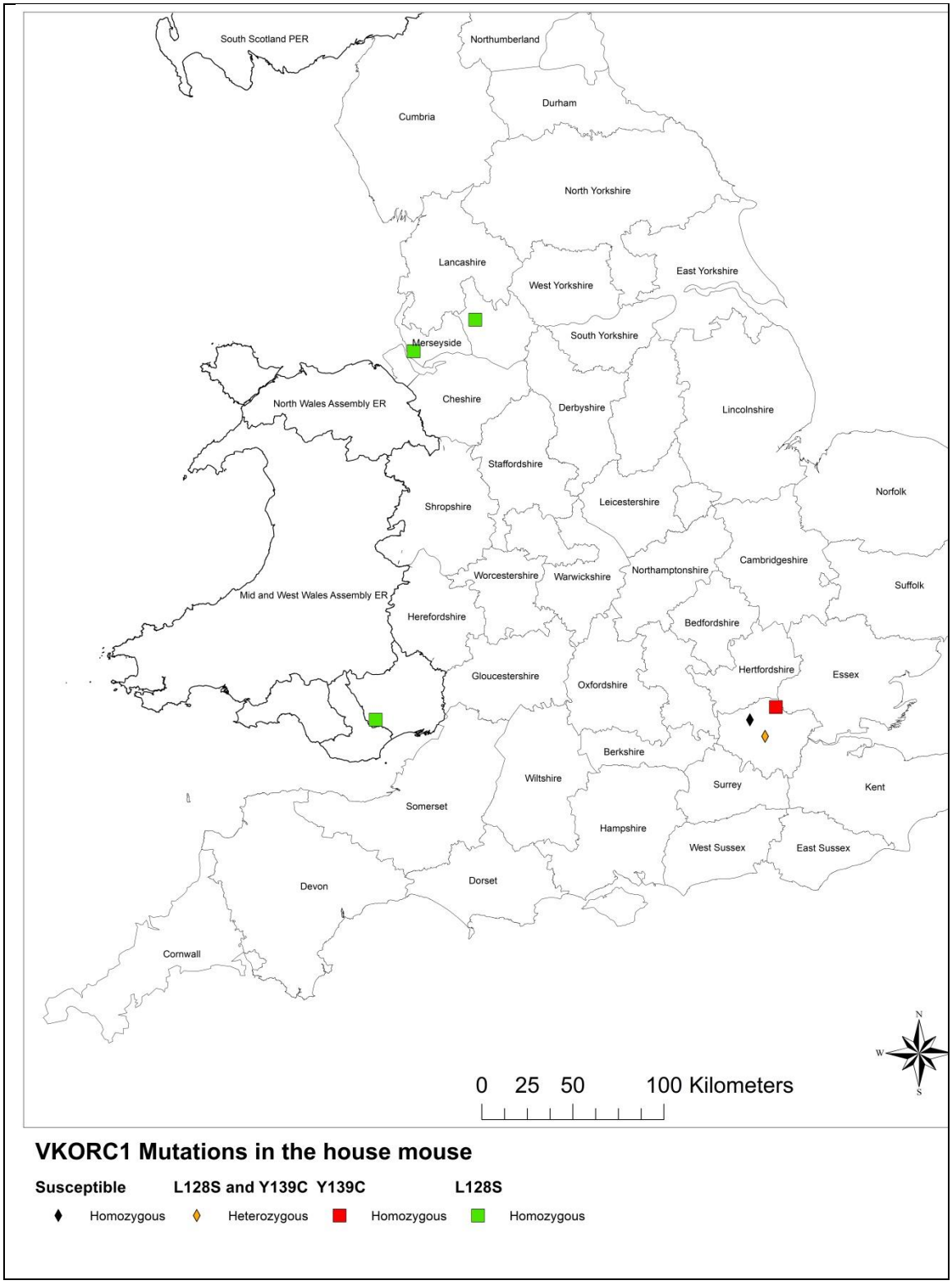
