The Current Status of Anticoagulant Resistance in Rats and Mice in the UK

Report from the Rodenticide Resistance Action Group of the United Kingdom to the Health and Safety Executive

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SUMMARY

Introduction: Resistance to anticoagulants in Norway rats (*Rattus norvegicus*) and house mice (*Mus domesticus*) has been studied in the UK since the early 1960s. In no other country in the world is the understanding the of resistance phenomena so extensive and profound. Almost every aspect of resistance in the key rodent target species has been examined in laboratory and field trials and results obtained by independent researchers have been published. It is the principal purpose of this document to present a short synopsis of this information. More recently, however, the development of genetical techniques has provided a definitive means of detection of resistant genotypes among pest rodent populations. Preliminary information from a number of such surveys will also be presented.

Resistance in Norway rats: A total of nine different anticoagulant resistance mutations (single nucleotide polymorphisms or SNPs) are found among Norway rats in the UK. In no other country worldwide are present so many different forms of Norway rat resistance. Among these nine SNPs, five are known to confer on rats that carry them a significant degree of resistance to anticoagulant rodenticides. These mutations are: L128Q, Y139S, L120Q, Y139C and Y139F. The latter three mutations confer, to varying degrees, practical resistance to bromadiolone and difenacoum, the two second-generation anticoagulants in predominant use in the UK. It is the recommendation of RRAG that bromadiolone and difenacoum should not be used against rats carrying the L120Q, Y139C and Y139F mutations because this will promote the spread of resistance and jeopardise the long-term efficacy of anticoagulants. Brodifacoum, flocoumafen and difethialone are effective against these three genotypes but cannot presently be used because of the regulatory restriction that they can only be applied against rats that are living and feeding predominantly indoors.

Our understanding of the geographical distribution of Norway rat resistance in incomplete but is rapidly increasing. In particular, the mapping of the focus of L120Q Norway rat resistance in central-southern England by DNA sequencing is well advanced. We now know that rats carrying this resistance mutation are present across a large part of the counties of Hampshire, Berkshire and Wiltshire, and the resistance spreads into Avon, Oxfordshire and Surrey. It is also found, perhaps as outlier foci, in south-west Scotland and East Sussex. L120Q is currently the most severe form of anticoagulant resistance found in Norway rats and is prevalent over a considerable part of central-southern England. A second form of advanced Norway rat resistance is conferred by the Y139C mutation. This is noteworthy because it occurs in at least four different foci that are widely geographically dispersed, namely in Dumfries and Galloway, Gloucestershire, Yorkshire and Norfolk. Once again, bromadiolone and difenacoum are not recommended for use against rats carrying this genotype and a concern of RRAG is that continued applications of resisted active substances may result in Y139C becoming more or less ubiquitous across much of the UK. Another type of advanced resistance, the Y139F mutation, is present in Kent and Sussex. This means that Norway rats, carrying some degree of resistance to bromadiolone and difenacoum, are now found from the south coast of Kent, west into the city of Bristol, to Yorkshire in the north-east and to the south-west of Scotland. This difficult situation can only deteriorate further where these three genotypes exist and resisted anticoagulants are predominantly used against them.

Resistance in house mice: House mouse is not so well understood but the presence in the UK of two resistant genotypes, L128S and Y139C, is confirmed. House mice are naturally tolerant to anticoagulants and such is the nature of this tolerance, and the presence of genetical resistance, that house mice resistant to the first-generation anticoagulants are considered to be widespread in the UK. Consequently, baits containing warfarin, sodium warfarin,

chlorophacinone and coumatetralyl are not approved for use against mice. This regulatory position is endorsed by RRAG. Baits containing brodifacoum, flocoumafen and difethialone are effective against house mice and may be applied in practice because house mouse infestations are predominantly indoors. There are some reports of resistance among mice in some areas to the second-generation anticoagulant bromadiolone, while difenacoum remains largely efficacious.

Alternatives to anticoagulants: The use of habitat manipulation, that is the removal of harbourage, denial of the availability of food and the prevention of ingress to structures, is an essential component of sustainable rodent pest management. All are of importance in the management of resistant rodents and have the advantage of not selecting for resistant genotypes. The use of these techniques may be particularly valuable in preventing the build-up of rat infestations. However, none can be used to remove any sizeable extant rat infestation and for practical reasons their use against house mice is problematic. Few alternative chemical interventions are available in the European Union because of the removal from the market of zinc phosphide, calciferol and bromethalin. Our virtual complete reliance on the use of anticoagulants for the chemical control of rodents in the UK, and more widely in the EU, calls for improved schemes for resistance management. Of course, these might involve the use of alternatives to anticoagulant rodenticides. Also important is an increasing knowledge of the distribution of resistance mutations in rats and mice and the use of only fully effective anticoagulants against them.

1. Introduction

The first case of anticoagulant resistance in Norway rats (*Rattus norvegicus*) was discovered in the United Kingdom in 1958 (Boyle, 1960) and research into this phenomenon has continued ever since. Indeed, UK scientists, first in the government science service and more latterly at several UK universities, have lead the way in research on almost all aspects of anticoagulant resistance. This has resulted in a vast published literature on this subject. Useful reviews have been produced by Greaves (1994) and Buckle (2006, 2012).

For many years our knowledge of the distribution of anticoagulant resistance in the UK was hampered by cumbersome, expensive and inhumane resistance detection measures, which involved the capture and maintenance of wild rodents in the laboratory for long periods (EPPO, 1995). However, new DNA sequencing technology was developed recently by scientists in Germany (Pelz et al. 2005; Rost et al., 2009) and this has revolutionised the detection of anticoagulant resistant rodent genotypes. The detection of a resistance mutation using DNA sequencing does not, however, permit any conclusions to be drawn about the impact of the possession of the mutation on our ability to control rodents that carry it. In order to do this we need to consider an array of additional information obtained from mechanistic studies. Fortunately, in the UK this information has already been provided by research conducted over a period of fifty years.

The patterns of use approved for anticoagulant rodenticide baits in the UK are influenced by a variety of factors. Of course, among these efficacy is a prime consideration. However, the ability of users to apply the most potent anticoagulants has been restricted by a long-term regulatory requirement that brodifacoum and flocoumafen, and more latterly difethialone, may be used only against rodent infestations that are living and feeding predominantly indoors. Consequently, these active substances are not used in the management of anticoagulant resistance Norway rat infestations because these, almost without exception, contain population components living outdoors. This restriction is applied because of regulatory concerns that these active substances may have a greater adverse impact on non-target wildlife than, for example, the less potent anticoagulants bromadiolone and difenacoum. The effects of this restriction on anticoagulant resistance management will be discussed in this report but it is beyond the scope of the document to examine the ecotoxicological justification for it.

The purpose of this report, therefore, is to bring together information currently available to the Rodenticide Resistance Action Group on the distribution and practical impacts of anticoagulant resistance in rodents in the UK.

2. Resistance Definitions

A document intended to provide information on pesticide resistance relies on a clear understanding of the definition of terms. The term 'resistance' has several different interpretations when referring to rodent individuals and populations that are capable of surviving applications of rodenticides. The guideline from the European and Mediterranean Plant Protection Organization (EPPO, 1995) describes anticoagulant resistance in rodents as an inherited trait which is expressed in the phenotype so that "rodenticide resistant rodents should be able to survive doses of rodenticide that would kill 'normal' or 'susceptible' conspecifics". This definition is perhaps adequate for first generation anticoagulants, where resistance factors are very high, but in many instances it is not appropriate for second generation anticoagulants where resistance factors may be low, and levels of resistance may be identified that have no impact on practical rodent control. For example it might be that, although some individuals are able to survive doses of anticoagulant that would kill fully susceptible animals, populations could still be controlled in practice.

Greaves (1994) proposed the following definition for rodent resistance to anticoagulants that includes a practical consideration:

> "Anticoagulant resistance is a major loss of efficacy in practical conditions where the anticoagulant has been applied correctly, the loss in efficacy being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant."

To comply with this definition genetic, toxicological and operational information is required and this has therefore been referred to as '*practical resistance*' (Buckle, 2006). Low-level resistance, which may be detected by resistance testing methods such as laboratory feeding tests and blood clotting response (BCR) tests (EPPO, 1995), but which has no obvious practical effect on the outcome of rodenticide applications, is called '*technical resistance'*. The term 'low-grade resistance' has also been used in situations where resistance is considered largely inconsequential in terms of the outcome of practical control treatments (Gill *et al*., 1992; Greaves, 1994). The definition of Greaves (1994) is widely adopted (*e.g.* RRAC, 2003; RRAG, 2010, 2012) and is applied in this document to describe 'practical resistance'.

Even when the term practical resistance is applied to either individual rodents or rodent populations, it must be understood that resistance is quantitative rather than qualitative. Two rodent populations may both be resistant but one may be more resistant to a particular anticoagulant rodenticide than the other. The strength, or degree, of resistance possessed by rodent populations is normally expressed as a '*resistance factor*' or '*resistance ratio'*. This is the resultant, at a specified lethal or effective dose percentile (usually the LD/ED_{50} or the LD/ED_{99}), of the measured susceptibility of a strain of resistant rodent divided by the equivalent measure for a strain of susceptible animals. The resistance ratio may be derived either from measurements of the oral toxicity of anticoagulants (*e.g.* Greaves and Cullen-Ayres, 1988) or from BCR tests (Prescott et al., 2007).

