University of Reading

Anticoagulant Resistance in Norway Rats Conferred by VKORC1 Mutations in South-East England

Thesis for Degree of Doctor of Philosophy

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Abstract

Anticoagulant resistance was discovered in Norway rats in the United Kingdom nearly 70 years ago, and since then the number of resistance mutations and resistance foci has increased. In no other country are mutations that are thought to confer serious practical resistance so widespread. Numerous studies have shown multiple resistance genotypes and phenotypes across south-east England. Despite this, the extent of these resistance foci and the resistance factors possessed by these rats are not known. The experiments described herein were designed to reveal the extent and impact of resistance mutations in south-east England.

304 tissue and faecal samples were taken from rats across the study area, in order to identify and delimit foci of VKORC1 resistance conferred by mutations in the VKORC1 gene. The L120Q resistance mutation was found to be prevalent across the study area, with the majority of rats possessing the homozygous form of the mutation, suggesting that for years, resistance has been selected for through use of ineffective second generation anticoagulant rodenticides (SGARs). Two strains of L120Q-resistant laboratory rats were dosed with SGARs and their prothrombin times (PT) measured, and resistance factors generated by comparison with data from susceptible strains. L120Q in its strongest form was found to confer practical resistance to bromadiolone and difenacoum, but not to resistance breakers such as brodifacoum. Finally, field trials were carried out in the L120Q resistance focus of central-southern England, which showed conclusively that brodifacoum is efficacious against L120Q-resistant rats in both Berkshire and Hampshire.

The UK rodenticide stewardship scheme, proposed by the Campaign for Responsible Rodenticide Use (CRRU) will see “resistance breaking” SGARs available for use against Norway rats only by competent users as of April 2017. Results indicate this development is both timely and necessary for proactive Norway rat control in the UK.

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Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

David James Rymer

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Chapter 1: Introduction

1.1 The Norway rat

1.1.1 Early history

The Norway rat (*Rattus norvegicus*), also known in the scientific literature as the sewer rat and brown rat, is thought to have originated in the grassy steppes north of the Caspian Sea before undergoing a population explosion and migrating outwards, eventually boarding ships and spreading throughout Europe (Lund, 2015). It was first recorded in the United Kingdom in 1728 (Brooks and Jackson, 1973). Along with another major commensal (lit. "sharing one's table" – Meehan, 1984) rodent, the house mouse (*Mus musculus*), it is a major pest in the UK. The black rat (*Rattus rattus*), also a commensal pest species, was introduced in 1100 but has disappeared from much of the UK since the introduction of the Norway rat and is consequently less of a concern in the UK at the present time (Corbet and Southern, 1977); this appears to be the case in many areas where the Norway rat has arrived subsequently to the black rat (Lund, 2015).

1.1.2 Norway rats as pests

1.1.2.1 Food spoilage and consumption

Norway rats are ubiquitous in the agricultural environment and may take advantage of poor hygiene, storage and husbandry practices. In the UK, losses of crops in the field due to Norway rats are limited compared to the levels of damage, for example, in Asia as the result of the activity of other, similar, species such as the buff-breasted rat *Rattus flavipectus* (Huang *et al.*, 2011), Polynesian rat *Rattus exulans* (Tobin, 1994), lesser ricefield rat *Rattus*

losea, ricefield rat *Rattus argentiventer* and black rat (Buckle, Yong and Abdul Rahman, 1985; Stenseth *et al.*, 2003; Brown *et al.*, 2005; Meerburg, Singleton and Leirs, 2009). The greatest economic losses to Norway rats in the UK are conversely not due to direct consumption, but the associated damage to packaging and contamination of food products with hair, urine and faeces. An adult Norway rat may produce 14,600 droppings and 5.4 litres of urine a year (Smith and Meyer, 2015), contaminating far more food than they can consume. Norway rats in California were found to very rarely leave droppings in their burrows, which increases the likelihood that these contaminants will find their way onto and into human produce (Pisano and Storer, 1948). Furthermore, the potential financial losses to a farm or manufacturer are far greater should the contamination go unnoticed until the product reaches consumer level; at this point estimation of financial losses would be purely speculative, but could easily result in loss of custom, complaints and compensation, and additional health inspections.

1.1.2.2 Disease transmission

In the UK, the most direct impact the Norway rat has as a pest is as a reservoir for zoonotic pathogens such as hantavirus, with the recent discovery in the UK of Seoul hantavirus, known to cause haemorrhagic fever with renal syndrome (HFRS), linked to Norway rats on farms in the north of England (Jameson *et al.*, 2013, 2014). Diseases such as salmonellosis (Richards, 1989), leptospirosis (Michna and Campbell, 1970; Webster, Ellis and Macdonald, 1995), campylobacter (MacIntyre, unpublished) and foot-and-mouth virus (Capel-Edwards, 1970) are all found in rats in the UK, and all may act as zoonotic pathogens, as well as having significant economic and welfare impacts due to their effects on farm animals. Due to the proclivity of Norway rats to leave droppings in areas where feed is stored and / or distributed to livestock, these may act as a source of transmission to farm stock feeding at the same location. In addition to the risks posed to livestock and humans in rural environments,

Norway rats also pose a disease risk to humans in the built environment including in their own homes as well as in food outlets A recent study in New York suggests that urban Norway rats may carry multiple strains of multiple diseases known to cause gastroenteritis in humans (Firth *et al.*, 2014).

The actual impacts of food spoiling and consumption are limited in Europe (Meerburg, Singleton and Leirs, 2009) compared to the potential direct suffering caused by zoonotic diseases for which Norway rats act as reservoirs (for recent details of these Zoonoses in England, see Public Health England, 2015). In the UK at least, Norway rats do not present a physical threat to people unless cornered and unable to escape (Meehan, 1984). Rodents as vectors and / or reservoirs of zoonotic infections, and the direct and indirect routes by which these pathogens may be spread, are explored in detail by Meerburg, Singleton and Kijlstra (2009).

1.1.2.3 Ecological impacts

Norway rats, along with other members of the *Rattus* genus and house mice, are also viewed as pests due to their impact on native species when they become established as introduced species in areas to which they are not native, normally as a stowaway on human shipping. They may compete with native fauna for food or nest sites, indirectly harm native fauna by harming plants used as shelter, or directly prey upon native fauna and flora (Howald, Ross and Buckle, 2015). The best known of these is the depredations of invasive rodents upon the nesting sites of ground-nesting birds. An exhaustive review of Norway rat impacts on island seabirds is not possible here, but see Jones *et al.* (2008). Examples of these direct impacts by Norway rats in the UK are rare beyond their displacement of the similarly invasive black rat (Corbet and Southern, 1977), but both species were successfully eradicated from Lundy in

the Bristol Channel after their presence was linked to the loss of breeding Manx shearwater *Puffinus puffinus* and Atlantic puffins *Fratercula arctica* from the island (Lock, 2006).

1.1.3 Adaptations for commensalism

Norway rats are generalist omnivores, capable of surviving alongside humans anywhere in the world (Lund, 2015). They are highly athletic, being able to swim for 72 hours before tiring, and smaller individuals have been seen scaling sheer brick walls (personal observation). Norway rats are highly neophobic, displaying fear and avoidance of new objects in their environment, making them difficult to trap and / or poison (Barnett, 1958). This neophobia apparently differs between populations; in some areas of Hampshire, control of Norway rats using chemical rodenticides has failed entirely due to heightened neophobia (Brunton, MacDonald and Buckle, 1993). Due in part to their neophobia, Norway rats may travel several kilometres a night in order to access a trusted food source (Fenn, Tew and MacDonald, 1987). Norway rat burrows are typically shallow, but contain multiple concealed exits or "bolt holes" which allow egress when the burrow is disturbed (Pisano and Storer, 1948), thus making it difficult to cover all exits during the use of gas or ferrets to eliminate a burrow.

Norway rats have a physical predisposition towards being successful pests of man, but their reproductive systems also contribute to their enormous impact. Rodents such as voles and the murine rats and mice tend to be *r*-strategists (MacDonald, Fenn and Gelling, 2015), with high reproductive rates but usually poor survival of offspring. Norway rats produce a large number of offspring due to the combination of several factors within their reproductive behaviour: firstly the female gives birth after a gestation period of just 20 – 23 days (Meehan, 1984), resulting in the creation of a new generation within three weeks. Litter sizes are

typically from seven to nine but as many as sixteen is possible. In ideal conditions (i.e. not food or harbourage limited), females undergo a post-partum oestrus: they are ready to mate within hours of giving birth. This causes a slight delay in egg implantation (two to five days – Meehan, 1984), meaning that individuals have the potential to give birth to a new litter every 24 days. This is aided by the short weaning time: a minimum of 20 days. Furthermore, Norway rats reach sexual maturity at 8 – 12 weeks. This means that not only can one female produce a large number of offspring in a short amount of time, but generation time is minimal; at peak reproduction, this allows for seven new generations in two years. These two facets of their physiology enhance their potential to become pests, and to make them difficult to control, along with the neophobic behaviour they display (Barnett, 1958).

1.2 Pest control and pesticides

Pest control comes in three forms: physical, biological and chemical. Physical control includes directly hunting the pest species (or chopping down and burning in the case of plants), using traps to kill or capture individuals, or excluding pests from areas wherein they can cause harm to humans or damage crops. In the case of most rodents (and even smaller organisms), traps and hunting are often of only limited use due to the number of target individuals involved. Biological control involves introducing predators, parasites or pathogens of the pest species into the affected area. As a reduction of a pest's impact is the end desire of pest control rather than its extermination (Smith and Meyer, 2015), by this definition, the use of dogs and other aggressive domesticated animals as predator deterrents by livestock herders would be considered a form of biological pest control.

Lethal control tends to provide the fastest results in terms of measurable reduction of pest damage to goods, property and foodstuffs, resulting in a heavy emphasis on chemical

control: direct applicationof synthetic or biological chemicals designed to kill the target species. The first recorded forms of chemical pesticides were insecticides. By 2500 BC, the Sumerian people were using sulphur as an insect repellent, and records from 1200 BC apparently reveal the use of various insecticides to protect seeds from granivorous arthropods in China (Banaszkiewicz, 2010). Despite their potential advantages, however, chemical pesticides are also associated with a range of problems and limitations.

1.2.1 Problems arising with chemical control (including biopesticides)

Chemical insecticides, in their wide variety and lengthy history, provide many examples of the problems inherent with the use of chemicals (of both synthetic and biological origin) to control pests, in particular impacts on non-target species and resistance to the chemical agents themselves. See, for example, the various non-target impacts of the organochloride DDT (Carson, 1962; U.S. Department of Health and Human Services, 2002), and the numerous orders of insects now displaying resistance to the modern Pyrethroids (Dong, 1997; Schuler *et al.*, 1998; Chen *et al.*, 2010). Genetically modified (GM) maize, modified with the addition of the Cry1Ac gene from the bacterium *Bacillus thuringiensis* (utilisation of a micro-organism as a form of biopesticide), has been shown to cause toxicological effects in non-target monarch butterflies *Danaus plexippus* (Losey, Rayor and Carter, 1999; Mendelsohn *et al.*, 2003), while target species such as pink bollworm *Pectinophora gossypiella* (Bagla, 2010) and corn earworm *Helicoverpa zea* (Tabashnik et al., 2008) have shown resistance to the toxic effects of the gene. Target species resistance is not limited to just insects, or to direct chemical applications. Glyphosate is the active ingredient of many proprietary herbicides worldwide, resistance to which has been discovered in the pigweed *Amaranthus palmeri*, a pest of soybean, cotton and peanuts in the USA, sufficient to prevent 12 times the

recommended application rate achieving full control (Culpepper *et al.*, 2006). Controversy has surrounded recent evidence suggesting the chemical is linked to carcinogenic effects in mammals (Guyton *et al.*, 2015), despite previous evidence claiming it was safe (Giesy, Dobson and Solomon, 2000). Methicillin-resistant *Staphylococcus aureus*, a bacterium resistant to the penicillin antibiotics, was typically associated with hospital settings, until the mid 1990s when it was found in people across Europe, North America and Australasia who had not contracted it in hospitals, or been exposed to those who had (Raygada and Levine, 2009). Resistance to Myxomatosis, a disease caused by the *Myxoma* virus (another example of biopesticide), has been present in UK rabbits *Oryctolagus cuniculus* since at least the 1950s (Ross and Sanders, 1984). Because the virus could be passed from rabbit to rabbit (unlike chemical pesticides), there could be no regulation of its application, and therefore no conservation of susceptible individuals as in the refuge strategy sometimes employed concurrently with insecticide application (e.g. Tabashnik *et al.*, 2008; susceptibility is a required resource if chemical applications are to continue for any given pest species). The issues mentioned here indicate that further independent research is required to ensure both the safety and efficacy of many chemical pesticides.

1.3 Control of Norway rats

1.3.1 Traps and other physical control measures

Because of the very serious effects that Norway rats have as commensal pests, a wide variety of methods have been used to control them. The most effective, in terms of reducing their impact, is to proof any premises they might attempt to enter. This requires that: all materials they might come into contact with (including by digging, swimming, climbing and jumping) are harder than 5.5 on the Mohs scale (MacDonald, Fenn and Gelling, 2015); any apertures

be at most 25 mm wide (to prevent adult rats squeezing through, although doing so would not exclude house mice); all debris, cover and overhanging vegetation be removed; and that climbing guards be placed anywhere that is scalable (Smith and Meyer, 2015).

Another option is to remove the food incentive for commensal rodents, thereby reducing the scale of the infestation, and the survival rate of individual rats. For example, stored grain on farms is significantly negatively correlated with bait consumption rates during rodenticide trials (Quy *et al.*, 1992). However, making such changes is expensive, and for most farm buildings would require a complete rebuild; new residential and commercial buildings are typically easier to adapt or build to resist rodent invasion, but the removal of food from human housing and food outlets in urban areas is not an option. Therefore, this form of control is largely impractical on farms, and can be limited in more urban buildings. Conversely, the simple removal of vegetation and other farming equipment (e.g. pallets and other debris) that afford rats shelter through good housekeeping practices has the potential to significantly and cost-effectively reduce rat populations by increasing competition between individuals for food and refugia (Lambert *et al.*, 2008).

Shooting and trapping are still carried out in the UK and are certainly effective against individual rats, but provide no guarantee of gaining complete control of a Norway rat population. The combination of these two methods proved efficacious during the eradication of escaped coypus (*Myocastor coypus*) in East Anglia (Gosling and Baker, 1987, 1989), but in this case the target species was up to three times larger than the Norway rat, and inhabited a specific habitat type in a limited area of the country. In comparison, Norway rats are found throughout the country and are much smaller, more numerous and breed at a faster rate, making the likelihood of killing an entire population via trapping and shooting low. In addition, the operatives hired to extirpate the coypu in East Anglia were assured of a significant financial bonus upon completion; it could be argued that continuous control of

Norway rats represents a great deal of work for typical pest control officers (PCOs), and their complete extirpation might not actually be desired by the very people usually tasked with their control (Greaves, 1995). For individual farmers capable of effectively utilizing guns, traps and / or dogs (see below), combining these methods with good housekeeping practices (Lambert *et al.* (2008) may have some positive impacts. Recent evidence regarding the extent of Norway rat migratory behaviour is mixed (Haniza *et al.*, 2015; Berny *et al.*, in prep), but the threat of reinvasion suggests that where possible, physical control measures should be undertaken.