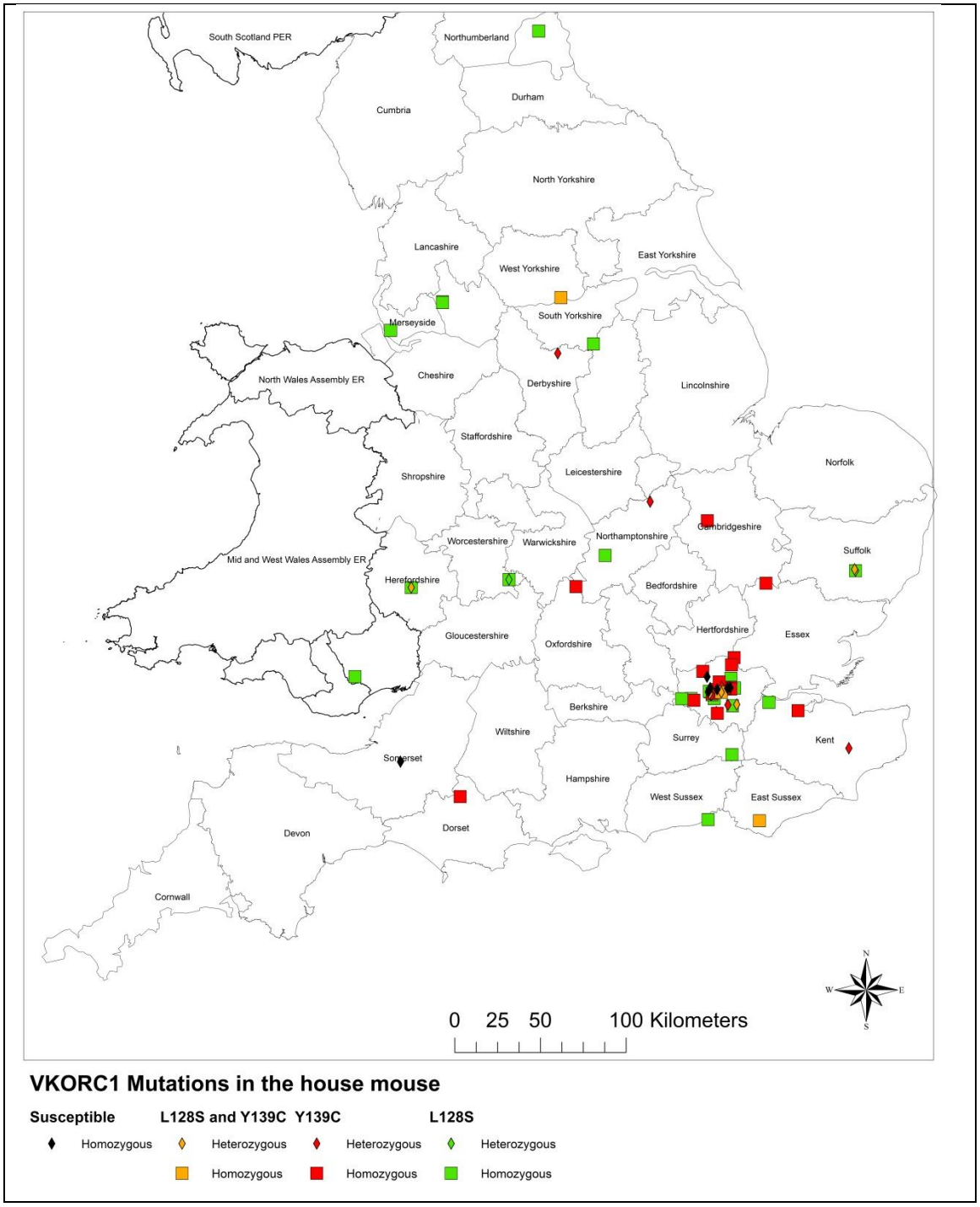