Normally only a portion of individuals in a population possesses a resistance genotype and the term '*incidence of resistance*' refers to the proportion or percentage of animals within a population that carry the resistance mutation. Historically, the incidence of resistance has not taken into consideration the genotype of the resistant animals, but with recent developments in

molecular methodologies this is changing, and it is now possible to consider the proportion of individuals that are homozygous and heterozygous for a resistance gene (Clarke, 2012).

3. Resistance Testing

Conventional techniques for testing rodents for resistance were reviewed by EPPO (1995) and therefore will receive only superficial attention in this report.

Early anticoagulant resistance testing methods relied on laboratory no-choice feeding tests in which bait, containing the normally-used concentration of the active ingredient under investigation, was offered to groups of individually-caged rodents for different numbers of days (WHO, 1982). Baseline tests were conducted for each rodent species using susceptible strains and the resulting dose/response lines were subject to probit analysis to obtain lethal dose percentiles, expressed in terms of the numbers of days of continuous feeding required to kill different percentiles of susceptible populations. Individuals that survived the lethal feeding period required to kill 99% of susceptible animals (i.e. the LFP_{99}) were considered resistant. Although conducted in the laboratory, these tests could be interpreted in terms of the practical outcome of rodent control treatments because resistance was defined in terms of the duration of feeding on commercially-used baits required to kill a high percentage of a rodent population.

A drawback with lethal feeding period tests is that they are time-consuming and, because mortality as the end-point, are questionable on grounds of humanness (Prescott and Buckle, 2000). Alternative tests were designed to overcome these difficulties using the blood clotting response (BCR). In BCR tests, the ability of the blood to clot in the presence of measured doses of an anticoagulant is determined in susceptible animals. Animals are said to be resistant when their blood continues to clot when a dose of anticoagulant is administered that would prevent clotting in susceptibles. For early BCR tests, Norway rat baseline data are available for a number of anticoagulant compounds (e.g. Gill *et al*., 1993; Prescott and Buckle, 2000). More recently, Norway rat and House mouse BCR base-line data have been made available for many first- and all second-generation anticoagulants by Industry's Rodenticide Resistance Action Committee using a novel and consistent BCR test methodology developed at the University of Reading (Prescott et al., 2007). A major difficulty of the early BCR test method was relating resistance determined by these test methods to practical treatment outcome. The novel RRAC BCR test methodology has overcome this particular difficulty, by permitting the calculation of resistance ratios from the BCR test data.

Both LFP and BCR resistance tests require the capture of wild rodents for screening in the laboratory for resistance. This is costly and time-consuming and severely restricts our ability to monitor the development and distribution of resistance in a cost-effective way. However, new advances in our understanding of the genetics of anticoagulant resistance now appear to offer the promise of cheap and rapid tests for resistance that overcome these drawbacks. Recent work (Rost *et al*. 2004) has identified mutations in the gene coding for vitamin K_1 epoxide reductase in both Norway rats and House mice that are responsible for anticoagulant resistance in a number of resistance foci in Europe. This information has made it possible to develop molecular-biological techniques for the identification of mutant resistance genes in DNA extracted from small pieces of rodent tissue, and even from faecal pellets (Pelz, 2007). Such quick and cheap tests, for the first time, permit more detailed mapping of resistance foci which, in turn, will assist in the management of anticoagulant-resistant rodent infestations. However, the severity of resistance conferred by the different SNPs, and therefore their importance in terms of practical rodent pest management, still require interpretation using mechanistic studies.

4. Resistance Mechanisms

4.1 Introduction

It is beyond the scope of this report to provide a comprehensive explanation of current theories on the possible mechanisms of resistance. Only a brief summary is presented here to permit an understanding of the following sections.

To obtain an insight into the mechanisms of anticoagulant resistance an understanding of the process of mammalian blood clotting is necessary, in particular the role of vitamin K. Activated, or functional, blood clotting factors are required to bind calcium ions to provide a substrate for the formation of blood clots. The reduced form of vitamin K, hydroxyquinone, is a cofactor for the enzyme gamma-glutamyl carboxylase which catalyses the conversion of glutamate residues to gamma-carboxyglutamate (Gla), in the production of functional prothrombin (clotting factor II) and several other essential blood clotting factors (Factors VII, IX and X). Vitamin K is a micronutrient in mammalian diets and for viability molecules of the vitamin must be recycled (and thereby reactivated). This is achieved in two reduction steps; firstly vitamin K epoxide to vitamin K quinone and secondly the quinone to the hydroxyquinone, with dithiol as a dependent cofactor in both reactions. This recycling allows each vitamin K molecule to be utilised 1,000-10,000 times (Thijssen, 1995). Some authorities state that both reduction stages are catalysed by the enzyme vitamin K-2, 3-epoxide reductase (VKOR) (*e.g.* Thijssen, 1995), though previously a second enzyme, vitamin K reductase, had been recognised (MacNicoll, 1985; Thijssen *et al*., 1988; Thijssen *et al*., 1989). Rost *et al*. (2004) refer to a 'vitamin K epoxide reductase multiprotein complex', all components of which may not yet have been identified.

Warfarin and its related substances, including both its congener first-generation anticoagulants and the second-generation anticoagulants, are thought to act by binding to the VKOR and inhibiting the two reduction stages of the vitamin K cycle. The precise mechanism of this interaction remains uncertain. The result of the anticoagulant-enzyme binding is the interruption of the vitamin K cycle and the production of non-functional (*i.e.* under-carboxylated) prothrombin and other clotting factors. Circulating, functional clotting factors degrade at differing rates and have different threshold levels of activity, and it takes some time before haemostasis is compromised, but eventually normal blood clotting fails and haemorrhage can occur. The depuration and regeneration of clotting factors II, VII and X in Norway rats are described in detail by Kerins and MacNicoll (1999).

Three different mechanisms have been hypothesised to explain the ability of some rodents to survive doses of anticoagulants that would prove fatal to their susceptible conspecifics (Suttie, 1980; MacNicoll, 1985; Thijssen, 1995). Two of them, pharmacokinetic and dietary-based resistance, will be considered briefly because they are now thought to be of only subsidiary importance in certain resistant rodent strains. The third, pharmacodynamic resistance, has been shown by recent research to be the likely prime mechanism of anticoagulant resistance in rats, mice and humans (Li *et al*., 2004; Rost *et al*., 2004; Rost *et al*., 2009).

4.2 Pharmacokinetic-based resistance

Chemical kinetics plays an important part both in the process of blood clotting and in the ability of anticoagulant rodenticides to prevent it. Certain compounds (*e.g.* acenocoumarin) are effective as anticoagulants in one species and ineffective in others and such differences are

sometimes due to an enhanced ability of animals to clear anticoagulant compounds. Further, it has been shown that phenobarbitone, a drug commonly used to increase metabolic capacity, reduces the effects of anticoagulants in rats and rabbits, probably by inducing enhanced clearance. Conversely, inhibition of one of the major mammalian metabolic systems, the cytochrome P450 enzymes, strongly potentiates the effects of anticoagulants. Therefore, enhanced clearance may play at least a part in some resistances (MacNicoll, 1985) and has been thought to be particularly significant in House mice (Misenheimer *et al*., 1994).

4.3 Dietary-based resistance

Reduced anticoagulant sensitivity may be the result of enhanced vitamin K availability. Several strains of resistant rats have been shown to be able to synthesise vitamin K from the pro-vitamin menadione sodium bisulphite (vitamin K_3) (MacNicoll and Gill, 1993), although warfarin susceptible rats, and both resistant and susceptible mice, were unable to process the compound in the same way. Vitamin K_3 is present in many proprietary animal feeds and this may partly explain the levels of resistance seen in certain rat strains but is not considered to be a major mechanism of resistance.

4.4 Pharmacodynamic resistance

Early work focused on the Welsh and Scottish resistance strains and showed that the two differed subtly in their mechanisms of resistance (MacNicoll, 1985). Both were found to involve altered VKOR enzymes. In the case of Welsh resistant rats, VKOR activity is considerably less sensitive to warfarin inhibition than in warfarin-susceptible rats. VKOR in Welsh rats is, however, sensitive to inhibition by difenacoum and other related compounds (Thijssen, 1995). In the case of Scottish resistance the altered VKOR enzyme is as sensitive to warfarin as in susceptible individuals but the binding of the enzyme-anticoagulant complex is reversible (Thijssen, 1987). The principle mechanism of anticoagulant resistance in Norway rats is now known to be associated with altered structures of the VKOR enzyme (Rost *et al*., 2004; Pelz *et al*., 2005; Rost *et al*., 2009), it is also suspected that this mechanism is of prime importance in House mice (see below).

Early work on the genetics of resistance in rodents was carried out by, among others, MacSwinney and Wallace (1978) in House mice and Greaves and Ayres (1976) and Greaves and Cullen-Ayres (1988) in Norway rats. However, recent research, enabled by previous work on rodents (Kohn and Pelz, 2000), has resulted in a more thorough understanding of the genetics of resistance.