1.3.2 Biological control

Vertebrate predators of rats are widespread in the UK, and may target Norway rats outside of urban areas and farmsteads, but there are few species large enough to hunt rats regularly that live closely alongside humans. Domestic cats are often numerous in urban areas and on farms but it is unlikely that they can access refugia such as burrows and sewers. Although cats will kill and sometimes eat rats, it has been shown that the size of Norway rats prevents large adults being taken (Childs, 1986). In northern England, dogs are still used to hunt rats on farms but, as with shooting, there is no guarantee of killing every single rat in the population. Repeated visits might reduce numbers sufficiently for the rat impact to be negligible, but because of the relative effort required (both in time and manpower), it is unlikely that dogs are the future of rat control in the UK. Use of parasites or bacterial or viral diseases is not desirable, due to the risk this would present to domestic animals and humans. Given the breeding rates of rats and the inability to control the application of infectious diseases, it is possible that a situation would arise similar to that of the Myxoma virus in rabbits (see above).

1.3.3 Chemical control

Due to the greater number of individual rats that can be killed by a feeding at a single bait station (as opposed to a single kill trap), chemical rodenticides have proven to be the most popular, effective and cost-efficient method of lethal control (Hadler and Buckle, 1992). There are three main categories of rodenticides: acute, subacute and anticoagulant (Buckle and Eason, 2015). The modes of action of the acute and subacute rodenticides are highly varied, as are their active ingredients. Extensive summaries of the most commonly used chemical agents, including their modes of action and their histories, can be found in Meehan (1984) and Buckle and Eason (2015). Acute rodenticides are typically highly toxic and can, therefore, be extremely dangerous. For instance, thallium sulphate can be absorbed through the skin, and zinc phosphide is so toxic that it was originally with emetics (rats being incapable of emesis) in order to prevent death of non-target animals and humans during the targeting of rats (Krieger, 1952). It is still in use under the name "Ratron," registered for use in the control of field voles (*Microtus agrestis*). The quick acting effects of acute rodenticides means, however, that an individual ingesting a non-lethal dose would cause it to become sick rapidly but allow recovery; as a consequence, surviving animals learn to associate the symptoms they have suffered with the new food ingested (i.e. the bait) and avoid it in the future ("bait shyness"). Because of their toxicity and speed of effect, acute rodenticides represent a severe danger to non-target organisms including humans and domestic animals. Even the foul taste of rodenticides such as red squill is not a guarantee of non-target safety (Fitzpatrick, McGirr and Papworth, 1955). Most acute and subacute rodenticides also have no available antidote (e.g. "1080"; Krieger, 1952), or antidotes that can only be administered with a short time period after exposure (e.g. Sodium Fluoracetate can be treated by barbiturates within three hours; McGirr and Papworth, 1955). Due to these issues, the acute and subacute rodenticides are, for the most part, not authorised for use in the

UK (Buckle and Eason, 2015) or indeed the rest of the EU (European Community, 1998). Those that are, are usually ineffective against rats (see Buckle, 2013a) or, like calciferol, not widely used despite some success against resistant rats in Wales (Rennison, 1974) and more recent studies suggesting that in combination with coumatetralyl, it is effective against bromadiolone-resistant rats in Germany (Endepols *et al.*, 2017). As such, rodenticides used against rats in the UK are almost exclusively anticoagulants.

1.3.4 Anticoagulants: discovery and use

The discovery of anticoagulant rodenticides is rooted in a series of cattle deaths in Canada almost a hundred years ago. These cows were initially suspected to have a communicable haemorrhagic septicaemia (Schofield, 1984) but, in the absence of any causative bacteria, the deaths were linked to the consumption of spoiled sweet clover silage. The "sweet clover disease", as it was initially known (Link, 1959), was replicable by feeding cattle or sheep improperly cured sweet clover hay, after which their blood clotting power was reduced, and fatal haemorrhage occurred with 30-50 days. After years of studying spoiled sweet clover, a small spoonful of crystalline dicoumarol was extracted in 1939 (Link, 1959). It was established that coumarin, a naturally occurring bitter-tasting compound in many plants, formed a 4-hydroxycoumarin when oxidised; when naturally fermented (during the process in which sweet clover became spoiled), or when formaldehyde is added in the laboratory, dicoumarol is formed. During the ensuing years, dozens of compounds were synthesised from dicoumarol, including sodium warfarin in 1948, then known as Compound 42 (Mills, 1955). Warfarin was found to be much more potent as an anticoagulant than dicoumarol, and was promoted for use as a rodenticide. Because of its potency and the antidotal effects of vitamin K (Link, 1959), it was also promoted for use in humans, and in 1955 the President of the

United States, Dwight D. Eisenhower, was treated with warfarin after suffering a heart attack. Warfarin was found to be efficacious against Norway rats in formulations of only 0.005% (Bentley, Hammond and Taylor, 1955), and other anticoagulant rodenticides, of varying effectiveness, soon followed (Bentley and Rowe, 1956; Bentley and Larthe, 1959).

Furthermore, anticoagulant rodenticides do not possess many of the weaknesses associated with acute poisons. Their delayed and chronic mode of action (animals usually take at least two days to suffer symptoms) means that animals fail to associate symptoms of poisoning with the bait they have consumed, (Drummond and Rennison, 1973). The slow onset of symptoms also allows rodents to keep feeding on the bait until a large enough dose has been consumed to cause mortality (ideally by the onset of symptoms the rodent will have consumed a lethal dose). Therefore it is to be expected that if a pest animal overcomes its neophobia and finds an anticoagulant bait palatable, it will eat this bait until it dies. Anticoagulant rodenticides are also much safer because their slower mode of action increases the amount of time available to seek medical or veterinary attention and the antidote (Vitamin K) is readily available.

1.3.5 Anticoagulants: mode of action

1.3.5.1 Vitamin K cycle

Anticoagulants interfere with the vitamin K cycle, the process by which the body recycles vitamin K in its various forms (Figure 1.1), which takes place largely in the endoplasmic reticulum of liver cells (Olsen, 1984). The reduced form of vitamin K, Vitamin K hydroquinone, is a cofactor in a reaction in which a carboxylase enzyme catalyses the conversion of glutamate residues (Glu) in substrate proteins to gamma-carboxy-glutamate

(Gla) (Thijssen, 1995), thus beginning the coagulation cascade (see Figure 1.2), which results in the production of insoluble fibrin, forming a blood clot (Stenflo and Suttie, 1977). As part of its catalytic action, the carboxylase oxidises the hydroquinone, forming Vitamin K epoxide. The epoxide is recycled to vitamin K and then to hydroquinone by the enzyme Vitamin K-2, 3-epoxide reductase (VKOR); each molecule of vitamin K may be recycled in this manner up to 10,000 times. Anticoagulants act as antagonists for complex subunit 1 (VKORC1) of Vitamin K-2, 3-epoxide reductase (Rost *et al.*, 2004). The 4-hydroxycoumarins form a tightly bonded complex with the reductase which is effectively irreversible, because the enzyme is normally reactivated by a reduction reaction (Fasco *et al.*, 1983). Once the target animal's liver has become saturated and there are no more free enzymes in either anticoagulant-susceptible stage of the vitamin K cycle, the absence of hydroquinone will reduce the effectiveness of the carboxylation of the Glu residues, thereby preventing the initiation and completion of the coagulation cascade. The vitamin K antidote is efficacious because alongside the Vitamin K-2, 3-epoxide pathway there is a low-affinity quinone reductase enzyme which directly reduces vitamin K to the required hydroquinone (Whitlon, Sadowski and Suttie, 1978). The low affinity of this pathway, and the fact that when its use is required there is no recycling of hydroquinone, means that it is only of use when vitamin K can be obtained in high doses in order to stave off anticoagulant-induced haemorrhage. Both the enzyme (VKOR) and substrate (Vitamin K epoxide) can be limiting factors e.g.; the symptoms of anticoagulant poisoning can be stimulated in rats prevented from consuming their own faeces, an important source of dietary vitamin K (Mameesh and Johnson, 1959).

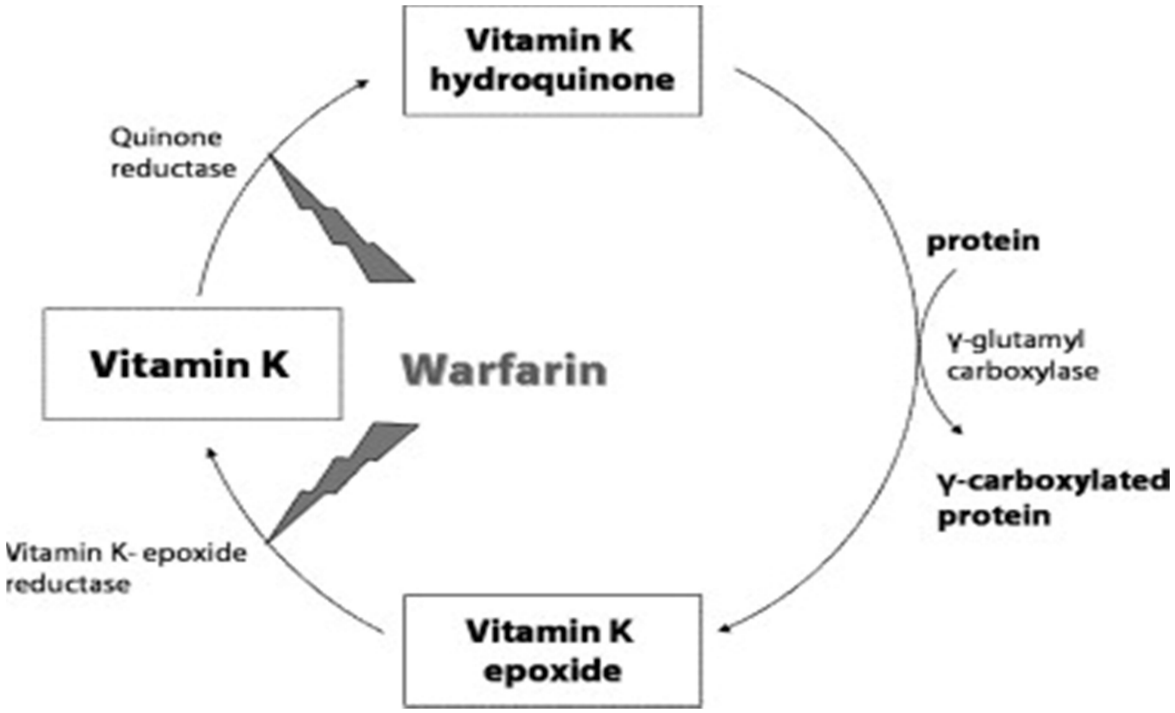

Figure 1.1: A simplified diagram of the anticoagulants' interference in the Vitamin K Cycle (from Kaesler *et al.*, 2012)

1.3.5.2 Coagulation cascade

The normal function of the vitamin K cycle starts the coagulation cascade, a series of reactions in which the inactive zymogens Factors II, V, IX and X are activated in turn to protein sereases Factors IIa, Va, IXa and Xa (Figure 1.2). Glu residues that are activated as part of the coagulation cascade are also found on the surface of proteins S, C and Z. The gamma-carboxylated residues on each clotting factor chelate Ca^{2+}, thus enabling them to bind to phospholipid bilayers on platelets, ensuring that clotting takes place at the damaged site. Each activated clotting factor then cleaves the inactive zymogen next in the cycle, thereby activating it and allowing it to bind to Ca^{2+}, thus continuing the series of reactions. The final stages of the reaction cascade, in which the extrinsic and intrinsic pathways converge, see Factors Va and Xa form a complex (prothrombinase) which activates prothrombin, forming thrombin. The activation of thrombin forms part of a positive feedback loop, activating Factors VIII and V. Thrombin also cleaves fibrinogen by proteolysis, forming small chains of

fibrin monomers. The fibrin monomers bind to adjacent chains, leading to polymerisation (Davie, Fujikawa and Kisiel, 1991). Finally, Thrombin also activates Factor XIII, which forms the covalent bonds that link fibrin polymers together, resulting in a blood clot. By preventing the carboxylation of Gla residues, anticoagulants cause an increase in the number of precursor zymogens (Harauchi *et al.*, 1986) because the activation process does not occur. When the Vitamin K-2, 3-epoxide reductase binding sites in the liver are saturated with anticoagulants, the multi-step coagulation cascade is not fully initiated. Therefore, any clotting factors circulating in the body decay, with varying half-lives (Kerins and MacNicoll, 1999). The delayed action of the anticoagulant rodenticides (which prevents bait shyness being engendered in target organisms) is a function of this decay; clotting time is not extended until any of the clotting factors falls below 10% of normal levels. The saturation in the liver also functions to extend the action of anticoagulant action of these poisons by forcing the liver to clear the anticoagulants in a biphasic elimination; excess anticoagulant must be cleared from the body before hepatic binding sites can release 4-hydroxycoumarin molecules without binding more of the same molecules (Huckle, Hutson and Warburton, 1988), thereby extending the anticoagulant effect.