Figure 4. Map showing all available data on the occurrence of resistance mutations among house mice in the UK.



The relative few house mouse tissue samples submitted in the period covered by this report add little information to that already obtained and shown in Figure 4. The L128S mutation appears to be very widely distributed across much of England, from Tyneside in the north-east to the Channel coast of East and West Sussex. New records in the 2019-20 samples for L128S were found in Monmouthshire and Merseyside. The prevalence of resistance among house mouse in the London area was further emphasised with a single Y139C record and another hybrid resistant mouse carrying both L128S and Y139C. However, as before, we still lack data for the house mouse and many of the records are for either single animals or very small samples.

Earlier reports provided information on a total of 88 house mouse samples and these are now augmented by a further 6 samples. Among the previous 88 a total of 82 (93.2%) carried one or more resistance mutations. With the addition of the six samples reported here, five of them resistant, the prevalence of resistance in UK house mice is now 87 resistant individuals out of a total of 93 (93.5%).

4. Discussion

This report is the fourth in a series compiled for CRRU UK by the Vertebrate Pests Unit of the University of Reading to document the distribution and frequency of resistance to anticoagulants among Norway rats (*Rattus norvegicus*) and house mice (*Mus musculus*) in the UK. The sampling period, which comprised September 2019 to February 2020, was curtailed by restrictions implemented by the University of Reading which prevented laboratory work during the 'coronavirus lockdown'.

Among the 54 tissue samples of Norway rats received in the sampling period, 40 carried one or more of the known resistance SNPs (Table 1). This gives a frequency of Norway rat resistance in this sample of 74.1%. As stated in previous reports, this is unlikely to be representative of the UK Norway rat population as a whole, because samples are generally received from those who conduct rodent pest management, are experiencing some difficulty in obtaining full control of an infestation and suspect that resistance may be present. However, a further consideration that affects the percentage of the sample that is found resistant is the fact that some samples were not selected for DNA extraction and sequencing because they were taken from within 5 km of a known resistance focus.

The small sample size has limited the new information that can be provided in this report. However, some interesting observations are possible from these few records. The surprising finding of Y139S ('Welsh resistance') in North Yorkshire in last year's survey was repeated in the 2019-20 data. Indeed, the known scope of the focus was extended over a wider area of the county and across the border into County Durham (Figure 1). All the second-generation anticoagulants are considered to be effective against this SNP, although some doubt exists about the efficacy of bromadiolone (Buckle *et al*., 2007).

Two Norway rat samples were homozygous resistant for the severe L120Q mutation, one from the Wiltshire-Somerset border and the other from central Gloucestershire (Figure 1). These records add to our understanding of the western and northern spread of the large central-southern England focus of this SNP (Figure 2). Only the most potent second-generation anticoagulants brodifacoum, difethialone and flocoumafen are fully effective against Norway rats carrying this mutation. Field trials of bromadiolone, difenacoum and brodifacoum against L120Q rats in Hampshire and Berkshire have confirmed the partial or complete ineffectiveness of the two former active substances. However, brodifacoum offered effective control of L120Q-resistant rats, with considerably less active substance being emitted into the environment during treatments using that compound (Buckle *et al*., 2020). The trials, much delayed by the 'indoor only' restriction on the use of brodifacoum, demonstrated the benefits for both the environment and resistance management of the use of fully effective anticoagulant rodenticides against resistant rodents.

As in previous reports, records of Norway rats carrying the Y139C mutation were widely scattered. A single heterozygous resistant rat was found on the borders of Derbyshire and Staffordshire, a first for both counties and far removed from the nearest known occurrence of this SNP in Central Manchester. The mutation was also found on the border of Surrey and West Sussex, a known focus, but also for the first time on the south coast of Sussex, near Shoreham. This observation brings to three the number of different mutations known to be present in the counties of Sussex, Surrey and Kent. These SNPs, L120Q, Y139C and Y139F, are the most severe resistances in the Norway rat currently known. This may presage even greater difficulties

in conducting rat control in the south of England in the future than now exist. The occurrence of the Y139F mutation among rats in central London was again confirmed.

A remarkable finding in the data for the period 2019-2020 is the apparently sudden emergence of Norway rats possessing two different resistance mutations – i.e. 'hybrid resistance'. Because resistance SNPs may occur at several gene loci in rodents, in particular those at positions 120, 128 and 139 of exon 3 of chromosome 1 in Norway rats, it is possible for animals to carry more than one resistance mutation. Up to this point, hybrid resistance was found in the UK only in a single L120Q/L128Q hybrid from Scotland. However, in our sample of 54 rats we report no fewer than eight that are hybrid-resistant. This is likely to have occurred as the result of resistance foci, which were previously discrete, meeting, merging and interbreeding. Previous reports in this series have documented the apparent spread of resistance across the UK. This is the first time that evidence has been recorded of the widespread coalescence of previously discrete resistance foci. At first, individuals that result from resistance interbreeding would be expected to be heterozygous for the two SNPs concerned, the offspring inheriting one copy of each mutant gene from each resistant parent; and that is what we see among all eight hybrid-resistant rats in this sample. However, if hybrid-resistant rats become more common, they are likely to breed both among themselves and with other resistant individuals. Some of these offspring might then be expected to be homozygous for, perhaps, several resistance mutations. This phenomenon is the predictable consequence of a regulatory policy, in place for 30 years in the UK but nowhere else, in which the most efficacious resistance-breaking anticoagulants could not be used to control Norway rats (Buckle, 2013). What is less predictable is the future impact of hybrid resistance on rodent pest management and, consequently, public health in the UK.