Li *et al*. (2004) and Rost *et al*. (2004) have, by different experimental strategies, identified a major VKOR gene in humans, rats and mice. The gene, which encodes for the small transmembrane protein found in the cell endoplasmic reticulum mentioned above, was given the name vitamin K epoxide reductase complex subunit 1 (VKORC1) by Rost *et al*. (2004) and VKOR by Tie et al. (2005). The gene has been mapped to chromosome 1 in Norway rats, chromosome 7 in House mice and chromosome 16 in humans. Uncertainty remains, however, as to whether or not this protein is the sole source of all VKOR activity in these species (see Tie *et al*, 2005; Rost *et al*., 2009).

Mutations in this gene were first found to be responsible for two heritable human diseases associated with abnormal blood clotting and resistance to drugs used in the prevention of

thrombo-embolic events (Rost *et a*l., 2004; Li *et al*., 2004). Mutations to the same gene were found in resistant rats and mice and confer resistance to anticoagulant rodenticides in rodents. Further research identified the amino-acid sequences of the mutant VKOR genes (Table 1) in many known resistant rodent strains in Europe (Pelz, *et al*., 2005).

Analysis of the DNA of anticoagulant-resistant strains of rats and mice from around the world has recently shown that several more resistance mutations of the VKORC1 gene occur, some of them having an impact on activity of the altered enzyme (Rost *et. al*., 2009). A total of 25 mutations, or single nucleotide polymorphisms (SNPs), have now been found in Norway rats and, of these, at least eight are known to have an adverse effect on the efficacy of anticoagulant rodenticides. In mice, nine mutations are known, three conferring a resistance effect on the animals that possess them (Pelz *et al*., 2011). Of these two mutations are identical to mutations found in Norway rats (Rost, *et al*., 2009).

The availability of this information permits better understanding of the relationships between the different resistant rat and mouse strains across the EU. This research supports the hypothesis that many independent mutation events have resulted in resistance in Norway rats and House mice in Europe. However, this research has also shown that all rodent resistance phenomena are not explicable using a single model based on altered VKORC1 enzymes (see also Kerins and MacNicoll, 1999, Heiburg, 2009).

From this analysis it is evident that the possession by individual rodents of a specific SNP is not necessarily a "marker" for resistance of either the practical or technical type. However, from work done in the UK, France and Germany it is possible to relate with certainty the practical implications for rodent pest management of all major resistance mutations found in the UK, both in rats and mice. This will be dealt with in more detail in subsequent sections of the report.

4.5 Pleiotropic costs of resistance

In some resistant rodent strains, the altered VKORC1 enzymes derived from mutant VKOR genes are less efficient in carrying out the reduction steps in the vitamin K cycle. This leads to an increased dietary requirement for vitamin K. This defect has been identified in resistant Norway rat strain from Wales, Hampshire and Berkshire (Gould, 2001), and in resistant house mice from Berkshire (Prescott, 1996, Gould, 2001), and results in vitamin K requirements in heterozygotes and homozygotes that are, respectively, about twice and 20 times as great as that of susceptibles (Greaves and Cullen-Ayres, 1988). There is some evidence that the possession of altered VKOR enzymes may impair other vitamin K-dependent calcium-binding enzymes, for example in bone, and Welsh and Scottish resistant Norway rat strains have been found to grow more slowly than their warfarin-susceptible counterparts (Smith *et al*., 1991; Greaves, 1994).

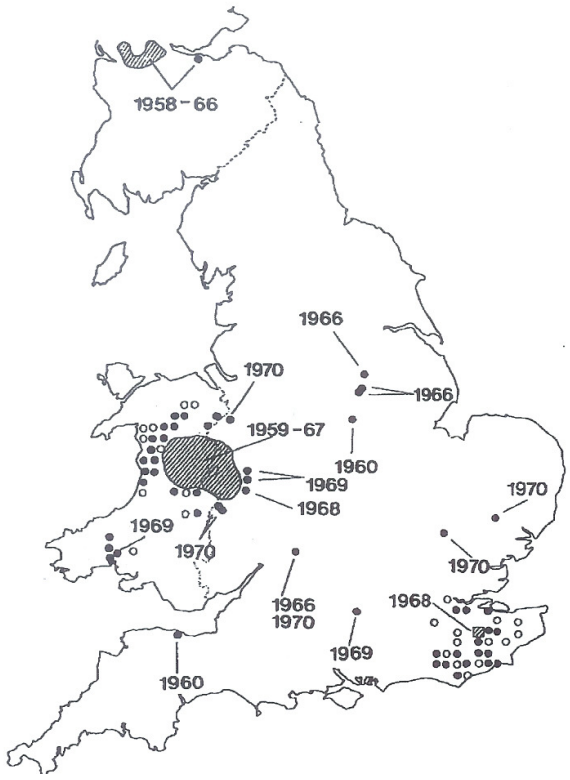
The pleiotropic costs of resistance may play an important role in creating balanced resistance polymorphisms in rodent populations, which would allow susceptible genotypes to recover dominance in the absence of the use of anticoagulant rodenticides. Practical evidence for the occurrence of this effect is equivocal however (Greaves, 1994).

5. Resistance in Norway rats

5.1. Background

Surveys of anticoagulant resistance in Norway rats in the UK have been conducted since the 1960s. Results from surveys conducted during the period 1959 to 1970 are shown in Figure 1. Many different resistance foci were identified at a very early stage and the majority of these exist to this day. Another early review of the distribution and significance of resistance among Norway rats in England and Wales was provided by MacNicoll *et al*., (1996).

Figure 1. Sites of anticoagulant resistance in the UK from surveys conducted during the years 1959 to 1970. Filled symbols show where resistant Norway rats were found, open symbols where resistance was not found. From Greaves and Rennison (1973).



Laboratory and field experiments conducted on Norway rats from these foci confirmed that the nature of resistance differed at many of them. Laboratory breeding programmes resulted in strains of rodents that were thought to carry, in more or less pure form, the genetic material from the resistance foci in Wales, Scotland, Hampshire and Berkshire. It was quickly recognised that, for example, resistance in the extensive focus on the Anglo-Welsh border was less severe than that found in central southern England. However, although different genetic mechanisms were postulated and arguments were refined to the stage that a number of different resistance genes were proposed, the structures of the resistance genes were unknown.

This situation changed with the pioneering work of H-J Pelz and his co-workers (Pelz et al., 2005) in which the genetic mutations at many of the historic UK resistance foci were determined for the first time. Their work relied on foundations from earlier studies by a number of researchers in the field of human medicine (e.g. Rost et al., 2004; Li et al., 2004; Tie et al., 2005).

A total of nine different SNPs have been found in Norway rat infestations the UK (Table 1). Of these, five are known to have significant detrimental impacts on anticoagulant efficacy. With the exception of Y139S which is only known from the UK, all the others (i.e. L128Q, L120Q, Y139C and Y139F) are found elsewhere in the EU and are known to have similar practical impacts on rat control (Buckle, 2012). In other words they are reliable markers for practical resistance to one or more anticoagulant rodenticides. We know from historical laboratory and field studies the approximate scope of some of the resistance foci in which these SNPs are found in the UK.

Table 1. Known VKORC1 mutations in Norway rats in UK. From: Pelz *et al*. 2005; Rost *et al*. 2009; Prescott *et al*. 2010; Clarke, 2012.

Mutation	**Abbreviated mutation name**	**Where present**
Leucine128Glutamine	L128Q†	Central Southern Scotland, Yorkshire, Lancashire
Tyrosine139Serine	Y139S†	Anglo-Welsh border
Leucine120Glutamine	L120Q†	Hampshire, Berkshire
Tyrosine139Cysteine	Y139C†	Gloucestershire, Norfolk, Lincolnshire, Yorkshire, SW Scotland
Tyrosine139Phenylalanine	Y139F†	Kent
Argenine33Proline	N33P‡	Nottinghamshire
Phenylalanin63Cysteine	F63C*	Cambridge/Essex
Tyrosine39Asparagine	Y39N*	Cambridge/Essex
Alanine26Threonine	A26T#	Cambridge/Essex

† 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

So far as we currently know, some of these SNPs are restricted in the UK to single foci. For example, Y139S is restricted to a large area of the West Midlands, the Anglo-Welsh border and central Wales and Y139F is restricted to Sussex and Kent. Other SNPs, such as Y139C, L128Q and L120Q are more widely dispersed. An ongoing project, involving field sampling and DNA screening, will be described in a subsequent section of the report (see Clarke, 2012).

5.2. L128Q ("Scottish resistance")

This SNP was the one found at the site of the first occurrence of Norway rat anticoagulant resistance in Scotland (Figure 1) and has been subsequently found in rats in parts of the north-west of England and in Yorkshire. L128Q appears to confer strong practical resistance to warfarin and diphacinone, with warfarin resistance factors for males and females of 51.5 and 115.9 respectively (Greaves and Cullen-Ayres, 1988). At first, coumatetralyl was found to retain some effectiveness against such rats but resistance factors were high, 34.0 in males and 56.2 in females (Greaves and Cullen-Ayres, 1988) and efficacy is certainly impaired. Second-generation anticoagulants are considered to be effective against this resistance strain. Evidence for this is provided by laboratory tests of difenacoum conducted at UK government laboratories (Hadler, et al., 1975) and resistance factors for difenacoum, bromadiolone and brodifacoum are all below 3.4 (Greaves and Cullen-Ayres, 1988). However, no evidence from field testing has been published to corroborate these laboratory studies.