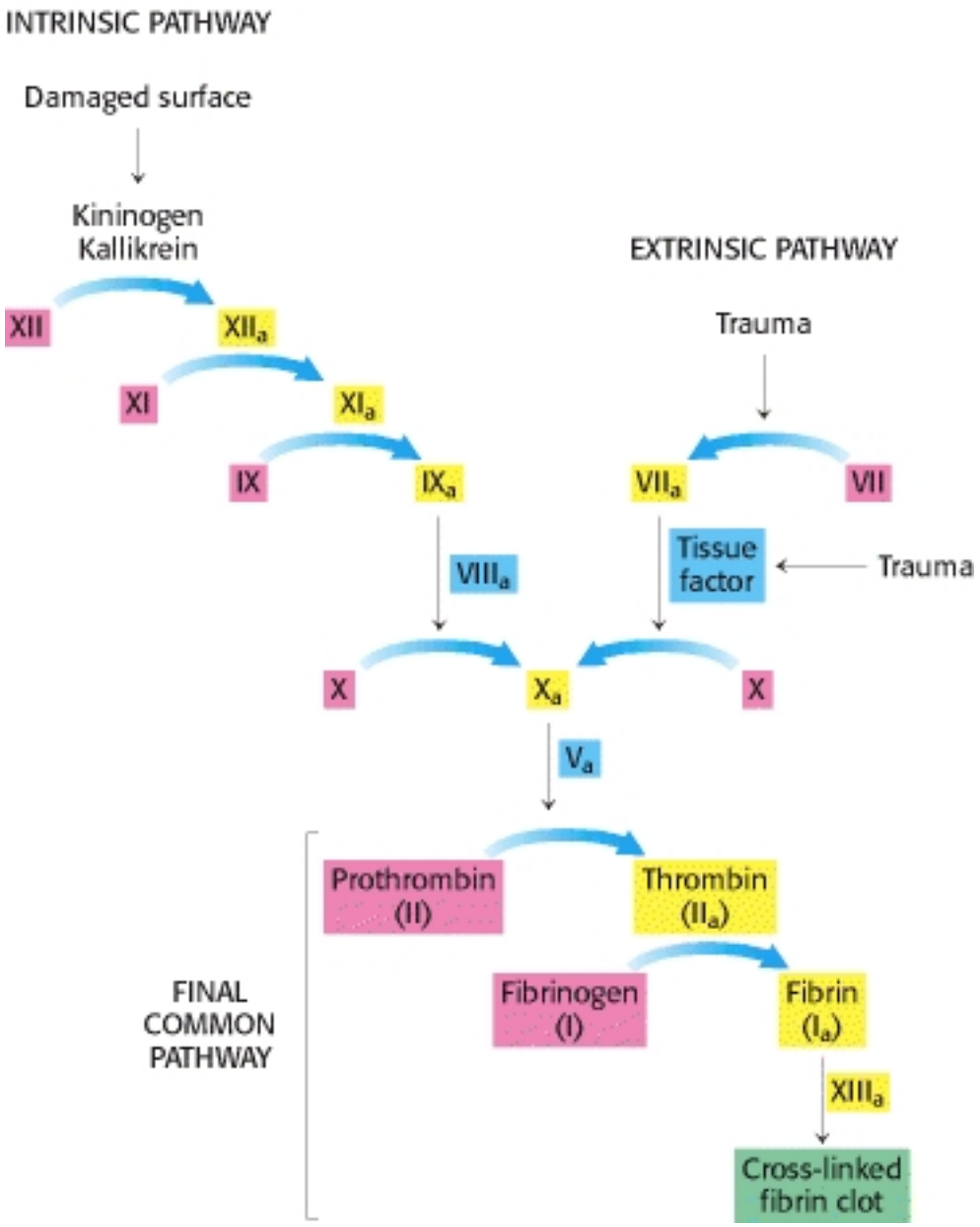

Figure 1.2: The coagulation cascade (from Berg, Tymoczko and Stryer, 2002)

1.4 Anticoagulant resistance in Norway rats

1.4.1 History of resistance to anticoagulant poisons

Boyle (1960) described how a population of Norway rats in Scotland were treated with diphacinone and warfarin, but despite good acceptance of bait, control was not achieved. Crucially the paper states that the treatments were conducted properly, leading to the

conclusion that the rats were not behaviourally resistant (i.e. bait shy), nor did the treatments fail due to poor housekeeping on the farm in question. Therefore, it is accepted that control of the population was not achieved because the animals in question were able to tolerate the anticoagulant to a previously unseen degree. The population in question was eventually eradicated but, within 18 months, a new infestation was found that also displayed warfarin resistance (Cuthbert, 1963). Rats from this infestation were captured and subjected to laboratory choice tests; although many were in fact susceptible, and died within a few days of feeding on 0.005% warfarin bait, the five (of 91) surviving rats went on to survive a 24-day period of no-choice feeding on 0.005% warfarin bait. Warfarin-resistant house mice were also found in Harrogate (Dodsworth, 1961) in the same year. A decade later, 20 farms were sampled randomly from within a 20 km^2 area centred on the original site of resistance in Scotland; every site which had rat infestations had rats which were, after warfarin feeding tests, classified as resistant (Brodie, 1976). Subsequently, many foci of resistance to the anticoagulants were found throughout the UK, with 14 foci of varying sizes identified (Greaves and Rennison, 1973).

Shortly after the discovery of resistance in Scotland, a large resistance focus was discovered on the Anglo-Welsh border. Resistant rats from this focus were identified through survival of a six-day no-choice feeding test with 0.005% warfarin (Bentley, 1968), and the level of resistance displayed was such that tests against warfarin and Vitamin K against the two strains, as well as their inter-bred offspring, confirmed through observation of grouped clotting times that Welsh and Scottish resistances were phenotypically and genetically different, although the full mechanisms of resistance were not yet understood (Greaves and Ayres, 1982). This form of resistance conferred such strong tolerance to the anticoagulants available that a containment zone was implemented around areas of confirmed resistance (Drummond, 1966). This belt of land had a total circumference of 160 miles and, within this

area, zinc phosphide was used as a countermeasure to the warfarin-resistant rats (Pamphilon, 1969). These measures appeared to be successful in preventing the spread of Welsh resistance, which otherwise appeared to spread outwards at the same rate that resistant rats could migrate (Drummond and Bentley, 1965; Drummond, 1970). Unfortunately, the 1967-68 outbreak of foot-and-mouth disease forced the cessation of the containment action and resistant populations of Norway rats were found outside the former perimeter shortly afterwards.

During the attempts to control the Welsh resistance outbreak, a six-day feeding test with warfarin bait at 0.005% strength was proposed as definitive test procedure for confirming resistance in individual rats (Drummond and Wilson, 1968); this was quickly adopted as the standard resistance test (Bentley, 1968; Telle, 1971; Brooks and Bowerman, 1973). Overall, 98% of anticoagulant-susceptible rats were shown to die after feeding periods of 3-4.82 days (LFP_{98}; Brooks and Bowerman, 1974). During the ten years following the first discovery of resistance in Scotland, resistance was also found in many sites in Denmark. In 1962, rats from two farms in Jutland were found to be resistant to warfarin when taken into the laboratory and subjected to feeding tests, with only a 33% mortality rate over multiple five-day feeding tests with 0.005% warfarin, and those that died survived longer than susceptible rats would typically (Lund, 1964). Resistance was found in more farms that year, with six individuals from these areas surviving warfarin feeding tests for 51 days (Lund, 1988). Further tests confirmed that Danish resistant rats were not simply warfarin-resistant; they possessed cross-resistance to all anticoagulants to some degree (Lund, 1966). By 1972, warfarin was no longer being used in areas of known resistance in Denmark (Lund, 1988). For ten years, anticoagulant resistance in Norway rats was not recorded outside of Denmark and the UK. Soon after this, however, multiple records of anticoagulant resistance in Norway

rats had been reported in the US (Brothers, 1972; Brooks and Bowerman, 1973), Germany (Telle, 1971) and the Netherlands (Ophof and Landeveld, 1969).

The first generation anticoagulant rodenticides (FGARs, including warfarin, coumatetralyl, chlorophacinone and diphacinone: reviewed by Brooks and Jackson, 1973) were used widely across Eurasia and North America, with different countries favouring different active ingredients (Bentley, 1972); where they failed, the acute rodenticides were still available as a last resort. During this early period, coumatetralyl was increasingly seen as superior to warfarin where resistance was occurring, but 100% control was not guaranteed (Bentley, 1968; Lund, 1969). Differences were noted between Norway rats and house mice, the two most problematic commensal rodents in areas where resistance was likely to be found, in their response to anticoagulants. Chlorophacinone was seen as very efficacious against house mice compared to warfarin (Lund, 1971), whereas against Welsh-resistant Norway rats it performed no better than warfarin (Bentley, 1968). However, as with Norway rats, coumatetralyl was shown to be more toxic to mice than warfarin, but could not be fully recommended against resistant animals (Rowe and Redfern, 1968). The situation was not helped by the slow and variable progression of the symptoms of FGARs, which caused a great deal of intra-strain variability in the response of rats to consumption of anticoagulant poisons, even among susceptible animals (Brodie, 1976; Chmela, Rupes and Privora, 1978).

By 1992, it was thought that the use of warfarin in most of Western Europe was unlikely to result in control (Myllymäki, 1995). As more resistance foci were discovered, and the severity of the problem was realised, the number of products available for use increased, and in the 1970s a more potent second generation of anticoagulant rodenticides (SGARs) were marketed (Hadler and Shadbolt, 1975); the first of these were difenacoum (Hadler, Redfern and Rowe, 1975) and bromadiolone (Marsh, 1977), both of which were claimed to be effective against animals resistant to FGARs. By the 1990s, however, many of the

differing foci had been shown to also be resistant against one or both of these SGARs (Redfern and Gill, 1978; Greaves, Shepherd and Gill, 1982; Greaves, Shepherd and Quy, 1982; Greaves and Cullen-Ayres, 1988; Cowan *et al.*, 1995; Quy *et al.*, 1995).

In addition to difenacoum and bromadiolone there are three other second generation anticoagulants: brodifacoum (Rennison and Dubock, 1978), difethialone (Lechevin and Poche, 1988) and flocoumafen (Bowler, Entwhistle and Porter, 1984). These are sometimes known as "resistance breakers" (Daniells, Prescott and Buckle, 2011; Buckle, 2013a) due to their higher toxicity and the fact that, to date, no Norway rats have been found with practical resistance to these compounds (Myllymäki, 1995). Due to their very high toxicity and persistence in the bodies of target animals (HSE, 1986), and consequently the perceived risk of primary and secondary poisoning of non-target species, in the UK they were limited only to use against rodents living primarily indoors until recently. Given the mobility of Norway rats and their wide-ranging feeding behaviour, this has made these more potent SGARs effectively illegal for the control of Norway rats. This is reflected in survey data from both urban and rural areas (Mcdonald and Harris, 2000; Dawson and Garthwaite, 2004; Dawson, Bankes and Garthwaite, 2003). The excessive use of frequently non-efficacious rodenticides has had severe consequences promoting resistance.

1.4.2 Mechanism of anticoagulant resistance

Resistance is a very technically precise phenomenon, and has been best summarised by Greaves, (1994): "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." This definition naturally precludes dietary based resistance wherein

anticoagulant-susceptible rats fed high protein diets have significantly reduced clotting impairment 53 hours after a lethal dose of anticoagulant, compared with rats fed normal diets (Colvin and Wang, 1974). There is also no room for naturally low susceptibility in this definition: For instance, wild-type susceptible house mice are naturally less susceptible to FGARs than are Norway rats and can survive higher doses of these rodenticides (Fisher, 2005). See Song *et al.* (2011) for in depth discussion of the Algerian mouse (*Mus spretus*), genetic introgression of which has been shown to confer a further form of genetic resistance to house mice. Tests using anti-bacterial drugs to remove gut bacteria which produce Vitamin K_2 showed that they do not influence the resistance status of individual rats (Lund, 1964), and that, therefore, the resistance mechanism interfered with the effect of the anticoagulants themselves, rather than bypassing them.

For over 40 years after the first discovery of anticoagulant resistance, the location of the resistance genes and their structures were unknown, and it was not possible to allocate a rat of unknown origin to a known resistance focus with certainty. However, a critical discovery was made when the location of the gene which causes resistance in rodents was identified. Pharmacodynamic resistance, in which animals display genetic mutations in the vitamin K-2, 3-epoxide reductase gene (VKORC1), is now thought to be the major mechanism of anticoagulant rodenticide resistance in Norway rats (Li *et al.*, 2004; Rost *et al.*, 2004, 2009). With the exons of the gene sequenced, a range of single nucleotide polymorphisms (SNPs) – mutations in the code caused by a single base pair changing and therefore coding for a different amino acid – were identified (Pelz *et al.*, 2005). Subsequently, the scientific literature has been largely concerned with sampling rat tissue and identifying the mutation(s) underlying some of the resistance foci across Europe and the UK and, where possible, determining the susceptibility of these individuals to the various anticoagulants

available (Lasseur *et al.*, 2007; Grandemange *et al.*, 2009, 2010; Prescott *et al.*, 2010; Baert *et al.*, 2012; Buckle, Klemann and Prescott, 2012; Meerburg *et al.*, 2014).

Nine SNPs have been found in wild Norway rats in the UK, and five of these are known to have a significant impact on the efficacy of some anticoagulants; no other country has populations of rats of each resistance strain established in the wild. This is likely explained by the fact that in other EU countries in which resistance is present, no heavy restrictions on SGAR use such as those in the UK have been in place (Buckle, 2013a). The five mutations are described here in the context of their known presence in the UK, and summarised in Table 1.1:

(1) Also known either as "Berkshire" or "Hampshire" resistance, the L120Q mutation confers a very strong form of resistance, known to be responsible for cases of practical resistance to bromadiolone and difenacoum (Redfern and Gill, 1978; Greaves, Shepherd and Gill, 1982; Gill *et al.*, 1993, 1994; Quy *et al.*, 1995), and technical resistance (resistance detectable in the laboratory, but with no practical effect in the field) to brodifacoum (Gill, Kerins and MacNicoll, 1992; Gill *et al.*, 1994). However, despite having an identical SNP, historically Norway rats from Hampshire and Berkshire were reported to have different levels of resistance to a variety of anticoagulant compounds (see Section 2.2.2.1). Recently, in a Hampshire site where treatments with large amounts of difenacoum and bromadiolone had failed over the course of two years, brodifacoum was used to quickly eradicate the population of rats in question (Meyer, 2009). Tests to determine the effect of this mutation on vitamin K requirement in rats have shown the Hampshire and Berkshire forms of this resistance to be very different in their effect (see Section 1.4.3). This mutation has recently been found in continental Europe (e.g. see Baert *et al.*, 2012).

(2) Also known as “Scottish” resistance, the L128Q mutation caused the initial resistance focus reported in 1959 when anticoagulant resistance in Norway rats was first identified. It is apparently unique in that the altered active site of Vitamin K epoxide reductase allows for a reversible bond (Thijssen, 1995) with some FGARs: the enzyme is reactivated in the same manner as it is after interacting with Vitamin K epoxide. In contrast, the other mutations described in this section reduce the likelihood of a bond forming at all, thereby increasing the dietary requirement for vitamin K. The SGARs are all thought to retain their efficacy against rats with this mutation (Hadler, Redfern and Rowe, 1975; Greaves and Ayres, 1982; Greaves and Cullen-Ayres, 1988); the altered Vitamin K epoxide reductase, when exposed to SGARs, has been shown not to reactivate (Thijssen, 1995). This mutation has also been found in parts of France (Grandemange *et al.*, 2010). Rats that possess this mutation are known to have a very high vitamin K requirement (see Section 1.4.3).

(3) Also known as “Gloucester” resistance, most of what is known of the impact of the Y139C mutation comes from work conducted in continental Western Europe. It has been found in Germany (Pelz, Hänisch and Lauenstein, 1995), the Netherlands (Meerburg *et al.*, 2014), France (Grandemange *et al.*, 2010) and Denmark (Lodal, 2001). As with L120Q, rats with this mutation appear to be largely resistant against bromadiolone and the FGARs (Endepols *et al.*, 2012) and, to a lesser extent, difenacoum (Buckle *et al.*, 2013) but can be controlled using brodifacoum baits (Buckle, Klemann and Prescott, 2012). It is believed that difethialone and flocoumafen baits would also be fully effective against this strain of rats. There have been numerous studies on the pleiotropic effects of this mutation, showing wildly different results (see Section 1.4.3).