Hybrid resistance might be most likely to occur in areas such as central southern England where the majority of rats in our samples are already homozygous for one resistance SNP. However, Pelz and Prescott (2015) summarised the pleiotropic costs of certain resistance mutations for the animals that carry them. In some cases, VKORC1 mutations result in higher dietary requirements for vitamin K, more so in homozygous animals than in those that are heterozygous, presumably because they have a detrimental effect on the action of the vitamin K epoxide reductase enzyme. It seems possible that hybrid resistance may not be viable with certain severe SNP combinations, such as L120Q and Y139F, because they will prevent vitamin K epoxide reductase from functioning properly. This may explain why we have not yet found hybrid resistance with these SNPs in south east England, although Norway rats that are homozygous for them are common and foci are in close proximity (Figure 1).

Our current knowledge of the practical impacts of the different resistance mutations depends on two lines of research. Firstly, many different anticoagulants have been applied within known resistance UK foci and their efficacy has been determined (see Buckle, 2013). In the second line of research, resistance factors have been derived from laboratory blood clotting response tests, both at the University of Reading and the Julius Kühn Institute at Münster in Germany, using methods developed at the University of Reading (Prescott *et al*, 2007); with the work funded in part by the Rodenticide Resistance Action Committee (RRAC) of CropLife International. Information from these studies on resistance factors for L120Q, Y139C and Y139F Norway rats and for L128S and Y139C house mice is provided at the RRAC website (see https://guide.rrac.info/aim-and-authors.html) and permits understanding of the relative severity of these resistance SNPs. However, both these lines of research provide information only on single resistance mutations. It seems unlikely that any hybrid-resistant individuals that are heterozygous for two mutations, such as all those reported here, would be more resistant to anticoagulants in practice than an individual that is homozygous for the most severe L120Q mutation, although this cannot be declared with certainty. However, the consequences for resistance management of a

Norway rat individual that is homozygous for more than one resistance SNPs is difficult to predict.

A total of 14 rats were found to be susceptible individuals, many of these were once more reported in the counties of the Midlands. In addition to these susceptible rats, and additional 34 rats were heterozygous for one or more resistance SNPs, thus giving 65% of resistant rats with some susceptibility remaining in their genomes.

The very small sample of house mouse tissues that were submitted for testing in the period September 2019 to February 2020 does not provide substantially improved understanding of resistance in this species. As might be expected from previous data, susceptibility was not common among the house mice studied; only one of the six individuals was susceptible. Previously, we have reported that London is a 'hotspot' for anticoagulant resistance in house mice; animals that are homozygous for both the L128S and Y139C mutations being prevalent in the capital (Jones *et al*., 2019). Three of the six samples submitted were from London, one was homozygous Y139C, one was susceptible and the third was hybrid-resistant L128S/Y139C. Anecdotal reports of the failure of baits containing one of the most potent anticoagulants, difethialone, to control mice in the centre of London could be attributable to these hybrid-resistant animals. More resistance testing and laboratory evaluation of the hybrid-resistant mice strain would be required to confirm this.

These few reports again draw attention to a regulatory anomaly. The predominant method for the management of house mice in all commercial and (especially) in food storage/preparation/sale premises is the use of permanent tamper-resistant mouse bait boxes containing anticoagulant baits. However, we draw attention to rules on permanent baiting, embodied in current product labels, which only permit the widely resisted bromadiolone and difenacoum to be used in permanent baiting programmes (CRRU, 2019). It seems contrary that we have just emerged from the virtual 'ban' on the use of effective resistance-breaking anticoagulants against Norway rats, which has undoubtedly contributed to the massive spread of resistant Norway rats in the UK, and now find ourselves in a similar contrary regulatory position with House mice. If this situation continues it seems likely that the already severe situation of house mouse resistance in the UK will further deteriorate.

It is with regret that we confirm the closure of the Vertebrate Pests Unit of the University of Reading and the fact that this will be the last report of this kind written by its staff. Continuity of resistance UK monitoring will be provided to CRRU from the laboratories of the Animal and Plant Health Agency (APHA) at Weybridge in Surrey, under the direction of Dr Richard Ellis. Those wishing to submit rodent tissue samples for DNA extraction and sequencing, for resistance characterisation, should visit the CRRU website for further information and advice (https://www.thinkwildlife.org/about-crru-uk/).

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