L128Q was also found in the sample of Norway rats taken for DNA resistance testing in France (Grandemange et al., 2010).

5.3 Y139S ("Welsh resistance")

Resistance was found in Norway rats on farms on the Anglo-Welsh border centred on the town of Welshpool soon after the original discovery of resistance in Scotland (Figure 1). Welsh resistant rats carry the Y139S mutation and have very high resistance factors to the first-generation anticoagulants warfarin and coumatetralyl. Before the second-generation compounds were introduced, coumatetralyl was though to have some limited utility against Welsh resistance but is not now recommended (RRAG, 2010). Extensive fieldwork was conducted in an attempt to curtail the spread of this focus, but the work was ineffectual and was eventually abandoned (Greaves, 1995).

Evidence for the effectiveness of the second-generation anticoagulants against Y139S is extensive, from both laboratory and field studies, because this resistant strain of Norway rats was the one mainly used to evaluate difenacoum, bromadiolone, brodifacoum and flocoumafen for their effectiveness against resistance (Table 2).

The second-generation compounds are considered to be effective against Welsh resistant rats although bromadiolone may be the least effective with a resistance factor for female Y139S resistant Norway rats of 6.9 (Greaves and Cullen-Ayres, 1988). To date this mutation has only ever been found in the original focus but the extent of its spread is no longer known.

Trials were conducted using restricted placements of baits containing bromadiolone, difenacoum and brodifacoum against field infestations of Y139S Norway rats (Greaves et al., 1988). The results showed that bait points containing 50 g of brodifacoum bait (0.005%), replenished weekly or twice weekly, gave complete control of Y139C resistant Norway rats in 14-25 days.

The current geographical extent of this focus is unknown. However, it is unlikely to cover a smaller area than that shown in Figure 1. Indeed, it is likely to extend over a large part the counties of Powys and Shropshire, and to extend to portions of the counties of Gwynedd, Herefordshire and Staffordshire.

Table 2. Laboratory and field studies of the efficacy of second-generation anticoagulants against Y139C.

Active substance	Laboratory study	Field study
difenacoum	Hadler et al., 1975	Rennison and Hadler, 1975
brodifacoum	Redfern et al., 1976	Rennison and Dubock, 1978
bromadiolone	Redfern and Gill, 1980	Richards, 1981
flocoumafen	Bowler et al., 1984	Buckle, 1986

5.4 Y139C ("Gloucestershire resistance")

Anticoagulant resistant Norway rats have been present in Gloucestershire since 1969 (Figure 1) and are now known to carry the Y139C mutation. This mutation is also found in the UK in such widely separated areas as Yorkshire, Norfolk and south-west Scotland (Table 1 and Clarke, 2012). We know little about the development of these resistance foci and no research has been published on Y139C from studies conducted in the UK. However, this SNP has also been present for decades over large parts of Jutland, and elsewhere, in Denmark (Lodal, 2001) and in North-west Germany around the city of Műnster (Pelz, 1995). It is now also found in The Netherlands (van der Lee et al., 2011). Therefore, most of what we know about the efficacy of anticoagulants against rats carrying the Y139C SNP comes from work carried out in Germany and Denmark. The fact that Y139C genotypes are similar from Denmark, Germany and the UK was confirmed by Pelz et al. (2005).

In Denmark and Germany, Y139C confers strong practical resistance against the first-generation anticoagulants; for example, resistance factors to coumatetralyl in Germany are 34 for males and 54 for females (Endepols et al., 2012). The strain shows resistance to the second-generation anticoagulants, particularly where there is a high incidence of resistance, and a high frequency of homozygous animals. The efficacy of bromadiolone, particularly, is poor against animals carrying this mutation (Endepols et al., 2012) and, although difenacoum is generally more effective, acceptable control may be difficult to achieve (Buckle et al., 2012 accepted). These studies, and practical experience in the UK, have resulted in RRAG advice that bromadiolone and difenacoum should not be used against Norway rat populations possessing this mutation.

Table 3. The quantities of anticoagulant baits used and estimated efficacy when bromadiolone, difenacoum and brodifacoum were used to control Y139C resistant rats on farms in the Münsterland

Active substance	Site number	Maximum daily pre-treatment census bait take (kg)[#]	Quantity of bait consumed (kg)	Estimated % efficacy[*]
Bromadiolone[†]	1	2.66	9.95	71.50
	2	2.14	43.40	0.00
	3	1.51	25.50	20.00
	4	4.52	38.38	69.00
Difenacoum[‡]	1	6.89	28.20	86.80
	2	1.62	8.10	59.90
Brodifacoum[§]	1	2.98	4.00	99.20
	2	1.63	1.45	100.00

[*] maximum daily census bait used to estimate efficacy

[#] provides a relative estimate of initial rat population size

[†] from Endepols *et al.*, 2012
[‡] from Buckle *et al.,* 2012 accepted
[§] from Buckle and Prescott, 2012

This, of course, brings about a considerable practical problem for effective rat control in the widespread UK foci of Y139C resistance because restrictions on the use of potentially effective rodenticides, brodifacoum, flocoumafen and difethialone, mean that these cannot be used in most

practical circumstances. Thus, currently in the UK, it is impossible to obtain practical control of Y139C Norway rat infestations where they occur.

It has been confirmed in field experiments conducted at the German focus of Y139C resistance that applications of 50 g placements of baits containing 0.005% brodifacoum are fully effective against Y139C Norway rats (Buckle and Prescott, 2012). Using the 'pulsed baiting' application technique, very small quantities of brodifacoum bait were required for complete elimination of Y139C resistant rat infestations. It is to be anticipated that the same would be the case in the UK. A summary of the German Y139C field trials of bromadiolone, difenacoum and brodifacoum is given in Table 3.

The extent of the various foci of this resistance mutation is unknown. However, rats carrying this SNP have been identified in the counties of Gloucestershire, Yorkshire, Lincolnshire and Norfolk. Additionally, Clarke (2012) records Y139C rats from Gwynedd, from sites in the West Midlands and from Stranraer (Dumfries and Galloway). The multiple foci of these resistant Norway rats makes this the most widely dispersed mutation in the UK (Clarke, 2012).

5.5 Y139F ("Kent resistance")

Resistance in Kent once covered a large part of that county and neighbouring East Sussex (Figure 1). A recent practical failure of a bromadiolone treatment resulted in the DNA sequencing of tissue samples from rats at the site. The Y139F mutation, never before found in the UK, was identified (Prescott et al., 2010). This SNP is found in several European countries including France, Belgium and The Netherlands (Buckle, 2012). There is little published evidence, however, about the practical effectiveness of anticoagulants against Y139F. Grandemange et al. (2009) conducted laboratory experiments using BCR testing to determine the effectiveness of chlorophacinone, bromadiolone, difenacoum, and difethialone against Y139F. From this, the authors recommended that the first-generation compounds and bromadiolone should not be used against Y139F, and that difenacoum might be effective, but its use would be expected to increase the frequency of the resistance mutation. They proposed that the highly potent compounds, such as difethialone, may be effective against the strain. The work of Grandemange et al. (2009) was used to develop RRAG recommendations that neither first-generation anticoagulants nor bromadiolone and difenacoum should be used where rats are found to be anticoagulant-resistant in Kent and East Sussex.

Once again, there are severe practical difficulties for those attempting to conduct effective rodent control on Norway rats, potentially over large parts of Kent and Sussex, because of the indoor restrictions on the use of potentially effective rodenticides, brodifacoum, flocoumafen and difethialone. Thus, as was the case in the Y139C foci, it is currently impossible to obtain practical control of Y139F Norway rat infestations where they occur in the UK.

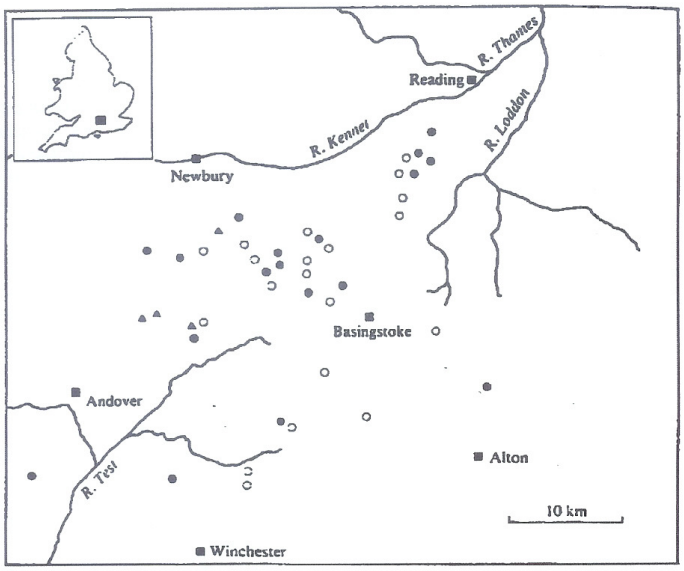
Once again, the current extent of this resistance in the UK is unknown. It is unlikely to be less in extent than the area shown in Figure 1. Rats carrying this mutation have been confirmed in the county of Kent (Prescott et al., 2010) and most recently in East Sussex.