(4) Only recently discovered in the UK (Prescott *et al.*, 2010), the Y139F (or “Kent”) mutation is widespread in North-Western Europe (Buckle, 2013a). Most of what is known

about this mutation's impact on efficacy comes from work conducted in continental Western Europe. Rats with this mutation are known to be resistant to FGARs, bromadiolone and difenacoum (Grandemange *et al.*, 2009). An older study showed that infestations of resistant rats in Kent could be controlled with difenacoum (Rennison and Hadler, 1975), but because this experiment took place 30 years before the discovery of the Y139F mutation, it cannot be confirmed that the rats in question possessed it or not.

(5) Until recently, the Y139S mutation had only ever been found in one focus on the Welsh-Shropshire border, and consequently has become known as "Welsh" resistance. One individual with this mutation was found in Yorkshire (Haniza *et al.*, 2015), but the authors admit this might have been misidentified. Due to repeated failures to control it upon its first discovery (Bentley, 1968; Greaves, 1994), it is not known how large this focus is. Rats with this mutation are known to be resistant to FGARs but are effectively controlled with all SGARs in the field (Rennison and Hadler, 1975; Rennison and Dubock, 1978; Richards, 1981; Greaves and Cullen-Ayres, 1988; Buckle, Endepols and Prescott, 2007). As with the L128Q mutation, this mutation is associated with serious pleiotropic costs to rats that possess it (see Section 1.4.3).

Table 1.1: Five mutations of exon 3 of VKORC1 that confer practical resistance to Norway rats

Original location in UK	**Mutation**	**Wild-type amino acid (nucleotides)**	**Mutation amino acid (nucleotides)**
Berkshire/Hampshire	L120Q	Leucine (Cytosine-Thymine-Guanine)	Glutamine (Cytosine-Adenine-Guanine)
Scotland	L128Q	Leucine (Cytosine-Thymine-Guanine)	Glutamine (Cytosine-Adenine-Guanine)
Gloucester	Y139C	Tyrosine (Thymine-Adenine-Thymine)	Cysteine (Thymine-Guanine-Thymine)
Kent	Y139F	Tyrosine (Thymine-Adenine-Thymine)	Phenylalanine (Thymine-Thymine-Thymine)
Wales	Y139S	Tyrosine (Thymine-Adenine-Thymine)	Serine (Thymine-Cytosine-Thymine)

1.4.3 Pleiotropic costs of resistance

The altered state of vitamin K-2, 3-epoxide reductase in resistant rats means that coumarin derivatives such as warfarin no longer act as antagonists, but it also affects the animal's vitamin K cycle in the absence of anticoagulants. In many cases, resistant animals suffer deficient vitamin K metabolism due to the VKOR enzyme's reduced activity (Pelz *et al.*, 2005) or binding affinity for both anticoagulants and vitamin K epoxide (Bell and Caldwell, 1973). They therefore have a greater dietary requirement for vitamin K than susceptible animals (Greaves and Cullen-Ayres, 1988; Markussen *et al.*, 2003), with animals homozygous for resistance mutations affected to a much greater extent than heterozygous individuals (Greaves and Cullen-Ayres, 1988; Jacob *et al.*, 2012).

The pleiotropic costs of resistance mutations are highly variable between strains. Welsh-resistant rats (Y139S) have been extensively studied in this regard. When left to breed freely, the number of males in Welsh-resistant litters very quickly starts to drop significantly compared to the number of females (Bishop, Hartley and Partridge, 1977). When this strain of rats is maintained on a low vitamin K diet and blood-prothrombin levels measured, concentrations in resistant animals diminished at a significantly faster rate than did those of susceptible animals (Bishop, Hartley and Partridge, 1977). In similar experiments, Welsh heterozygotic, homozygotic and susceptible rats were maintained on varying dietary levels of vitamin K in order to determine the effect on their resting one-stage prothrombin time (PT); it was found that heterozygotes required 2-3 times more vitamin K in their diets than susceptible rats in order to maintain a similar PT, while homozygotes required more than 10 times as much (Hermodson, Suttie and Link, 1969). These experiments were replicated and shown to be even more profound in the case of male homozygotes, some of which required up to double the dietary vitamin K of female homozygotes. These experiments also showed that coprophagy is an important source of vitamin K for anticoagulant-susceptible and

heterozygous Norway rats, but this behaviour could not supply the requisite vitamin K for homozygous-resistant animals. A heavily resistant population of Welsh-resistant rats in the field showed a significant decline in the proportion of resistant animals present during an 18-month hiatus in the application of FGARs (Partridge, 1979).

Further experiments showed that litters of Norway rats from two heterozygotic-resistant parents suffered significantly more mortality than those from one susceptible and one heterozygotic-resistant parent (Partridge, 1980). Scottish-resistant (L128Q) subadult males rats were found, even when supplied with vitamin K_3 (menadione) in their water, to suffer from reduced growth rates compared with Welsh-resistant male rats of the same age, which in turn had reduced growth rates compared with susceptible male rats (Smith, Townsend and Smith, 1991). An earlier study showed, however, that Scottish resistant rats (known then as HS rats) require lower levels of dietary vitamin K than do Welsh resistant rats (known then as HW rats) in order to prevent prothrombinaemia (Greaves and Ayres, 1973).

There have also been several studies on Danish resistant rats (Y139C aka "Gloucester resistant"). Here, evidence for pleiotropic costs is mixed. One study has suggested that the mutation results in reduced litter sizes (Jacob *et al.*, 2012); resulting in a gradual reduction in the proportion of resistant animals in a population (Lund, 1966). However, a further study on resistant rats from Denmark found no costs associated with the breeding of resistant rats in the absence of treatment (Heiberg, Leirs and Siegismund, 2006), while a population study that took place over two years found that the prevalence of resistance did not fall for the duration (Siegismund, Heiberg and Leirs, 2002); however, it was acknowledged that the menadione in the lab-pellets used may have influenced this.

Despite the increased survivability and population fitness conferred by vitamin K-rich food sources, it has been shown that rats from Germany which have very high pleiotropic

costs conferred by their specific resistance mutation (despite having the same resistance mutation as rats from Denmark - see Section 3.1.4.3; Jacob *et al.*, 2012) do not select food sources based upon their vitamin K content, and therefore do not preferentially seek out dietary vitamin K despite their high requirement for it (Jacob and Freise, 2011). Hampshire-resistant rats (homozygous for the L120Q mutation) have a dietary vitamin K requirement up to four times greater than that of Welsh-resistant rats (Greaves and Cullen-Ayres, 1988). Studies previously carried out at the University of Reading contradict these findings: suggesting that Hampshire-resistant rats, when fed a vitamin K deficient diet, suffer less prolonged prothrombin times than do Welsh-resistant rats on the same diet; and that Berkshire-resistant rats suffer prolonged prothrombin times when fed vitamin K deficient diets (Pelz and Prescott, 2015). A subsequent study showed resistant rats from southern England (their exact provenance is not stated) suffered no size-related costs compared with susceptible animals (Smith *et al.*, 1993), indicating that they are capable of social dominance in populations of mixed resistance status (Brooks and Jackson, 1973). To compound the confusion around this, it has been clearly shown that any pleiotropic costs of resistance are readily offset by the typically high levels of menadione in animal feed on farms, and that farm animal feed even increases the selection of resistant rats in the presence of anticoagulant treatment, because it has no antidotal effect on susceptible rats (MacNicoll and Gill, 1993b).

1.4.4 Practical impacts of resistance

The greatest, most immediate, impact of anticoagulant resistance is the reduction in effectiveness of anticoagulant treatments (Cowan *et al.*, 1995; Quy *et al.*, 1995; Meyer, 2009). Failure to control rodent populations can result in spoilage and loss of foods and goods, zoonotic disease transmission and direct harm to vulnerable species as described

above. Invariably, subsequent attempts to control rodent outbreaks are implemented such that, regardless of the success or failure of these further attempts, more money will be spent on eradicating or controlling resistant populations than susceptible populations. In this manner, anticoagulant resistant Norway rats cost farmers and landowners money both directly and indirectly. The prevalence of resistance resulted in the development of the SGARs, and the requirement for new baiting techniques utilising more rodenticide bait than ever before, such that concerns have been raised that resistance is actually a financially lucrative problem for the pesticide industry, at the cost of those people living with rodent infestations in their homes and / or workplaces (Greaves, 1995).

As with many chemical pesticides, the use of anticoagulant rodenticides has led to both lethal and non-lethal exposure of non-target organisms due to accidental misuse and / or deliberate abuse of these baits. Because most vertebrates have similar physiologies, pesticides targeting vertebrates often have non-target impacts (Smith and Shore, 2015). Mammals and birds have the same coagulation pathway (Gentry, 2004), albeit with varying levels of the blood clotting factors described above, and are therefore vulnerable to non-target poisoning. Due to their high toxicity and the perceived danger they present non-target species, the resistance breaking SGARs were, until recently, restricted solely for use against rodent infestations in the UK that lived primarily indoors, making them effectively illegal to use against Norway rats. Bromadiolone and difenacoum, on the other hand, were authorised for use “indoors and outdoors”. This lack of restriction on their use resulted in massive use of these compounds in rural areas, (Mcdonald and Harris, 2000; Dawson, Bankes and Garthwaite, 2003). This over-use may have been further exacerbated by their ineffectiveness in many areas of the UK, thereby resulting in severe exposure of non-target wildlife.

Victims of primary non-target poisoning (whereby animals directly consume anticoagulant rodenticides; Smith and Shore, 2015) are usually rodents that, due to the

relatively large size of Norway rats, are able to access any bait point / box that a rat can. Bank voles *Clethrionomus glariolus*, field voles and wood mice *Apodemus sylvaticus* are all potential victims of primary poisoning, and their numbers fell by an average of 56% in a series of field trials using coumatetralyl to target Norway rats at farms and pheasant *Phasianus colchicus* feeding sites (overseen by gamekeepers) in the UK (Brakes and Smith, 2005). However, it is important to note that the use of coumatetralyl in this experiment, selected to reduce the potential risk to birds, may have exacerbated the impact on non-target animals; three of the VKORC1 mutations present in Norway rats in the UK are thought to confer practical resistance to coumatetralyl (Y139C; Endepols *et al.*, 2007; Y139S and L128Q; Buckle, 2013a), and the other two are known to confer strong resistance to SGARs (e.g. Y139F; Grandemange *et al.*, 2009; L120Q; Gill, Kerins and MacNicoll, 1992) Therefore, it is unlikely that coumatetralyl would effectively control the rat population, leading to an increase in the volume of coumatetralyl used, thereby exacerbating the impact on non-target animals.

Victims of secondary non-target poisoning are predators or scavengers that hunt, or consume, the bodies of animals that have themselves consumed anticoagulant rodenticides (Smith and Shore, 2015). Anticoagulants are highly persistent in mammalian tissue, particularly the liver. For example, brodifacoum has a half-life of 307.4 days in the liver of house mice, and other SGARs have liver half-lives of between 28.1 and 93.8 days (Vandenbroucke *et al.*, 2008). Therefore, animals which survive the consumption of rodenticide baits (a figure which is directly influenced by the level of resistance in commensal species in the UK) present an amplified risk to predators and scavengers (Shore *et al.*, 2015) as do other animals that consume baits and survive (or die). Resistant rats from south-east England, which are more likely to survive exposure to anticoagulants than are susceptible rats, have also been shown to absorb and retain (due to higher consumption)

significantly more difenacoum than susceptible conspecifics (Atterby, Kerins and MacNicoll, 2005).

Species living in close proximity to humans are especially at risk; red kites *Milvus milvus* have been shown to be highly active around farm buildings, typically scavenging dead rats in one farm site within 12 hours (Ntampakis and Carter, 2005). Of 23 red kites found dead and handed in to the Predatory Bird Monitoring Scheme (PBMS) between 1994 and 2005, 17 (73.9%) contained residues of at least one anticoagulant rodenticide, with an average concentration in the liver of 0.166 mg/kg. Summary data from The Toxicology Laboratory of the College of Veterinary Medicine (Lyon, France) 1992 – 2002 in France are presented by Berny and Gaillet (2008), and further suggest a correlation between anticoagulant rodenticide use and residue presence in birds of prey carcasses submitted for analysis. Red foxes *Vulpes vulpes* are also predators of commensal rodents and, depending upon available prey, may frequently have SGAR liver residues (Tosh *et al.*, 2011a). Non-target animals that consume rodenticides may also provide a route of contamination for secondary poisonings; given the rate of exposure of non-commensal rodents (Brakes and Smith, 2005) and the distances they can carry an anticoagulant load (sufficient to leave the boundaries of the farm on which they were exposed – Tosh *et al.*, 2012), it is not surprising that species such as polecats *Mustela putorius* (Shore *et al.*, 1996), barn owls *Tyto alba* (Walker *et al.*, 2008), weasels *Mustela nivalis* and stoats *Mustela erminea* (McDonald *et al.*, 1998) are all frequently found with liver residues of SGARs.

In addition, although rarely active during the day, Norway rats have been shown to lose their normal nocturnal behaviour when suffering from anticoagulant poisoning, resulting in their being readily available as prey (Cox and Smith, 1992) for both diurnal and nocturnal predators. This may therefore be the causal factor in the high numbers of kestrels (diurnal hunters of mice and voles) that have been were found to contain SGAR residues (65% of

individuals analysed between 2001 and 2005; Shore *et al.*, 2007); wood mice, known to be able to carry up to 0.64 mg/kg brodifacoum in their livers (Tosh *et al.*, 2012), and bank voles respond in the same manner (Brakes and Smith, 2005), thereby representing a contamination pathway for diurnal predators. This does not, however, explain the number of sparrowhawks *Accipiter nisus* found with anticoagulant contamination in Scotland between 2000 and 2010; despite restricting their predation to birds, they had similar rates of anticoagulant contamination to species that prey on rodents (Hughes *et al.*, 2013). The absorption of environmental anticoagulant rodenticide residues by earthworms may represent a pathway of exposure from passerines to sparrowhawks (Booth *et al.*, 2003; Liu *et al.*, 2015).

Furthermore, the use of liquid chromatography mass spectrometry (LCMS) has enabled researchers to identify SGAR residues in invertebrates (Brooke *et al.*, 2013), and suggests that they represent a contamination pathway for insectivorous mammals such as the European hedgehog *Erinaceus europaeus* (Dowding *et al.*, 2010). The increased sensitivity of this methodology, relative to high performance liquid chromatography (HPLC), may also come to reveal the extent of non-target poisoning in the UK to be worse than previously thought (Dowding *et al.*, 2010). A recent study utilising HPLC has shown slugs to represent a high-risk secondary exposure pathway hedgehogs, common shrews *Sorex araneus* and starlings *Sturnus vulgaris* (Alomar *et al.*, 2018).