5.6 L120Q ("Hampshire" and "Berkshire" resistances)

In 1969 (Figure 1), rats from a farm in north-east Hampshire were found to be resistant to warfarin but, in addition, to have very low resting levels of blood clotting factors when compared

to rats of the Scottish and Welsh strains, suggesting Hampshire resistance is of a different character. Subsequently, rats were tested for resistance using feeding tests (EPPO, 1995) for warfarin and, later difenacoum, and were found to be resistant to both compounds (Greaves and Cullen-Ayres, 1988). A laboratory breeding programme was carried out to product a pure line of Hampshire resistant rats called the Homozygous Hampshire (HH) strain. Later investigations (Figure 2) showed that rats putatively resistant to difenacoum were prevalent over an area of 1,200 square kilometres, including parts of the neighbouring counties of Berkshire and Wiltshire (Greaves et al., 1982: MacNicoll and Gill, 1987).

Figure 2. The distribution of farmsteads providing samples of rats that contained difenacoum-resistant (filled circles), warfarin-resistant (open circles) and non-resistant (closed triangles) Norway rats. From Greaves *et al*. (1982).



In 1982, rats from a farm in this area (northwest Berkshire) were found to possess low-grade resistance (*i.e.* technical resistance) to brodifacoum, in addition to resistance, determined by a BCR test, to difenacoum (Gill et al. 1992). A breeding programme was conducted to establish the resistance gene and the derived strain was called the Homozygous Berkshire (HB) strain.

Subsequent investigations on another, nearby farm demonstrated unequivocally that Berkshire resistant rats show practical resistance to bromadiolone (Quy *et al*., 1995). No data are available from published sources on resistance factors for anticoagulants against male HB rats. However, in feeding trials conducted at the University of Reading, thirteen female rats survived doses of between 22.2 and 44.2 $mg.kg^{-1}$ of bromadiolone, and ten male rats survived doses of between 14.9 and 30.1 $mg.kg^{-1}$ of bromadiolone (Hussain, 1998). Furthermore, resistance factors for female Berkshire resistant Norway rats have been estimated to be: 18 for difenacoum and 35 for bromadiolone, but only 5 for both brodifacoum and flocoumafen (MacNicoll, personal communication, 2004). The HB strain is the most extreme form of anticoagulant resistance currently known, but still only exhibits a low degree of resistance to brodifacoum and flocoumafen (i.e. technical resistance).

The genetics of these two resistance strains is uncertain. Both carry the L120Q mutation but it is postulated that Berkshire resistance is conferred by the presence of this mutation as well as some

other factor(s), possibly because of other genes, or because of the combined effects of pharmacodynamically-based resistance (altered biochemistry of the target enzyme) and enhanced clearance (i.e. pharmacokinetically-based resistance) (Thijssen, 1995). However, laboratory stocks which posses the L120Q mutation are labile in respect of their resistance factors and separation between the two strains is uncertain. Recently, work conducted at the University of Reading on rats from sites in Hampshire has shown them to exhibit levels of resistance that are more characteristic of the Berkshire strain. It may be that the prolonged use of ineffective anticoagulants in this area has enhanced physiological resistance so that the old, and once separate, Hampshire and Berkshire foci can no longer be defined with certainty.

Field trials of bromadiolone and difenacoum were conducted by workers from the University of Reading recently on farms near Newbury (Berkshire) and Winchester (Hampshire), where the rat infestations where almost entirely L120Q homozygous resistance. Very large quantities of bromadiolone and difenacoum baits were used at these sites and poor levels of control were achieved.

What we can say with confidence about the use of rodenticides in the Hampshire/Berkshire area is, firstly, that the first-generation anticoagulants are totally ineffective. Secondly, that bromadiolone (and probably difenacoum) are largely ineffective in extensive parts of this focus. A recent, carefully-monitored practical treatment has shown that brodifacoum is fully effective against L120Q rats (Meyer, 2009). Although the site was in central-southern Hampshire (Winchester), previous treatment records show the complete failure of difenacoum and bromadiolone baits and suggest that the resistance there was of the advanced Berkshire type. A total of 213 kg of (mainly) difenacoum and bromadiolone was used over a period of two years at this small site without any observable effect on the rat infestation. An application of 3.4 kg of brodifacoum bait, made under an emergency extension of approval, eradicated the infestation in 18 days.

Until recently the focus of L120Q resistance in central southern England was thought to comprise a single contiguous focus. The extent of the focus comprised very large portions of Hampshire and Berkshire, with parts of the neighbouring counties of Wiltshire, Oxfordshire and Surrey also involved. Recent work by Clarke (2012) has revealed the presence of L120Q Norway rats also in Sussex and Avon. It is not currently possible to say whether these foci are contiguous with the main one. An outlying focus has also been identified in south-west Scotland.

A noteworthy feature of the L120Q focus is the frequent observation that few, if any, rats carry the wild-type genotype exist among resistant infestations. This implies that current rodenticide applications in the resistance area have operated an extreme selection pressure in favour of L120Q resistant genotypes.

Consequently, bromadiolone and difenacoum are no longer advocated for use anywhere in this area against L120Q (RRAG, 2010). Once again, brodifacoum, flocoumafen and difethialone are recommended for use against L120Q but their use is largely precluded by the regulatory restrictions upon them to indoor use only. No satisfactory and cost-effective control of Norway rat infestations is therefore currently feasible over a large part of central-southern England, and increasingly in other parts of the UK.

6. Resistance in House mice

6.1 Background

The house mouse (*Mus domesticus*) possesses a degree of natural resistance to anticoagulant rodenticides. This means that these chemicals are generally less effective against house mice than they are against Norway rats. True resistance to anticoagulants, conferred by genetical mutation, has been known among house mice in the UK since the 1960s. Resistance is now so widespread it is often said that it is harder to find susceptible house mice than resistant ones. Consequently, no approvals for biocidal products containing first-generation anticoagulant active substances are in place in the UK. The RRAG supports this regulatory strategy. However, second-generation anticoagulants are still widely and successfully used against house mice in the UK.

The study of resistance to anticoagulants in the house mouse has long been a 'poor relation' in comparison to the quantity and quality of available information on anticoagulant resistance in Norway rats. Consequently, there are a number of important unanswered questions about resistance in UK house mice. In particular we remain uncertain about the precise nature of the genetics of the phenomenon and, probably more importantly, no map of the distribution of anticoagulant resistance in house mice in the UK has ever been produced, due at least in part to its assumed widespread occurrence.

Recently, in Germany, a study of the distribution of resistance in house mice has been conducted using the new system of DNA sequencing for the detection of anticoagulant resistant mutations (Pelz et al., 2011). It revealed that resistant house mice are very widespread and frequent in Germany. More than 90% of the mice examined carried genetical resistance mutations and resistance was found at 29 of the 30 locations sampled. The two resistant house mouse strains were found in the German study are also known to be present in the UK, thus suggesting that a similar situation may exist here.

A brief explanation of the taxonomy of the species is required. For many years, and in particular during the era of early research on anticoagulants, the house mouse in the UK was known by the scientific name *Mus musculus*. The species was then considered in Europe to be variable and to have several different 'forms'. However, in 1998 it was determined that there actually exists in Europe several full species that had previously gone under the 'umbrella' name *Mus musculus*. The species present in the UK became known as *Mus domesticus*. True *Mus musculus* is now considered to exist only in the eastern parts of the European mainland. *Mus domesticus* is thought to have been the ancestor of all laboratory albino mice and all fancy mice found in the pet trade. House mice in this report are therefore referred to as *Mus domesticus* (Berry et al., 2008), although the specific name *M. musculus* has been used in some of the original references.

6.2 Tolerance, natural "resistance" and the early anticoagulants

The first anticoagulant extensively tested against house mice was warfarin. Groups of anticoagulant-naïve mice were offered in the laboratory 0.025% warfarin bait. It is apparent that complete mortality of house mice was not obtained unless the animals fed on warfarin bait, without choice, for very long periods (Rowe and Redfern, 1964). The data were used to calculate a series of values for the toxicity of warfarin expressed as lethal feeding periods (LFP). These are defined as a number of days of continuous, no-choice feeding required to kill a given percentage

of the mice tested. For example the LFP_{50}, LFP_{90} and LFP_{99}, and these values are analogous to the more well-known LD_{50}, LD_{90} and LD_{99} based on lethal doses. The analysis revealed that the LFP_{50} for 0.025% warfarin for house mice was 4.8 days and the LFP_{99} was 29.5 days. These results, in comparison with similar results obtained for Norway rats (*Rattus norvegicus*) whose LFP_{50} and LFP_{99} are 1.7 and 5.8 days respectively, showed that house mice possess a remarkable degree of tolerance to warfarin (Rowe and Redfern, 1965). This does not conform to the definition of resistance given above and is sometimes known as *tolerance* or *natural "resistance*".