The actual effect of the extensive non-target contamination on its victims in the UK is, however, ambiguous. The number of residues found is influenced by a variety of factors, including operational bias. The Wildlife Incident Investigation Scheme (WIIS) is a statutory organisation set up to investigate instances of suspected poisoning of UK wildlife (Brown *et al.*, 1996), but due to the nature of its investigations, only animals that died of poisoning (or appeared to have died of poisoning) are recorded, potentially inflating the apparent rate of non-target poisoning in the UK. Meanwhile, PBMS data for 2007-2008 state that of 98 barn

owls examined in those years, 79 contained residues of at least one SGAR, and 53 contained residues of multiple SGARs. Of these 98, >80% had sum liver residues of <0.08 mg/kg of all SGARs (Walker *et al.*, 2010). These data rely on opportunistic sampling, and therefore cannot be used as an estimate of total populations exposed to anticoagulant rodenticides. In laboratory tests, it was found that the lethal range for SGAR residues in barn owl livers began at > 0.2 mg/kg (Newton *et al.*, 1999), however it is possible that sub-lethal liver concentrations do impact individual fitness and therefore indirectly contribute to mortality. Furthermore, barn owls are one of many species secondarily exposed to SGARs, and data from France show wild foxes, buzzards and red kites suffering mortality as result of SGAR exposure (Berny *et al.*, 1997; Berny and Gaillet, 2008).

In addition, according to Buckle, (2013b) none of the species often found to be victims of non-target poisoning in the UK are of conservation concern, or even thought to be decreasing (however, see Fournier-Chambrillon *et al.*, 2004 for evidence of residues found in critically endangered European mink *Mustela lutreola* in France). The lack of central (i.e. governmental) co-ordination among the various governmental schemes, NGOs and industry means that it is likely that instances of poisoning go under-reported in the UK (Sainsbury *et al.*, 2001). The negative effect of the misuse of anticoagulants in concert with Norway rat resistance is avoidable and, given the lack of humaneness associated with the symptoms and effects of anticoagulant poisoning (Mason and Littin, 2003), its prevention is desirable.

1.4.5 Stewardship scheme

With the impending (31st October 2014 – 31st January 2017) expiry of all anticoagulants from the Biocidal Products Directive (BPD; Buckle, 2013a), and the continued issues of resistance, a new system of rodent control was required. From the 1st April 2016, the UK rodenticide

stewardship scheme, co-ordinated by the Campaign for Responsible Rodenticide Use (CRRU), was put into effect (Buckle *et al.*, 2017). The concept behind the scheme is simple: by shifting the responsibility of responsible rodenticide use to the users, and requiring proof of competence from them, the capability of users to deal with resistant Norway rat populations would be enhanced, as would their knowledge on how to reduce incidences of non-target poisoning. It is thought that by enabling users to access any SGAR and changing their patterns of use, a greater impact can be had on rat infestations whilst simultaneously minimising negative environmental/non-target impacts (Eason *et al.*, 2001). At the time of writing (April 2017), the "use by period" (a grace period in which products purchased before proof of competence was required could be utilised) has ended. From this point on, all rodenticide products that fall under this stewardship regime will carry the following statement: "For supply to and use only by professional users holding certification demonstrating compliance with the UK rodenticide stewardship scheme requirements". A survey of farmers in Northern Ireland found that the majority of farmers who use rodenticides do not search for or remove rat carcasses following rodenticide treatments, and do not remove bait in response to rat population decline (Tosh *et al.*, 2011b). Only 1% of farmers surveyed had attended a training course on the safe use of anticoagulant rodenticides (Tosh *et al.*, 2011b), so the advent of the stewardship scheme was both necessary and timely.

Foxes in Ireland, where use of brodifacoum and flocoumafen is much more prevalent than in the UK, have a higher rate of flocoumafen and brodifacoum residues than do the foxes of the UK (Tosh *et al.*, 2011a). Given that data from the UK show that non-target residues are linked with anticoagulant usage (Walker *et al.*, 2010; Hughes *et al.*, 2013), it is reasonable to assume that incidences of non-target poisoning involving the resistance-breaking SGARs will have increased in the year since the stewardship scheme was introduced, regardless of the increased level of "competence" among users. As the sales of

brodifacoum, flocoumafen and difethialone increase, especially now that old rodenticide stock can no longer be used, monitoring this issue as it continues to develop will be an important measure of the success of the scheme in its aims. CRRU has already nominated the barn owl as its sentinel species for this work (Buckle *et al.*, 2017), but monitoring of other species will also be necessary in the coming years.

1.4.6 Anticoagulant resistance in Norway rats in the south-east of England

Loss of fitness due to pleiotropic costs as described above is thought to have little impact in areas where resistance is prevalent, because there are few susceptible animals present to compete with resistant individuals (CSL, 2002). The established presence of several of the resistance-conferring VKORC1 mutations in the south-east of England (Pelz *et al.*, 2005; Prescott *et al.*, 2010; Haniza *et al.*, 2015), in combination with the frequency of recorded treatment failures and apparently resistant populations (Greaves, Shepherd and Quy, 1982; Cowan *et al.*, 1995; Quy *et al.*, 1995; Meyer, 2009) suggests that anticoagulant resistance in Norway rats in the south-east of England has reached a point where susceptible rats are so few and far between that, with limited gene flow between populations (Haniza *et al.*, 2015), it can only be dealt with by successfully controlling the strongest forms of resistance in Norway rats. Prior to the commencement of the stewardship scheme described above, the Rodenticide Resistance Action Group (RRAG, 2012) suggested that in Hampshire, Berkshire and parts of several other southern counties, control of Norway rats by legal chemical means was impossible, and should not be attempted.

1.5 Aims of the current study

1.5.1 Locate and delimit resistance foci

It can with be stated with complete certainty that in the UK there are at least five resistance-conferring mutations in Norway rats that have significant effects upon efficacy of anticoagulant rodenticides in the field, and that the three mutations that confer the greatest level of resistance can all be found in the south-east of England. However, that is the limit of what can be said with confidence. Whilst we know where there is at least one focus of each of the three strongest mutations present in south-east England, what we do not know is the precise extent of each focus, and indeed exactly how many foci of each mutation there are. It is important to try to delimit each focus, in order to understand how much of this area is affected by these mutations. For instance it is known that the Y139F mutation is present in Kent, and it is thought to be impossible to achieve complete control over rat populations with this mutation with bromadiolone and difenacoum (Grandemange *et al*., 2009). Therefore it is suggested that knowing that this mutation (and others like it) occurs in Kent is not enough; the extent of this focus needs to be explored, as does the possibility that other foci of the Y139F mutation exist. If this mutation appears in – or spreads to – geographical locations in which the SGARs difenacoum and bromadialone are still currently used this will serve to increase the proportion of rats with the mutation in the local population, thereby reducing any chance for control in the near future and exposing non-target wildlife to an increased risk of lethal secondary poisoning. As stated above, it is currently thought that there are five mutations in the VKORC1 gene conferring significant resistance to wild Norway rats in the UK. It is necessary therefore to keep up to date with the locations of all of these mutations in the UK, as the presence of a mutation previously unknown in a given area could lead to treatments with inappropriate (i.e. non-effective) rodenticides and therefore result in an

increase in number of resistant rats and exposure of non-target wildlife to secondary and / or primary poisoning. Most, if not all, of what we know of the practical effects of resistance mutations comes from wild examples of animals from the various foci undergoing laboratory tests. For this reason we already have an indication of what effect a known mutation will have when a population of resistant rats undergoes anticoagulant rodenticide treatments. What is not known is the effect of two foci extending outwards and crossing over, allowing rats with different resistance conferring mutations to interbreed. This situation has already arisen in the field (Haniza *et al.*, 2015), and identifying further areas where this may occur, or has already, will be invaluable given the established presence of the strongest resistance mutations in the south-east of England (Pelz et al., 2005; Prescott *et al.*, 2010; Haniza *et al.*, 2015). Using maps to display the locations and size of resistance foci may also allow subsequent studies to utilise data from the PBMS and WIIS to quantify the effect that rodenticide resistance has on non-target exposure to rodenticides. The extent to which resistance exacerbates non-target effects is not yet known, and would be useful for the prevention and reduction of further harm to non-target wildlife.

1.5.2 Establish which anticoagulants are safe to be used

In order to directly control the highly resistant Norway rat populations of south-east England, the level of resistance conferred by the dominant mutation(s) in the region must be established. Within a decade of the discovery of anticoagulant resistance in Norway rats and house mice, wild and laboratory rodents were subjected to laboratory tests to quantify and qualify the effect of resistance (Drummond and Wilson, 1968; Rowe and Redfern, 1968). With the advent of the UK rodenticide stewardship scheme, the usage of the resistance breaking SGARs is likely to have increased, and to continue increasing. It is imperative

therefore that information regarding the effect of each of the SGARs on the resistant rats of south-east England is made available, in a standardised form, for the first time. These laboratory experiments will build on the work of Prescott *et al.* (2007) to provide resistance factors for resistant strains of rats, as well as effective doses of the relevant anticoagulants.

1.5.3 Confirm their impact in the field

Because exact SGAR doses (or the equivalent bait consumption) cannot be guaranteed in the field, and the genetic profile of a population of wild rats is unlikely to be as uniform as that of a laboratory strain, the results obtained in a laboratory test are not always an accurate representation of what will occur in the field. Utilising the results obtained in laboratory experiments to determine the resistance factors for the SGARs against the resistant rats likely to be found in the south-east of England, suitable locations and anticoagulant rodenticides will be identified for brodifacoum field trials. These field trials will explore the practical, real-world impact of resistance on rodent control using a variety of SGARs, including those only recently cleared for use under the stewardship scheme (Buckle *et al.*, 2017). By recording the volume of total active ingredient consumed during the trial and monitoring tracking patches in order to determine Norway rat activity, these trials will allow us to estimate the impact of the stewardship scheme on rodent control success throughout the UK. In addition to elucidating the feasibility of rodenticide use in the south-east of England, the results of these field trials may confirm the findings of the laboratory experiments – if they do, it will allow future users to safely rely on the results similar experiments using other SGARs and other strains of resistant rats to predict likely outcomes in the field.

Chapter 2: Methods

2.1 Identifying and delimiting resistance foci

2.1.1 Resistance identification

The first discoveries of anticoagulant resistance in Norway rats centred on failed rodenticide treatments in the field during attempts at control. Nearly 60 years later, this would still appear to be the method most commonly used by PCO (or other stakeholder) to establish that a wild populations of rodents contain individuals that are resistant to certain anticoagulant rodenticides; primarily because of financial and temporal constraints, PCOs are reluctant to delay attempts at controlling rodent populations in order to ascertain whether or not any of the animals on site carry a resistance mutation. This is an unreliable method of determining whether or not resistance is present, for a variety of reasons. Firstly, rats may avoid consuming anticoagulant bait due to neophobia or the presence of alternative food sources (Quy, Shepherd and Inglis, 1992); before resistance is confirmed, it must be established that the bait in question has been properly and effectively used and sufficient bait uptake to cause mortality has occurred (Quy *et al.*, 1992). Reinvasion by rats or the presence of other rodents may also provide false-positive evidence of survival of consumption of rodenticide bait. If during a rodenticide treatment it appears that resistance is present, the time taken to establish this via failure to achieve control despite following proper procedures, may be sufficient for negative impacts of the application to occur (such as selecting for resistant rats within the population and exposing predators of rats and non-target rodents to secondary poisoning). A further problem with this method is that it provides little information regarding the strength of the resistance conferred by the mutation, and the prevalence of the mutation within the

population. The only information provided is survivability at a single concentration of a single active ingredient, with an unknown feeding period.

2.1.2 Molecular techniques

For many years, the phrase “cross resistance” was used in reference to different strains of rats and mice for which resistance against multiple anticoagulants could be demonstrated. In 2004, three exons of the VKORC1 gene were identified and sequenced, and were believed to code for functional components of the enzyme vitamin K epoxide reductase (Rost *et al.*, 2004; see Section 1.3.5.1) and a series of anticoagulant resistance-conferring mutations were discovered. These mutations were shown to be responsible for the known resistance foci across Europe. Crucially, all the known major resistance mutations were subsequently found to be located on the third exon of VKORC1, confirming that every mutation that confers resistance to a given anticoagulant will confer resistance to all other anticoagulants. Some of these resistances may only be technical resistance, where there is no discernible effect on treatment outcome.

Molecular techniques are most often used to analyse a small amount of blood, tissue or faecal matter from a rodent. These methods are advantageous as they do not involve the use of live animals. Methods which rely on the response of animals to direct application of anticoagulants are both time consuming and relatively inhumane due to the stress caused by exposing wild animals to unfamiliar environments and handling by researchers. The molecular technique most often used is the Amplification Refractory Mutation System (ARMS) Polymerase Chain Reaction (PCR), which determines the presence or absence of a single mutation in wild rodents, as in studies from Germany (Pelz *et al*., 2005), Denmark (Heiberg, 2009), and Belgium (Baert *et al*., 2012). In the present case, all three exons of

VKORC1 were sequenced to determine for which (if any) VKORC1 mutations each rat was positive. Assuming that fresh tissue or faecal matter from wild rats is readily available, these tests are ideal in terms of humaneness, because no laboratory-housed animals are required whatsoever. From March 2009 to September 2015, tissue and faecal samples were collected by researchers with a focus on the south-east of England. VKORC1 exons 1, 2 and 3 were sequenced and subsequently generated chromatograms were analysed individually by eye for all SNPs. Primers were generated using Primer3web 4.0.0 (Koressaar and Remm, 2007; Untergasser *et al.*, 2012). Primers used are detailed in Section 3.2.5.