We also know that the feeding behaviour of house mice is such that they often do not feed consistently from any single food source and this characteristic would make it even less likely that warfarin would be fully effective against house mice. Research on anticoagulants continued after the invention of warfarin. Other compounds, such as coumachlor, diphacinone, chlorophacinone and coumatetralyl came to the market. However, it is generally accepted that none of these perform significantly better than warfarin against house mice. Therefore, the regulatory policy not to permit the use of these active substances against house mice in the UK is justified.

6.3 Resistance to first-generation anticoagulants

In 1961, just ten years after the introduction of warfarin, reports were received of the failure of this compound to control mouse infestations from a number of widely separated locations in the UK. A resistance test was developed in which survival after 21 days of continuous feeding on 0.025% warfarin bait was considered to be indicative of resistance (EPPO, 1995). Using this test, the presence of warfarin resistance was confirmed in mouse infestations from many parts of the UK. Tests of diphacinone and chlorophacinone against mice that had survived the 21-day warfarin resistance test showed that these compounds did not provide a solution to warfarin resistance in mice. Some time later, a population of resistant house mice was discovered in Cambridge. These had a distinctive coat colour and it appears that the gene for this attribute was linked to that of resistance. These 'Cambridge Cream' mice were held in the laboratory and much subsequent assessment of the activity of anticoagulants against resistant house mice relied on tests on the progeny from this original breeding stock.

6.4 Resistance to second-generation anticoagulants

Difenacoum and bromadiolone were the first active substances to be tested against resistant house mice. Laboratory tests showed a useful level of activity of these compounds and both appeared to be substantially more effective than warfarin (Hadler et al., 1975: Redfern and Gill, 1980). Two days of no-choice feeding of 0.005% difenacoum resulted in 87% mortality and ten days of similar testing of bromadiolone gave 80% mortality.

Subsequently, a series of pen tests was carried out using families of warfarin-resistant house mice and field trials against natural infestations were also conducted (Rowe et al., 1981). A result observed in these trials was the frequent inability of difenacoum and bromadiolone to provide complete control, both in the case of resistant family groups in pen tests and of wild infestations in the field. Indeed, mice survived in five of the 12 field trials conducted. These survivors were removed to the laboratory and later offered either 0.005% bromadiolone or difenacoum for 21 days. Respectively 43% and 18% of the mice survived in these bromadiolone and difenacoum tests. These results appeared to show that some mice, substantially resistant to bromadiolone and

difenacoum, were present in field infestations even before these two compounds came into widespread use. It is not clear whether this was just another manifestation of tolerance or whether resistance mutations were already present in some mouse populations. The tests also showed that, for what ever reason, control was likely to be more problematic in the case of bromadiolone than difenacoum and this has subsequently proved to be the case.

Two more second-generation anticoagulants, brodifacoum and flocoumafen, were subsequently introduced and these were shown to be substantially more potent than bromadiolone and difenacoum against house mice (Rowe and Bradfield., 1976; Rowe et al., 1978; Rowe et al. 1985). In the laboratory, complete mortality of resistant house mice was achieved with both these compounds after one- and two-day periods of no-choice feeding. Six field trials of brodifacoum against wild house mouse infestations resulted in an average of 98.8% control and ten of flocoumafen gave an average of 97.2% control. An advantage of these two compounds for resistant house mouse control is that only small quantities of bait are required to achieve a lethal dose, even of resistant mice, and this characteristic is important for house mice because of their sporadic feeding behaviour.

6.5 Genetics of anticoagulant resistance in House mice

The genetics of anticoagulant resistance in the house mouse is not well understood. It was initially thought that resistance in mice was similar to that in Norway rats, there being a single gene governing anticoagulant resistance (Wallace and MacSwinney, 1976). However, the outcome of classical genetical breeding studies failed to confirm this and it may be that house mouse resistance is complicated by the involvement of several genes, and perhaps even several different resistance mechanisms.

Since the early genetical studies, a very limited amount of research work has been done on house mouse resistance in the UK. The early work on resistant house mice was done on the so-called Cambridge Cream resistance strain. This was developed at the Central Science Laboratory (now the Food and Environment Research Agency), and has been used in resistance research in the UK since the 1980s. This is now known to carry the leucine128serine mutation, which is referred to by its abbreviated name L128S. It is likely that this mutation occurs widely in the UK, as it does in Germany (Pelz et al., 2011).

In the 1980s, a population of resistant mice was discovered in the Reading area and studies were conducted on them which resulted in the development of a laboratory homozygous strain of resistant house mice (Prescott, 1996). The mutation later found in this strain was tyrosine139cysteine (or Y139C). Once again, this resistance mutation was found in the geographical survey of resistance conducted recently in Germany (Pelz et al., 2011). This strain is considered to be fully resistant to the first-generation anticoagulants and to the second-generation compound bromadiolone.

Thus, we can say with reasonable certainty that we have in the UK at last two different house mouse resistance mutations in the UK. Little is known of their geographical distribution and there are few studies of the degree of resistance that these mutations confer.

6.6 Summary of recommended use of anticoagulants against house mice in the UK

It has long been a regulatory policy that anticoagulants such as warfarin, chlorophacinone, diphacinone and coumatetralyl should not be used for the control of house mice in the UK. Consequently, there are no current approvals for the use of rodenticide products carrying these active ingredients for mouse control. RRAG supports and endorses this regulatory policy. This is because the occurrence of resistance to them would be likely to render them widely ineffective and because the use of these substances is likely to increase the severity and spread of resistance among house mice.

We know that one of the two strains of resistant mice present in the UK (Y139C) shows a significant degree of resistance to bromadiolone. There are also many anecdotal reports of the failure of bromadiolone to control house mice. While it is likely that some infestations may be controlled, at least in part, by applications of bromadiolone, the use of this active substance against house mice in UK is not recommended as it may not result in an inadequate level of control and will exacerbate resistance problems.

The situation of difenacoum is more equivocal. This active substance is widely used in successful mouse control treatments. However, mice carrying the Y139C mutation possess a degree of resistance to difenacoum. The situation with L128S is more uncertain. What is certain, however, is that 30 years ago some individuals within mouse infestations could not be controlled with difenacoum baits, and it is unlikely that this situation has improved in the intervening period. It would therefore be prudent, in areas where resistance in house mice is suspected, not to use products that contain difenacoum.

Studies on the intrinsic activity of the second-generation anticoagulants demonstrate that brodifacoum and flocoumafen are the most potent active substances against susceptible house mice (Prescott et al, 2007). There is also good evidence from early field studies that brodifacoum and flocoumafen are effective against anticoagulant-resistant house mice. Furthermore, laboratory studies conducted on mice carrying the Y139C mutation at the University of Reading have confirmed that brodifacoum baits are effective against this type of resistant house mouse.

Currently, there are no anecdotal reports of the failure of either of these compounds to control infestations of house mice in the UK. Therefore, products containing brodifacoum and flocoumafen should be the rodenticides of choice when carrying out control treatments against house mice in the UK. This is because they offer the promise of the highest levels of control and are the least likely to result in anticoagulant-resistant mice surviving treatments.

Baits containing brodifacoum and flocoumafen in the UK presently carry a restriction on their use that they should be used only to control infestations of rodents ‘indoors’. Generally, house mouse infestations are known to live and feed predominantly indoors and this allows the use of brodifacoum and flocoumafen baits to be used against them.

Baits carrying the second-generation anticoagulant difethialone are new to the market in the UK. Literature produced by the manufacture claims that there is ‘no known resistance in mice’. Such claims, however, fall short of proof that difethialone is effective for the practical control of resistant house mice and RRAG is aware of no published difethialone field trials conducted in the UK against these animals.

The study mentioned earlier (Prescott et al. 2007) on the potency of difethialone and the other second-generation active ingredients against anticoagulant-susceptible house mice showed that difethialone falls somewhere between bromadiolone and difenacoum in terms of intrinsic activity. It should be held in mind, however, that difethialone baits contain 0.0025% of the active

substance, while those carrying brodifacoum and flocoumafen contain twice that concentration, namely 0.005%.

7. DNA Sequencing of Resistant Norway Rats in the UK

7.1 Background

The technique of DNA sequencing for the detection of rodents carrying anticoagulant resistance SNPs is relatively new. The pioneering work on this technology was done by scientists in Germany (Rost et al., 2004; Pelz et al., 2005). In collaboration with scientists in the UK, Pelz et al., (2005) sequenced DNA material from UK resistant Norway rats and house mice and, thereby, provided RRAG with an understanding of the SNPs underlying the majority of the established UK anticoagulant resistance foci.

DNA sequencing has been used recently for important surveys of the distribution of resistance in several EU countries including Germany (Pelz, 2007; Pelz et al., 2011), The Netherlands (van der Lee et al., 2011) and Belgium (Baert et al., 2011). In the UK, the technique was used for the first time to identify the resistance SNP present in the Kent resistance focus (Prescott et al., 2010).