2.1.3 Obtaining samples

2.1.3.1 Tail samples

As of March 2009, the University of Reading and the University of Huddersfield had entered into an agreement with industry representatives to analyse Norway rat tissue samples supplied by pest controllers. Samples sent to the University of Reading were to be from the west Midlands, East Anglia and central-southern England. Following the decision in 2012 for the University of Reading project to focus on a contiguous area in the south-east of England, on behalf of an industrial partner (Killgerm), the focus shifted to obtaining fewer samples from a greater number of sites, spread evenly across the south-east of England. While this increased the chance of recording false negatives (obtaining tail samples from susceptible rats in a population where a resistance mutation was present in some, but not all rats), it also maximised the chances of accurately delimiting resistance foci. From April 2012 until September 2015, tail samples were obtained from rats that had been live trapped and killed, found in kill-traps or killed directly using dogs or firearms. The last ~five cm of tail was removed with a clean blade, and stored in 5 ml vials. These were filled with industrial

methylated spirits (IMS) diluted to 70 – 80%. Vials were then frozen as soon as possible. Following this methodology, no more than two tails were taken from any one site, and never from two juvenile animals caught or killed in the same area due to the likelihood of their being related, and therefore having the same VKORC1 SNP profile. Initially, tails were sampled directly by researchers. Areas that had not been sampled were identified on a map and farms and other sites of likely rat infestation visited. Once permission to do so had been granted by the landowner, attempts were made to capture rats in cage traps, which were then humanely killed on site in a CO_2 chamber and their tails harvested. Due to the requirement to check traps daily, in areas increasingly further afield from Reading, it quickly became apparent that, in order to obtain samples from across the whole of south-east England, assistance would be required. Because the results of the study were to be made public by RRAC, in order to allow farmers, PCOs and other stakeholders to identify resistance foci covering their areas and respond to Norway rat infestations accordingly, it was hoped that they would in turn be invested in aiding attempts to obtain samples for testing. Therefore the editors of gamekeeper trade publications were contacted in order to publicise the study and request help in obtaining samples from areas outside Reading's immediate surroundings. This resulted in articles in the Winter editions of Keeping the Balance 2012 and 2013, and Countryman's Weekly in 2013 (see Appendix 1). In addition, pest control companies known to operate in the south-east were contacted, as were various industry contacts from among the members of RRAC. Those who expressed interest via phone calls and email were provided with a protocol for correct handling of tail samples and how many samples to take from a single site (see Appendix 1), as well as vials filled with 70 – 80% IMS free of charge. Interest was also generated due to several television appearances from those involved with the project, including ITV local news, The One Show and Ladykillers, and a Radio Berkshire broadcast. A large number of tail samples were obtained when a local borough council signed

a contract with the University of Reading to provide 100 tail samples from the central Reading conurbation, in return for all results obtained. Regardless of provenance, an exact postcode or x,y data were required with each sample in order to map results accurately. Despite the effort made to solicit the assistance of stakeholders who could contribute tail samples, very few individuals and companies made contact as a result, even fewer of which were located outside of the original "central-southern England" designation of Berkshire, Hampshire and Surrey, and it was decided that a different approach was required.

2.1.3.2 Faecal samples

Between September 2013 and December 2013, farms in Kent, East Sussex and East Anglia were visited, and Norway rat faecal samples collected for molecular analysis. Once permission to search the site for suitable samples had been obtained, rat droppings were examined visually, and those that appeared the most moist and glossy selected. Where droppings were very close together in a group or pile, only two were taken in an attempt to avoid taking too many samples from one individual. However, aside from grouping of droppings, there was no way to be entirely sure that droppings came from different individuals, so multiple droppings were taken from different areas of each site. Droppings were stored in vials and stored in a portable fridge within 30 minutes. Following a maximum of five days of collection, vials were transferred to a laboratory freezer and stored at -20^{0}C.

2.1.4 Molecular analysis

The DNEasy Blood and Tissue Kit (Qiagen Ltd, Crawley, West Sussex, UK) was used for DNA extraction from tail samples as follows:

1. Heat Buffer ATL to 55 ^{0}C.
2. Shave 2-3 mm of tissue off tail sample using a razorblade.

3. Add 180 µl of Buffer ATL and 20 µl of Proteinase K to tissue sample.
4. Place in incubator. Set temperature to 50-55 ^{0}C, and shaker speed to 50 RPM. Leave overnight.
5. Remove sample from incubator. Vortex for 15 seconds.
6. Make a mixture of 50% Buffer AL and 50% ethanol; Add 400 µl of this to each sample.
7. Vortex for 15 seconds.
8. Pipette sample into a spin column contained within a 2 ml tube.
9. Centrifuge at 8000 RPM for one minute.
10. Add 500 µl of Buffer AW1. Centrifuge at 8000 RPM for one minute.
11. Add 500 µl of Buffer AW2. Centrifuge at 13,000 RPM for three minutes. Put spin column in a new 1.5 ml Eppendorf.
12. Add 70 µl Buffer AE. Wait for two minutes.
13. Centrifuge at 8000 RPM for one minute.

The QIAamp DNA Stool Minikit (Qiagen Ltd, Crawley, West Sussex, UK) was used for DNA extraction from faecal samples as follows:

1. Weigh and separate 180-220 mg of stool sample.
2. Add 1.6 ml Buffer ASL. Vortex for one minute.
3. Centrifuge at 13,000 RPM for one minute and discard pelleted stool, retaining liquid supernatant.
4. Add one InhibitEX Tablet. Vortex for one minute.
5. Repeat Step 3 twice.
6. Extract 600 µl of liquid supernatant. Add to 25 µl Proteinase K.
7. Add 600 Buffer ASL. Vortex for 15 seconds.
8. Incubate at 70^{0}C for 10 minutes.
9. Add 600 µl of 96-100% ethanol to the lysate. Vortex for 15 seconds.
10. Apply 600 µl of the lysate from Step 9 to a spin column contained within a 2 ml tube. Centrifuge at 13,000 RPM for one minute and discard the 2 ml tube containing the filtrate.
11. Apply 600 µl of the lysate from Step 9 to the retained spin column, housed in a new 2 ml tube. Centrifuge at 13,000 RPM for one minute and discard 2 ml tube containing the filtrate.
12. Repeat Step 11.
13. Repeat Step 11 with 500 µl of Buffer AW1 instead of lysate.
14. Apply 500 µl of Buffer AW2 to the retained spin column, housed in a new 2 ml tube. Centrifuge at 13,000 RPM for three minutes and discard 2 ml tube containing the filtrate. Put spin column in a new 1.5 ml Eppendorf.
15. Add 20 µl Buffer AE. Wait for one minute.

16. Centrifuge at 13,000 RPM for one minute.

Extracted DNA was then amplified by PCR, and PCR products were evaluated by electrophoresis and examination under UV light. The PCR conditions were: two minutes at 94◦C; followed by 35 cycles of denaturation at 94◦C for 30 seconds, annealing at 57◦C for 30 seconds, elongation at 72◦C for 30 seconds; followed by a final extension at 72◦C for three minutes. Subsequently, the temperature was reduced to and held at 4◦C until the samples were removed. Primers are described in Section 3.2.5. DNA samples taken prior were amplified and analysed as described by Prescott *et al.* (2010).

The QIAamp PCR Purification Kit (Qiagen Ltd, Crawley, West Sussex, UK) was used to purify PCR products as follows:

1. Add Buffer PB at a ratio of 5:1 to PCR product.
2. Transfer to a spin column housed in a 2 ml tube. Centrifuge at 13,000 RPM for 40 seconds; discard flow-through.
3. Add 750 µl Buffer PE. Centrifuge for 40 seconds at 13,000 RPM. Discard flow through, and centrifuge again for one minute.
4. Transfer column to a 1.5 ml eppendorf. Add 25 µl Buffer EB. Wait for three minutes.
5. Centrifuge at 13,000 RPM for one minute. The resulting elution is the final DNA product.

Final DNA products were sent to Source Bioscience (Source BioScience plc, Nottingham, Nottinghamshire, UK) for Sanger sequencing. Sequences were received in the form of chromatograms which were individually analysed by eye in order to determine if there were any SNPs present, and their zygosity. Visual analysis was necessary firstly because the sequencing did not consistently recognise one nucleotide over another in a heterozygous base, and secondly because in chromatograms that show some evidence of contamination (which were frequent in the case of faecal samples) a human can still

sometimes determine the presence of a mutation in areas minor contamination that a chromatogram display application can't recognise.

2.1.5 Delimiting resistance foci

After successful sequencing, the postcode of each sample was converted to OS x and y grid references using an online postcode converter (www.streetmap.co.uk/gridconvert.html). These were then added to an OS basemap (Ordnance Survey Ltd, Southampton, Hampshire, UK) using ArcGIS (Esri UK Ltd, Aylesbury, Buckinghamshire, UK), and assigned symbology based on the presence or absence of VKORC1 exon 3 mutations and their zygosity. These spatial data could then be analysed in terms of distance between resistance foci and their presence/absence in specified counties.

2.2 Quantifying anticoagulant resistance in resistant strains of Norway rat

2.2.1 Blood clotting response tests

Very soon after the first discovery of anticoagulant resistance in rodents (Boyle, 1960) rats from populations that appeared to be exhibiting resistance (due to an apparent lack of efficacy of established anticoagulant poisons when applied to the populations in question) were often live trapped and subjected to laboratory feeding tests in order to determine whether physiological resistance was present, and if so to what degree. A lethal feeding period was established (Drummond and Bentley, 1965) with bait containing 0.005% warfarin for six days, during or after which 99% of susceptible rats are expected to die (LFP_{99}). Those that survived were classified as resistant. A similar test was established for difenacoum, when it became clear that it was efficacious against rats from the Welsh resistance focus but

questionable against rats from central-southern England (Redfern and Gill, 1978). Because different rats respond differently to captivity and to specific baits, and feeding usually takes place over several days, the LFP_{99} does not give an exact dose at which 99% of rats are expected to die (LD_{99}). In order to administer a known concentration of active ingredient, a system of limited bait intake must be applied, wherein the volume of bait supplied to an individual must be measured in terms of its concentration and the weight of the rat. This is typically much lower than the usual daily food intake of a rat in order to assure 100% consumption. Even so, 100% consumption, and therefore application of the full dose within the accepted time frame, can never be guaranteed. Another method of delivering a known concentration of active ingredient is through oral gavage or intraperitoneal injection. While this is a much more precise method than feeding tests, there is a requirement to wait for animals to suffer the symptoms of anticoagulant poisoning or die. This, along with the number of animals required for these tests, makes them both relatively impractical and inhumane. In due course, blood clotting response (BCR) tests were developed which allowed researchers to directly record the ability (via the time taken) of blood to clot. Using gavage or subcutaneous injection to administer the dose meant the entire dose could be delivered at once. This allowed blood to be taken at a fixed point, a known timeframe after the delivery of the entire dose. Blood samples taken in this way can be used to determine an effective dose (ED) of a given concentration of an anticoagulant. In the first of these tests, warfarin was dissolved in dimethyl formide and injected subcutaneously; blood was extracted retro-orbitally both before and after dosing, and prothrombin times measured using Diagen "Two-Seven-Ten" (Greaves and Ayres, 1967). This method was subsequently refined over many years for the identification of resistant rats (Martin *et al.*, 1979; MacNicoll and Gill, 1993; see Section 4.1.1 for details). Because of the small amounts of blood required, animals classified as resistant – and therefore likely to survive the anticoagulant dose administered – could later

be used in feeding tests to confirm the results of BCR tests (Greaves and Ayres, 1967) or in other tests entirely. With the establishment of laboratory-resistant strains of Norway rats, BCR tests increasingly focused on identifying ED_{99} values for susceptible rats, and then administering these doses to resistant rats in order to determine the likely impact of resistance in the field (Gill *et al.*, 1994; Prescott and Buckle, 2000). However, it was not until the standard methodology was established (Prescott *et al.*, 2007) that a system was created that would make all future data generated for the various strain-anticoagulant combinations comparable and therefore of use to everyone, providing the methodology was correctly followed. The new methodology, particularly its statistical analysis, also enabled the quantification of resistance (resistance factors, RF) conferred by the recently identified VKOR mutations (Pelz *et al.*, 2005). The history, implications and liabilities of these methodologies are discussed in greater detail in Section 4.1.1.

2.2.2 Strains of resistant Norway rat housed at Reading

2.2.2.1 Strain origins

Both feeding tests and oral gavage require live capturing wild rats and bringing them into a laboratory. Before tests can begin a mandatory treatment for ectoparasites and an acclimatisation period is necessary. Research on anticoagulant resistance in the UK has been aided by the establishment of various strains of laboratory rodents that are homozygous for an anticoagulant resistance mutation that was derived from a particular geographical location. Norway rat strains include the “Scottish strain”, the “Welsh strain”, the “Hampshire strain”, and more recently the “CSL Berkshire strain”, and house mouse strains include the “Cambridge creams” and the “Reading Berkshire strain”. The use of such strains overcame a number of problems associated with laboratory tests on wild caught animals, including: Extra

time required to acclimatise to laboratory conditions, inconsistency of feeding during test, difficulties of handling, stress-induced humaneness issues, unexpected mortality when maintained in the laboratory, and the uncertain nature of procuring a given number of wild rats in time for a predetermined experiment. The first resistant laboratory rats were descended from wild rats brought into the laboratory for feeding tests (see Section 2.2.1). Rats that survived the susceptible LFP_{99} and were classified as resistant were crossed onto susceptible laboratory rats and their offspring subjected to further feeding tests in order to obtain a strain of captive resistant rats. There are currently three strains of resistant rats housed at the University of Reading (see Section 4.1.3 for details), all of which underwent testing to quantify their level of resistance as part of this study.

The first strain of anticoagulant-resistant laboratory Norway rats were captured in Scotland (Boyle, 1960; Greaves and Ayres, 1973) and taken back to the laboratory. There, via feeding tests against anticoagulants, a group of animals was established to be homozygous for Scottish (L128Q) resistance. These animals were subsequently back-crossed onto susceptible laboratory rats over several generations resulting in a domesticated line, fully homozygous for Scottish resistance. The second to be generated came from a group of resistant rats from Wales (Bentley, 1968) which were captured and selected for homozygosity (Y139S) and back-crossed onto susceptible laboratory rats as described above. The third strain to be generated came from a site in Hampshire where rats were demonstrated to possess practical resistance to both warfarin and difenacoum (Greaves and Cullen-Ayres, 1988). Rats captured there were selected for homozygosity (L120Q) and back-crossed onto susceptible laboratory rats as described above. The fourth strain to be generated came from a site in north Berkshire where rats were discovered in 1992 to have a mutation that conferred technical resistance to Brodifacoum (Gill, Kerins and MacNicoll, 1992). These rats were taken to two different laboratories to be back-crossed onto susceptible rats, resulting in two separate strains of

"Berkshire" resistant laboratory rats (also L120Q). At the times that the resistant lines of laboratory rats were generated, it was established that the fully homozygous strains possessed differing levels of tolerance to anticoagulants (Greaves and Cullen-Ayres, 1988), as well as differing levels of dietary vitamin K requirement (Greaves and Ayres, 1973).

2.2.2.2 Maintenance of rats

All rats were housed in plastic cages with removable metal lids. Cage floors were covered in sawdust to absorb urine, faeces and water. Rats were presented with cardboard tubes and/or empty glove boxes for enrichment and hiding opportunities, along with shredded card bedding and wooden blocks for chewing. RM3 Laboratory Diet (Special Diet Services, Braintree, United Kingdom) and water were supplied ad libitum. Cages and all contents were changed weekly, with a small amount of bedding and sawdust retained in order to allow rats to become accustomed to their new cages. Tubes were also retained if they remained relatively clean and intact. Each cage was labelled with the number and sex of rats housed therein, along with their unique identity code and date of birth. Rats were always housed in same-sex groups of no more than four, and no less than two, siblings. The room housing cages was maintained at 18-23ºC, on a 12-hour light/dark cycle.