It is important to note that the identification of a specific resistance SNP does not confirm the presence of anticoagulant resistance, either of the 'technical' or 'practical' type. It merely demonstrates the presence of a rodent or rodents with genetic material that differs from that found in wild type animals. Further information is needed from other studies, preferably from well-designed laboratory tests or field efficacy evaluations, so that the impact of the resistant SNP on the outcome of practical applications of anticoagulants can be determined. Fortunately, in the UK and elsewhere in the EU, this essential basic research has been done for at least five of the most important resistance SNPs in Norway rats, as describe in the preceding sections of this report. Therefore, we can with a high degree of confidence predict the impacts on rodent control of the presence of the majority of SNPs found in UK rats and mice in particular the following: in rats L120Q, Y139S, Y139C, Y139F and L128Q, and with somewhat less confidence in house mice, Y139C and L128S.

7.2. UK DNA Norway Rat Resistance Surveys

7.2.1 Data Sources and GIS analysis

The information presented here comes from a variety of sources and had been made available to RRAG with permission for publication. The following sources are therefore gratefully acknowledged:

1. West Berkshire District Council. A coherent attempt has been made by the Council to examine the distribution of the L120Q SNP in the area around Newbury. DNA samples from that source were analysed at the Universities of Huddersfield and Reading.
2. University of Reading. The University has received samples of DNA for sequencing from a number of sources from the Hampshire/Berkshire and Kent/Sussex foci.
3. Food and Environment Research Agency. The results from preliminary surveys of resistance mutations from several sites in the UK were kindly made available for inclusion in this document. The samples were collected by the Central Science Laboratory, York, now the Food and Environment Research Agency, and analysed by full VKORC1 gene sequencing by Rost et al., (2009).
4. Syngenta AG, Basel, Switzerland. Field experiments conducted by University of Reading researchers on behalf of Syngenta have provided substantial samples for

DNA analysis from four sites in the Hampshire/Berkshire focus which are reported here. All samples from this source were analysed at the University of Reading.

5. The Universities of Huddersfield and Reading are collaborating with an industry consortium to obtain a structured analysis of the distribution of Norway rat resistance genotypes in the UK. The data obtained from samples analysed at the University of Reading within this project are presented here. Further data obtained by the University of Huddersfield, and analysed there, are presented in a separate report (Clarke, 2012).

These data have been entered onto a Global Information System (ArcGIS) database in which positional data for each rat DNA sample is entered either as a UK National Grid Reference or as a UK postcode. Additional data entered are: 1) resistance mutation found, 2) whether the sample was homozygous or heterozygous for the SNP found. The resistance maps prepared in this way, therefore, contain accumulated data from all data sources.

7.2.2 Materials and Methods

Because DNA analysis of the samples was conducted at several laboratories, including the Universities of Huddersfield and Reading, a variety of methods of DNA sequencing has been used. No attempt will be made here to provide details of all sampling and analytical methods at all of the laboratories providing data. However, the majority of the positional data presented in this report are derived from samples analysed at the University of Reading using the following methods.

Genetical material was obtained from the field in the form of tail tip samples. These samples were preserved in individual tubes of 80% alcohol and taken to the laboratory by surface mail or were delivered by hand. On arrival in the laboratory, they were frozen at -20°C until analysis. Genomic DNA was extracted using the Qiagen DNeasy tissue extraction kit following the manufacturer's recommendations (Qiagen Ltd., Crawley, West Sussex, UK). Briefly, approximately 4mm of tissue was shaved from each tail using a sterile sharp razor blade. The tissue shaving was placed in a 1.5ml microtube and 180 µl of pre-warmed extraction buffer ATL 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 nanodrop 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.

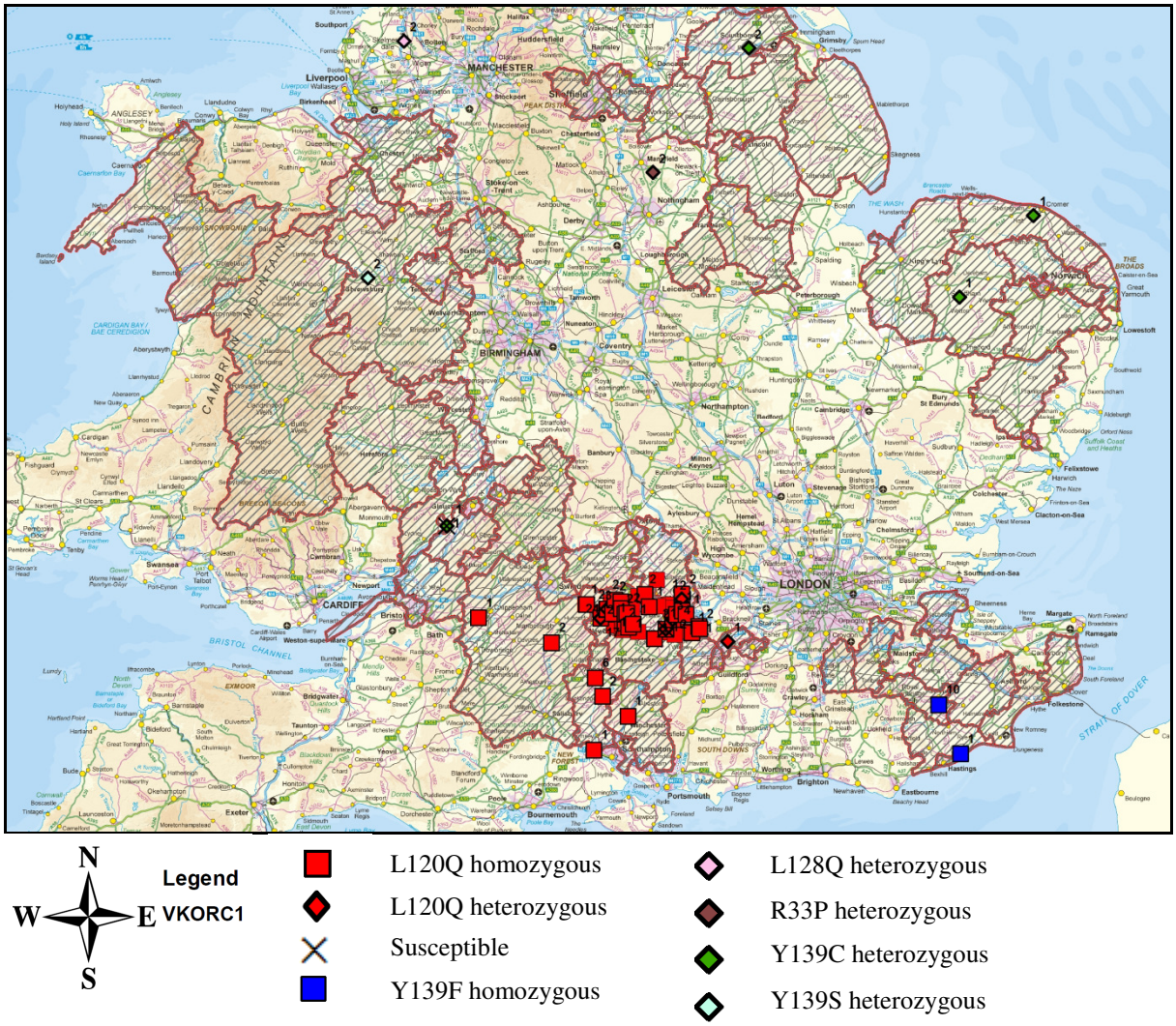
7.2.3 Results.

A map summarising all DNA sequencing results submitted to RRAG is given in Figure 3.

The results show the dispersed nature of the occurrence of the Y139C SNP. Samples positive for this mutation were analysed from two well separated sites in Norfolk. It is impossible to say whether the occurrence of the SNP in Norfolk is contiguous between these two localities, or

whether there are in fact two distinct foci. It seems highly likely that the former is the case. Samples positive for Y139C were also found in Yorkshire and Gloucestershire. It seems unlikely that these are contiguous foci, either with each other or with that in Norfolk, because they are separated by considerable distances. Y139C is also found in south-west Scotland, the West Midlands and Gwynedd (Clarke, 2012).

Figure 3. Combined results of DNA genotyping of tissue samples from Norway rats from various sources in the UK.

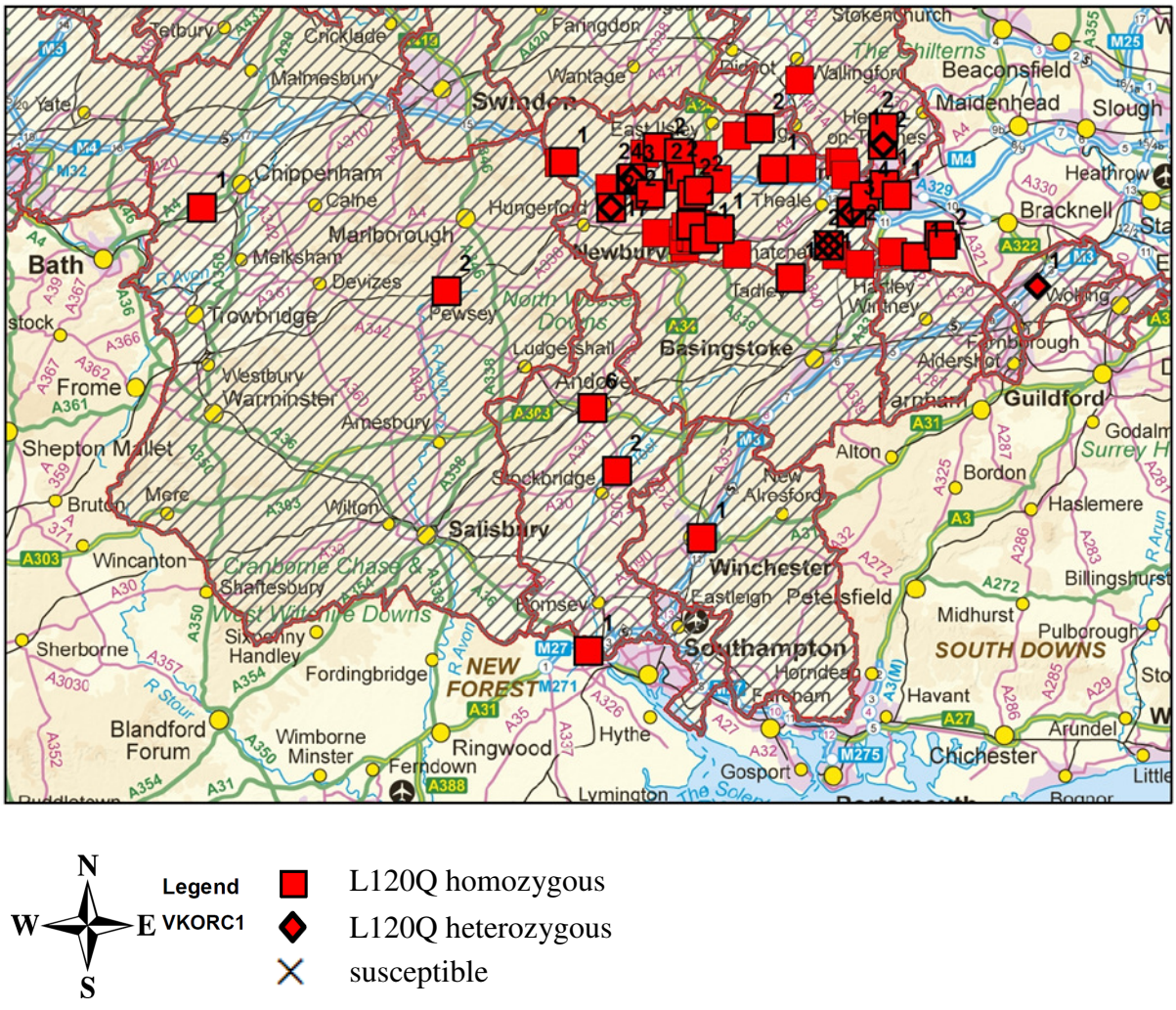


The very widespread occurrence of Y139C is of considerable significance to the future use of anticoagulants for rat control in the UK. Presently, the use of effective anticoagulants (i.e. brodifacoum, difethialone and flocoumafen) is virtually precluded at all these foci. Therefore, there is the predominant use of the resisted active substances bromadiolone and difenacoum. If this situation persists, the intensive selection pressure towards the resistant genotype will result in the geographical spread of the mutation and a tendency towards the homozygous condition of the genotype coming to predominate in resistant infestations. Given the widespread current distribution of the mutation, it is possible that, in the foreseeable future, rats carrying Y139C may be present over the larger part of the UK.

Two sites were found, one in Kent and another in East Sussex, were the Y139F mutation was present (Figure 3). Once again, we do not know if the two locations are a part of a single focus or are separate occurrences. However, the previous distribution of resistance in Kent and Sussex (Figure 1) suggests that the two are indeed contiguous and these observations are likely to be a part of a much larger focus. RRAG recommends that bromadiolone and difenacoum should not be used against Y139F Norway rats,

Samples analysed at Fera have confirmed the presence of the Y139S mutation in the vicinity of Shrewsbury in the extensive and well-known resistance focus on the Anglo-Welsh border. Also, L120Q in Lancashire and R33P in Nottinghamshire. The Y139S and L120Q genotypes are susceptible to bromadiolone and difenacoum. Very little is known of the phenotypic consequences of the R33P SNP.

Figure 4. Combined results of DNA genotyping of tissue samples from Norway rats in the central southern England focus of L120Q resistance.



The DNA sequencing data is most extensive for the L120Q mutation (Figure 4). This resistance was first found on farms in north-east Hampshire and west Berkshire (Figure 2) and for some time it was considered that the focus remained relatively confined. However, recent DNA

screening has demonstrated the presence of the mutation in over a very large part of central southern England. The data show that the mutation is present to the west as far as Corsham in Wiltshire and, to the east, it is present at least as far as Camberley, Surrey. Rats carrying this SNP have been found north of Reading, near to Wallingford in Oxfordshire, and in the south on the outskirts of the New Forest in Hampshire to the west of Southampton. Further analysis of samples submitted to the University of Huddersfield demonstrates L120Q in south-west Scotland and the Ashdown Forest (Sussex) (Clarke, 2012).

Figure 4 shows that the majority of L120Q samples reported here are from West Berkshire. However, the apparent high density of resistant Norway rats is probably an artefact of more intensive sampling in that area, due in large part to the activities of the staff of West Berkshire District Council. It is highly likely that the focus of resistance is contiguous over the area shown in Figure 4 and there is no evidence that the incidence of resistance in West Berkshire exceeds that in other neighbouring counties. It is important to note that resistant rats are present, and may even predominate, in the conurbations of Reading, Newbury, Winchester, Basingstoke, Andover and Salisbury. Once again, effective rodent control is severely compromised in this entire area. This has been demonstrated most clearly by recent experience of social impacts on communities living in houses in the south-west suburbs of Reading.

Once again, it is the view of RRAG that continued use of bromadiolone and difenacoum in this area will lead to the increasing spread of this severe form of anticoagulant resistance, will not provide any satisfactory level of rat control and constitutes unnecessary risk to wildlife because of the large quantities of ineffective anticoagulants that are entering the environment (see Meyer, 2009).

Further information on the distribution in the UK of anticoagulant-resistant Norway rat genotypes has been presented in another report (Clarke, 2012). The data presented here should be considered in conjunction with those additional data.

8. Alternative Rodent Control Techniques

The principal purpose of this report is to present information on the incidence and impacts of anticoagulant resistance in the UK. No practical guidance on resistance management is given as this is available in other RRAG publications (RRAG, 2010 and 2012). However, some brief mention of rodent pest management methods that are alternatives to anticoagulants rodenticides is warranted. 'Sustainable' is a watch word much used now in the regulation of pesticides and a strategy for the sustainable use of rodenticides, including anticoagulants, has been recently proposed (Anon., 2012).

It is axiomatic to all those involved in rodent pest management that chemical solutions to pest problems, on their own, seldom offer effective control in the long term. A combined approach to rodent control is essential to minimise the use of rodenticides, including the use of the anticoagulants, and this is sometimes called Integrated Pest Management (IPM). One of the classical planks of IPM, and essential in pesticide resistance management, is the rotation of alternative chemical modes of action. Unfortunately, because of regulatory decisions recently taken by the manufacturers of active substances such as zinc phosphide, calciferol and bromethalin, this option is largely unavailable for rodent control in the European Union because of the extreme paucity of alternatives to anticoagulants.

Habitat modification is an essential component in any balanced rodent control strategy (Lambert et al., 2008). This includes the removal of foodstuffs that might sustain rodent infestations, the prevention of ingress into structures by use of proofing measures and the denial of harbourage. The use of such measures may have an important part to play in the sustainable management of Norway rat infestations. However, those who engage in practical mouse control know how difficult it is to implement these measures thoroughly to prevent mouse infestation. House mice are capable of living from very limited food resources. They are also adept at getting into buildings through very small apertures and finding harbourage where none appears to exist. So, while all these measures always require consideration and often implementation, none is likely to preclude mouse infestation and is still less likely to remove existing infestations of house mice.

Unlike rats, house mice generally do not exhibit strong aversion to novel objects ('neophobia'). Therefore, in most circumstances, house mice are readily trapped. Trapping is a very useful tool in the control of mouse infestations, particularly where the operator has a good level of experience and skill and, if the infestation is substantial, large numbers of traps can be deployed.

Alternatives to anticoagulants as chemical interventions for rat control are largely unavailable in the UK because, as mentioned above, of the withdrawal from the market of zinc phosphide and calciferol and the unavailability of bromethalin. Some non-anticoagulant rodenticides are available in the UK for mouse control and the most well-known of these is alphachloralose. The use of baits containing this active substance provides good control of house mice in some circumstances.

These alternative measures, in the case of both resistant Norway rats and house mice, carry the very important benefit that they do not select for the anticoagulant resistance genetic trait because they act equally effectively against both susceptible and resistance rodents. Their use within a wider strategy of the control of rodent will serve to prevent the spread of anticoagulant resistance, as well as the removal of resistant infestations in certain favourable situations. However, our virtual complete reliance on the use of anticoagulants in the chemical control of rodents in the UK calls for improved schemes for resistance management. Certainly these will involve the use of alternatives to anticoagulant rodenticides where appropriate and

cost-effective. Also important will be projects that provide an increasing knowledge of the distribution of resistance mutations in rats and mice and then the use exclusively of only fully effective anticoagulants against them.

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