2.2.2.3 Breeding

Rats in sibling groups were assigned a code which identified their parents and which generation they came from. This information was used to avoid inbreeding. Up to two males and two females were assigned to new breeding pairs when necessary. A single male and a single female were removed from their respective cages and placed in a new cage together, with a small amount of bedding and sawdust from their original cages. The cages and maintenance for breeding pairs were identical to all other rats, except that their water was supplemented with menadione (Vitamin K_3). This was supplied as a precaution at a time

when breeding females are likely to haemorrhage, in order to overcome the higher dietary requirement of vitamin K that is known to occur in some resistant animals. Vitamin K-infused water was made by introducing 0.4 g of Vitamin K_3 (Menadione; Sigma-Aldrich Company Ltd, Dorset, England) to 20 litres of water. Fresh batches of this water were made up every two weeks. The RM3 Laboratory Diet provided is known to contain 4.14 mg/kg of menadione, which is more than required to prevent a vitamin K deficiency in the strains of rat present at this laboratory (Hussain, 1998). The female would typically become pregnant within a month, however this was highly variable. Once the female gave birth, the cleaning schedule was relaxed, and old sawdust replaced when necessary, rather than moving the parents and offspring into a new cage. However, despite attempts to minimise disturbance of females with very young offspring, some infanticide occurred (assumed, but not confirmed, to be committed by the males), and some females rejected and ate their young. In pairs where this occurred, during the next round of pregnancy males were removed and housed separately. If this failed to remedy the problem, the parents were not reunited, and a replacement breeding pair was made up. Six weeks after birth, surviving offspring were weaned and split into groups as described in Section 2.2.2.2. Two males and two females from each litter were retained for future breeding until another litter from the same parents was successfully weaned. Breeding pairs were separated after six pregnancies.

2.2.3 Dosing methodology

In order to determine the impact of rat size on Prothrombin time, 78 rats underwent BCR tests, as described above, without anticoagulant dosing. These rats all resulted from abandoned attempts to cross the CSL Berkshire and Reading Berkshire strains, and were therefore all homozygous for the L120Q mutation (although with unknown heritable traits

from the two parent strains). The males (N: 25) weighed between 106 and 465 grams. The females (N: 53) weighed between 91 and 324 grams. Linear Regression Models were used to determine whether any of the male, female and grouped datasets showed a significant PT response to individual weight. Animal weight did not have any significant effect on PT response time (see Section 4.3.1); rats of any size could be selected for BCR testing when required by the study, with no requirement to select for a particular body weight. Triethanolamine and PEG200 (polyethylene glycol, molecular weight 200) were obtained from Sigma Aldrich (Sigma-Aldrich Company Ltd, Dorset, England). The second generation anticoagulants brodifacoum, flocoumafen, difenacoum and bromadiolone were obtained in pure powder form to be processed into stock solutions of either 10 mg/ml (brodifacoum. bromadiolone and difenacoum) or 1 mg/ml (flocoumafen). Difenacoum and bromadiolone were supplied by Pelgar (Pelgar International Ltd, Hampshire, England), flocoumafen was supplied by Fluka (now Honeywell, Bucharest, Romania) and brodifacoum was supplied by Sigma Aldrich. These ingredients were supplied to Reading Scientific Services Ltd, who used the following methodology to produce the required stock solutions:

To produce a 1 mg/ml stock solution:

1. 0.25 g technical anticoagulant measured into a 250 ml volumetric flask.
2. 0.05 ml triethanolamine and 0.3 ml PEG 200 added.
3. Flask swirled in a 60ºC water bath, and ultrasonicated at 60ºC to aid dissolution.
4. After complete dissolution, the solution was made to its 250 ml volume with PEG 200.

To produce a 10 mg/ml stock solution:

1. 2.5 g technical anticoagulant measured into a 250 ml volumetric flask.
2. 2.5 ml triethanolamine and 7.5 ml PEG 200 added.

3. Flask swirled in a 60ºC water bath, and ultrasonicated at 60ºC to aid dissolution.
4. After complete dissolution, the solution was made to its 250 ml volume with PEG 200.

A brodifacoum 10 mg/ml stock solution was made and diluted by a factor of 10 to produce a 250 ml 1 mg/ml stock solution. All work was carried out in subdued lighting, and all containers were covered with aluminium foil in order to exclude light throughout the procedure.

These stock solutions were known to provide, when administered to rats at a rate of 0.5 ml per 100 g of bodyweight, a dose of 1% or 0.1% active ingredient, respectively. Stock solutions were then diluted by weight with PEG200 to the required concentrations for dosing via oral gavage. In order to preserve accuracy, no stock solution was ever diluted by more than a factor of 1 in 10. For instance, if a stock solution of 0.1 mg/ml bromadiolone was required, an initial stock solution of 1 mg/ml had to be created from the original 10 mg/ml solution. For ease of handling, rats were typically dosed when they weighed between 150 g and 400 g. Males were selected after eight weeks of age and before 3 months; females were selected after eight weeks of age but rarely grew to exceed 400 g. After 24 hours, rats were sedated under terminal anaesthesia with isoflurane (Merial, Harlow, United Kingdom), and 0.9 ml of blood withdrawn via cardiac puncture into 0.1 ml of 3.2% tri-sodium citrate (to prevent clotting). The latter was made by dissolving 3.2 g of pure tri-sodium citrate crystals (Sanofi UK, Guildford, United Kingdom) in 100 ml purified water. The blood samples were centrifuged at 5300 rpm for six minutes, resulting in plasma being separated from all other blood contents. Plasma was removed using a pipette and either processed immediately, or frozen at -21^{o}C for future processing.

2.2.4 BCR methodology

BCR tests were conducted using Amelung KC4 micro semi-automatic haemostasis equipment (Diagnostica Stago UK Ltd, Theale, United Kingdom). Four cuvettes were used for each plasma sample. Diagen freeze dried rabbit brain thromboplastin (Diagnostic Reagents Ltd, Thame, United Kingdom) was reconstituted with 5 ml purified water and left for 10 minutes, as per manufacturer's instructions.

With each cuvette:

1. a ball bearing was placed in each cuvette
2. 50 µl rabbit brain thromboplastin added
3. 50 µl plasma was added, and the cuvettes were allowed to heat in the Amelung for two minutes
4. 25 ml calcium chloride (Diagnostic Reagents Ltd) solution was added using an automated pipette which starts the Amelung timer
5. On clot formation the ball bearing was prevented from moving and the timer was stopped
6. The clotting time (or prothrombin time) was recorded

The calcium chloride initiated the clotting cascade by forming complexes with the activated clotting factors, which in turn activate further clotting factors in the coagulation cascade, resulting in the activation of prothrombin to thrombin. For each plasma sample, the four replicate values were recorded, and if three values were within a 10-second window, the average of these was taken as the final PT. If three values were not within a 10-second window, the process was repeated. If recorded PTs lasted beyond 100 seconds, this rule was relaxed.

2.2.5 Analysis of BCR results and application to real world pest control

Where numbers allowed, rats were dosed at single concentrations in groups of ~10. PT values over 47.5 s (equivalent to INR of >5) were classified as "responders"; PT values below 47.5 s (equivalent to INR of <5) were classified as "non-responders". Based on the size of the dose-groups and bias towards responders and non-responders, subsequent suitable doses for each of the anticoagulants were identified. The data were analysed by Probit Analysis (Statistical Analysis System, Marlow, United Kingdom), providing 95% fiducial limits for effective dose percentiles (ED_{01}-ED_{99}) when enough doses had been administered. Once ED percentiles were generated for an anticoagulant against both males and females of a strain, the two datasets were combined, and compared with one another via Genmod Analysis in SAS (Appendix 2). This established whether the response lines for males and females of the same strain were statistically separate, parallel or coincident, and based on this result, new effective dose percentiles for males and females were generated. These effective doses could then be compared with established effective doses previously recorded for susceptible laboratory animals (Prescott *et al.*, 2007). Using this methodology, the resistance statuses of the three strains of rat described above have been evaluated, and at the ED_{50} level, resistance factors generated.

2.3 Field trials of anticoagulant rodenticides in areas of known L120Q resistance

2.3.1 Site identification

The final stage in the evaluation of an anticoagulant rodenticide, prior to its approval by regulators in the EU, is a series of field trials in which the rodenticide is presented in a

systematic fashion and the volume of bait taken is carefully measured. This method was used in order to investigate the impact that the L120Q mutation has on real life attempts to control populations of Norway rats. Before this process can be started, suitable trial sites must be identified, and permission to use them obtained. Farms located within the Berkshire-Hampshire, and subsequently known as L120Q, focus in central-southern England were visited in order to ascertain their suitability for an anticoagulant rodenticide field trial. Sites were selected based on the obvious presence of Norway rat infestations. Sites were rejected if free-ranging poultry and livestock, domestic animals or wildlife would obviously be put at risk by the presence of rodenticides. Most farmers were receptive to the idea of hosting field trials due to their having trouble in controlling infestations, in the hope that the experimental trials might be "successful" – i.e. eradicate the Norway rat population. Due to the extremely thorough nature of scientific field trials (as opposed to standard treatments conducted by landowners or PCOs) some farms were rejected because access could not always be guaranteed, or farmers would not tolerate bait points in close proximity to their livestock. Four farm sites were identified as suitable locations to trial proprietary bromadiolone or difenacoum baits, and two farm sites were selected to host trials of experimental brodifacoum baits, resulting in six separate trial locations. The field trials of difenacoum and bromadiolone (2009 – 2010) were undertaken by the University of Reading several years before the brodifacoum trials (2016), and the total control achieved during the bromadiolone and difenacoum trials (according to census bait consumption – see Section 2.3.8) has been reported elsewhere (Daniells, 2011). Data from these trials have been explored in further detail herein in order to compare performance with that of brodifacoum baits.

2.3.2 L120Q confirmation

Rats at all trial sites were trapped and killed, and their tails removed, and in the case of the brodifacoum trials, fresh droppings were collected from sites. DNA from tissue samples taken from previous trial sites (henceforth referred to as Sites/Trials A, B C and D) was analysed following the methodology of Rost *et al.* (2004). DNA from tail and faecal samples from sites of brodifacoum trials (henceforth referred to as Sites/Trials E and F) was extracted, amplified and analysed using the same methodology as described in Section 2.1.4, in order to confirm presence and prevalence of the L120Q resistance mutation.

2.3.3 Bait types

Over the course of six field trials, four second generation anticoagulant rodenticide bait products were tested against resistant rats. Trials A and C were conducted with Ratak Pellets, containing 50 ppm difenacoum; Trials B and D were conducted with Contrac Pellets, containing 50 ppm bromadiolone; Trials E and F were conducted with experimental brodifacoum formulations, containing less than 30 ppm brodifacoum. All trials used whole wheat bait during pre and post-trial censuses.

2.3.4 Bait points

Three types of bait points were used in field trials: bait boxes, tyre baiters, and bait trays under natural cover. Bait boxes were wooden or plastic boxes with two entrance holes through which an adult Norway rat could pass. Within the box, a plastic tray (if required) was placed, containing the bait. The lids of the boxes could be removed in order to access the bait and measure bait take. Bait boxes had approximate dimensions of 31cm x 22cm x 14.5cm,

with a wall thickness of approximately 2cm and a floor thickness of 1 cm. The two rectangular entrances into the bait boxes were approximately 7.5cm x 5cm. Tyre baiters were metal stands with three-pronged bases, on which a car tyre was placed, holding the tyre a number of centimetres off the ground. In the centre of the base was a tall screw, onto which a lid was placed. Rats gained access by crawling underneath the tyre: thus preventing access and providing a degree of safety for domesticated animals. Researchers could remove the lid to access and weigh bait. Bait was placed in wooden or plastic trays. Wooden and plastic bait trays all had approximate dimensions of 16 cm x 11 cm x 5 cm, with rims of approximately 2 cm. Bait trays under natural cover were either wooden or plastic, and allowed bait to be positioned under cover, using materials and natural cover found on site or using wooden boards measuring approximately 60 cm x 30 cm x 1 cm. The cover was used to protect the baits against non-target species. Natural cover included wooden planks found on site, large feed and water troughs or disused farm equipment. Where necessary, bait was split between two bait trays in order to more safely accommodate large volumes without spillage.

At bait points where the bait had clearly not been touched, the data was labelled 'NT' (No Take) and recorded as 0. At bait points where there had been clear bait consumption, evidence of nibbling, or movement of bait, the remaining bait was weighed, and the figure subtracted from the original weight of laid bait, to obtain a measure of the amount of bait taken from each individual bait point. The recorded data was labelled 'P' (Partial). At bait points where no bait remained, the figure recorded was equal to the weight previously applied, and labelled CT (Complete Take). Using the saturation baiting methodology during Trials A, B, C and D, if a bait point was labelled CT, the subsequent volume of bait laid there was doubled or otherwise increased. During Trials E and F the pulse baiting technique was used, resulting in more frequent visits because the volume of bait could not be increased.

Based on weight of bait consumed and strength of formulation, weekly consumption of active ingredient was calculated.

2.3.5 Tracking patches

Tracking patches consisted of fine sand and were at least 100 mm x 150 mm, with sufficient depth to ensure a flat surface. Each day the site was visited, tracking patches were checked and assigned a score based on the following scale.

0 = no sign of rodent tracks
1 = 1-5 individual rodent footprints
2 = >5 footprints and up to 25% of patch covered with footprints
3 = 26% - 95% of patch covered with footprints
4 = > 95% of patch covered with footprints

Tracking patches were then smoothed over and fresh sand added if necessary.

2.3.6 Carcass searching

In order to comply with study protocols in line with those of the European and Mediterranean Plant Protection Organisation (EPPO, 1999a) carcass searches were carried out at Sites E and F. A preliminary carcass search was carried out in the first week of the trial in order to find carcasses of any animals in the trial site or its environs. Carcass searches were then carried out weekly throughout the trial, using the same route. The route encompassed the entirety of the active trial area, and where possible extended up to 400 m from the nearest bait point.

2.3.7 Stages

Pre-treatment censuses were initiated at a trial site once it had been established that the site was suitable. Between 50 and 94 bait points and between 35 and 54 tracking patches were established in suitable locations. The sites were visited on a daily basis over a four to six day period to record pre-treatment census bait take and tracking patch activity. Following the end of the pre-trial census, a pre-trial lag period of between five and 11 days ensued. During the pre-trial lag phase, bait points and associated bait were removed and trial sites were not visited. Tracking patches remained in place, in the same number, throughout the entirety of the trials. After the lag phase, the trial phase began with application of trial bait in bait points that were positioned in different locations to those used for the pre-treatment census. Trial sites were visited two or three times per week for a minimum of five weeks. Trial phases ended if, after five weeks, Norway rat activity had plateaued, begun to increase or ceased entirely. If rat activity was still falling, trials were allowed to continue until one of the above circumstances occurred. After trial phases ended, a second, post-trial lag phase ensued, lasting between two and four days. Post-trial lag phases were carried out in identical fashion to pre-trial lag phases. Finally, a post-trial census lasting either four or six days was carried out, in identical fashion to that of the pre-trial census.

2.3.8 Interpretation

Trial efficacy was quantified by comparing census bait take and tracking patch scores between the pre and post-trial censuses. Maximum nightly score for each activity census from the pre-trial census was compared with that of the post-trial census, utilising the following calculation (EPPO, 1999a):

$$\% \text{ efficacy} = \frac{\text{pre-treatment activity} - \text{post-treatment activity}}{\text{Pre-treatment activity}} * 100$$

The resulting values indicated as a percentage how much rat Norway rat activity decreased or increased over the course of the field trial.

Chapter 3: Locating and delimiting Norway rat resistance foci in south-east England

3.1 Introduction

3.1.1 History of resistance identification

In many UK sites where rodent pest control is attempted, the practical efficacy of anticoagulant rodenticides against Norway rats has steadily diminished over the last 50 years due to the occurrence and discovery of anticoagulant resistance. This resistance was first discovered in Norway rats in the UK in 1959, and reported shortly thereafter (Boyle, 1960). The intervening years have led to an increase in the number of resistance foci in the UK (Greaves and Rennison, 1973; Pelz *et al.*, 2005; Prescott *et al.*, 2010; Haniza *et al.*, 2015).

Over time it became clear that different resistance foci exhibited greater or lesser resistance to the various anticoagulants (or none at all), leading to individual rat populations being variously described as being resistant to individual anticoagulants, with no realisation that all resistance-conferring mutations in fact confer a level of cross-resistance (though often merely "technical" resistance, with little to no effect in real applications of rodenticides) to most, if not all anticoagulants. Thijssen (1995) describes differing sensitivity of VKOR enzymes, and differing modes of pharmacodynamics resistance, in two different resistance foci ("Scottish" resistance and "Welsh" resistance, see Section 1.4.2), and how different resistance types may confer resistance to some anticoagulants but not others, but no description of the underlying genetic mutations was available at the time.

3.1.2 Resistance identification methodology

Tests to determine anticoagulant resistance in an individual animal were costly both in terms of time and money, as they required capturing the animal alive and performing resistance tests in the laboratory (Drummond and Rennison, 1973). These Lethal Feeding Period (LFP) tests were based on the feeding period required to achieve 99% mortality in susceptible animals, as determined by Probit analysis. Suspected resistant animals were given the rodenticide for this feeding period as a checking test, and survival of these feeding tests were taken as initial evidence that the animals were resistant, although not all animals fed consistently during the feeding test, and it was often difficult to distinguish between resistance and poor feeding (RRAG, 2012) or resistance-induced vitamin K deficiency (Martin *et al.*, 1979). A quicker alternative is the blood clotting response (BCR) test. In these tests, animals are dosed by oral gavage with a known quantity and concentration of anticoagulant, and 24 or 96 hours later the ability of their blood to clot is tested. Test animals are considered resistant when their blood continues to clot despite being given a dose of anticoagulant that would prevent or delay clotting in susceptible animals. When used to determine whether or not an animal is to be considered resistant, these tests were merely a quicker and more humane alternative to the LFP tests described above. Initially they were validated by performing the BCR test to assign animals to either “resistant” or “susceptible” groups, and then the results were confirmed using traditional LFP tests (Greaves and Ayres, 1967). Subsequent refinement of the methodology has improved the consistency and precision of these tests, allowing the generation of Probit dose response data for many anticoagulant active ingredients against male and female Norway rats and house mice, thus determining the level of tolerance to various anticoagulants through the statistical generation of effective doses (Prescott *et al.*, 2007). This methodology is explored more thoroughly in Section 2.2.1, and used extensively in Chapter 4.

Despite the reduced time, reduced cost and reduced humaneness concerns (when compared with LFP tests), the BCR method cannot on its own determine which resistance mutation is involved when resistance is detected in an individual animal. Because the resistance gene is dominant (Greaves and Ayres, 1967), it is not always possible to determine whether the individual in question is homozygous or heterozygous for resistance. Furthermore, when wild rodents are tested in the laboratory for resistance using LFP tests or BCR tests, the researcher is exposed to an aggressive wild animal, with risks of bites, scratches, and the potential for disease transmission. The stress of extensive handling and being restrained for the rat is also a source of concern.

3.1.3 DNA analysis

A faster method for determining the resistance status of an animal is through DNA analysis, wherein fresh tissue or faecal matter is broken down so that the DNA material therein can be extracted, amplified, purified and examined (Pelz, Rost and Müller, 2007). Work done by Rost *et al.* (2004) identified the gene vitamin K epoxide reductase complex subunit 1 (VKORC1) in humans and Norway rats for the first time, finding it to be responsible for coding for functional elements of the vitamin K epoxide reductase multiprotein complex (VKOR, which recycles vitamin K epoxide through various stages into vitamin K hydroquinone, the cofactor which allows the carboxylation and activation of certain blood clotting factors in the body) and the mutations therein causing known, identifiable resistance to the anticoagulants in wild Norway rats. Subsequently, through sequence analysis of wild and laboratory Norway rats classified through BCR tests as either susceptible or resistant, a range of resistance mutations and their locations in the VKORC1 gene were identified (see Section 1.4.2). By expressing rat VKORC1 and five of the associated resistance-conferring

SNPs as membrane-bound proteins in yeast *Pichia pastoris*, Hodroge *et al.* (2011) and measuring associated kinetic and inhibition properties, the recombinant proteins were shown to carry out the same functions as the proteins involved in the Vitamin K cycle in rat liver microsomes, confirming the effect of the VKORC1 mutations. These five mutations are known to occur on the UK mainland and confer practical resistance to at least some first generation anticoagulants rodenticides (FGARs) if not some second generation anticoagulant rodenticides (SGARs) are the main focus of this study. All of these mutations are single nucleotide polymorphisms (SNPs): wherein a single codon (nucleotide triplet that codes for a single amino acid) changes due to a single nucleotide change. This mutant codon causes an amino acid change that results in a protein complex with an altered active site, affecting its natural function and conferring resistance to anticoagulants.

3.1.4 Stewardship scheme

Until recent changes in legislative procedures, the anticoagulants available for use against outdoor populations of Norway rats in the UK had reduced efficacy against some of these resistance foci, with a complete absence of efficacy being demonstrated at many sites. These include bait containing widely used and common active ingredients such as warfarin (Greaves and Cullen-Ayres, 1988) and the second generation anticoagulant rodenticides (SGARs) difenacoum (Greaves, Shepherd and Gill, 1982) and bromadiolone (Quy *et al.*, 1995). Before the advent of the rodenticide stewardship scheme implemented by CRRU (Buckle *et al.*, 2017), the use of the most potent anticoagulant rodenticides (difethialone, flocoumafen and brodifacoum) was restricted to use against populations of rodents that existed predominantly indoors, due to their perceived increased risk to non-target species. This effectively prevented their usage against Norway rats, which rarely restrict their

movements to indoor environments. These developments are relatively recent, and it is too early to determine if they have had any impact on the control of commensal rodents in the UK, or the prevention of non-target poisoning. With more options for rodent control available to all users, it is more important than ever that information on the status of anticoagulant resistance in the UK is made available in order to prevent misuse of rodenticides.

3.1.5 Identifying and delimiting resistance foci in south-east England

Since 2009 the University of Reading has, through funding from various bodies, been working to identify and delimit the various foci of anticoagulant rodenticide resistance mutations in the Norway rats of the UK. In order to continue this work and properly explore and delimit the extent of resistance-conferring VKORC1 mutations in Norway rats it was decided that the area initially covered should be contiguous, and therefore in 2012 a new project based at Reading University was started. The locations specified for this work were changed and expanded to include the whole of Surrey, Oxfordshire, Berkshire, West Sussex, East Sussex, Essex, Cambridgeshire, East Anglia, and London. The aim was to produce a map showing individual locations spread evenly over this contiguous area: each location on the map would be represented by a symbol which indicated the type (if any) of anticoagulant resistance mutation present, and its zygosity. These data will be made freely available to pest controllers through the web site of the Rodenticide Resistance Action Committee (RRAC) of CropLife International. The long term objective is to enable the identification of areas of known resistance, thereby preventing the application of ineffective rodenticides; which would exacerbate the resistance problem and increase the risk to non-target species. Displaying these results on a map would also facilitate future research into the repercussions of two different resistance foci encroaching into one another - with the possibility of interbreeding

between Norway rats from different resistance foci producing offspring with more than one resistance conferring mutation.

3.2 Materials and methods

3.2.1 DNA samples

In order to identify the spread of the various resistance mutations and delimit the foci wherein they were discovered, sites across the south-east of England were visited in order to obtain DNA samples. Due to ease of access and generally high Norway rat abundance, farms were most often visited. Farms were identified using Ordnance Survey maps (Ordnance Survey Ltd, Southampton, Hampshire, UK), and owners or tenants approached on site by the researchers. Initially sites with no recent history of anticoagulant rodenticide treatment were preferentially selected. This was to prevent inflation of the number of resistant animals discovered, as recent or ongoing treatments would kill off susceptible animals, assuming correct application of rodenticides. This practice was subsequently abandoned when it became clear that stakeholders applied rodenticides with a frequency that made finding rat populations that had undergone no recent chemical control untenable. Alternatively, stakeholders were contacted or made aware of the study, with the intention that they would supply DNA samples of their own volition. Stakeholders included landowners, gamekeepers, farmers, public health officials and PCOs. PCOs were contacted via industry contacts, and the study was publicised by various print and television media outlets.

3.2.2 Tissue samples

DNA samples were obtained from tail tips taken from recently killed Norway rats. Rats were caught by researchers in live traps and dispatched using a Home Office schedule 1 method. Tails tip were removed immediately and stored in 70 – 80% IMS and then frozen at -20^{0}C until analysis. Stakeholders were requested to follow the same methodology in order to prevent degradation of tissue. Stakeholders were requested not to take tail tips from rats found dead at sites where anticoagulant rodenticides were being used, and not to take tail tips from rats that had been dead for more than 24 hours. However, whether all stakeholders abided by these rules could not be ascertained.

3.2.3 Faecal samples

By 2013 it had become clear that most of the larger pest control companies – the main source of tails sent to the University – were hesitant to get involved with supplying samples, despite adverts in trade publications and repeated appeals from senior figures in the industry. In addition, despite clear instructions being repeatedly provided to suppliers of tissue samples, the number of samples where it was not possible to extract viable DNA due to degradation or contamination indicated that some tail samples were being handled or selected incorrectly. Due to the neophobic nature of Norway rats (Barnett, 1958; Inglis *et al.*, 1996) and the large study area, it was decided that having researchers attempt to make up the bulk of samples via direct captures of live rats would be inefficient. Therefore, from September until December 2013, researchers visited farms in Kent and East Anglia and collected faecal samples for analysis. Suitably fresh samples were identified by sight. Because it was impossible to ascertain which samples came from different individuals, selected droppings were collected together in vials. Once at the laboratory, faecal samples were frozen at -20^{0}C until analysis.

3.2.4 DNA extraction

DNA was extracted from tail tips using the DNeasy Blood and Tissue Kit following a modified version of the manufacturer's instructions (Qiagen Ltd, Crawley, West Sussex, UK). See Section 2.1.4. See Prescott *et al.* (2010) for details of samples taken prior to 2012.

DNA was extracted from faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen Ltd, Crawley, West Sussex, UK) following a modified version of the manufacturer's instructions for human faecal samples. See Section 2.1.4.

3.2.5 DNA amplification

Elutions resulting from DNA extraction of faecal and tail samples were amplified by PCR (see Section 2.1.4). Because the study was exploratory, with the presumed potential for any mutation to be exhibited by rats in any location, it was necessary to detect all the possible single nucleotide polymorphisms of the VKORC1 gene. In order to cover this range of sites of potential SNPs, primers were obtained from Invitrogen (Life Technologies Ltd, Paisley, Renfrewshire, UK) that flanked codons from before and after as many of the known SNP sites as possible in each of the three exons. Primers used from 2012 onward for detecting SNPs in exon 1 were F: GAG GAG CCC TGG ACG TTT and R: AGG AGA AGA CGC GGG AAC; primers used for exon 2 were F: GGT GGA GCA CGT GTT AGG AG and R: GGT CAC CAA GAC ATG AGG TG; primers used for exon 3 were F: TGA GTT CCC TGG TGT CTG TC and R: TTT TAG GGA CCC ACA CAC GA. PCR products were resolved by electrophoresis and the resulting gel examined under UV light. Providing the PCR was shown to be successful, PCR products were purified using the QIAquick PCR

Purification Kit (Qiagen Ltd, Crawley, West Sussex, UK). Primers used prior to 2012 were based on those used by Rost *et al.* (2004).

3.2.6 Sequencing and interpretation

PCR products were sent to Source Bioscience for Sanger sequencing (Source BioScience plc, Nottingham, Nottinghamshire, UK). Chromas Lite and Finch TV were used to open the resulting chromatogram files; with the majority of the three exons of the VKORC1 gene displayed onscreen, mutations could be identified simply by visual analysis of all relevant codons and comparison with those found in the genomic DNA of a wild-type Norway rat. In order to measure the size of confirmed mutation foci and identify areas where further samples were required, location data for every individual site where results could be obtained were displayed as point shapefiles on ArcGIS (Esri UK Ltd, Aylesbury, Buckinghamshire, UK).

3.3 Results

3.3.1 Exon 3

Between April 2012 and September 2015, exon 3 of the Norway rat VKORC1 gene was successfully analysed from genomic DNA that was extracted from 164 tail samples and 14 faecal samples. In addition, results for 126 tail samples taken prior to the revised agreement between the University of Reading and Killgerm (April 2012) are presented here. A further 41 tail samples and 20 faecal samples were tested but no results could be obtained. For the tail samples, this was most likely because of contamination or degradation of the DNA, as a result of poor storage prior to arrival at the laboratory. In the case of the faecal samples, it is

likely that the DNA is less stable than in tail samples, when stored for a prolonged time period at -20^{0}C.

Table 3.1: Total number of VKORC1 exon 3 mutations found in successfully tested samples.

Mutation	Total number of samples	Homozygous (% of total)	Heterozygous (% of total)
L120Q	224	173 (77.23%)	51 (22.77%)
L128Q	9	2 (22.22%)	7 (77.78%)
Y139C	9	1 (11.11%)	8 (88.89%)
Y139F	19	10 (52.63%)	9 (47.37%)
Y139S	2	0 (0%)	2 (100%)
L120Q and L128Q	6	0 (0%)	6 (100%)
Susceptible/wild-type (% of all samples)	35 (11.15%)		

Table 3.2: Presence-absence of L120Q, Y139C and Y139F in English counties (findings unknown prior to 2017 are highlighted in bold; asterisks indicate uncertainty due to the grouping of West and East Sussex by Haniza *et al.*, 2015).

County	L120Q presence	Y139C presence	Y139F presence
Berkshire	Yes		
Dorset	**Yes**		
East Sussex	**Yes***		Yes
East Yorkshire		Yes	
Essex	**Yes**		
Gloucestershire		Yes	
Greater London	**Yes**		
Hampshire	Yes		
Kent	**Yes**		Yes
Norfolk	**Yes**	Yes	**Yes**
Oxfordshire	Yes		
South Yorkshire		**Yes**	
Suffolk			**Yes**
Surrey	Yes	**Yes**	
West Sussex	**Yes***		
Wiltshire	Yes		

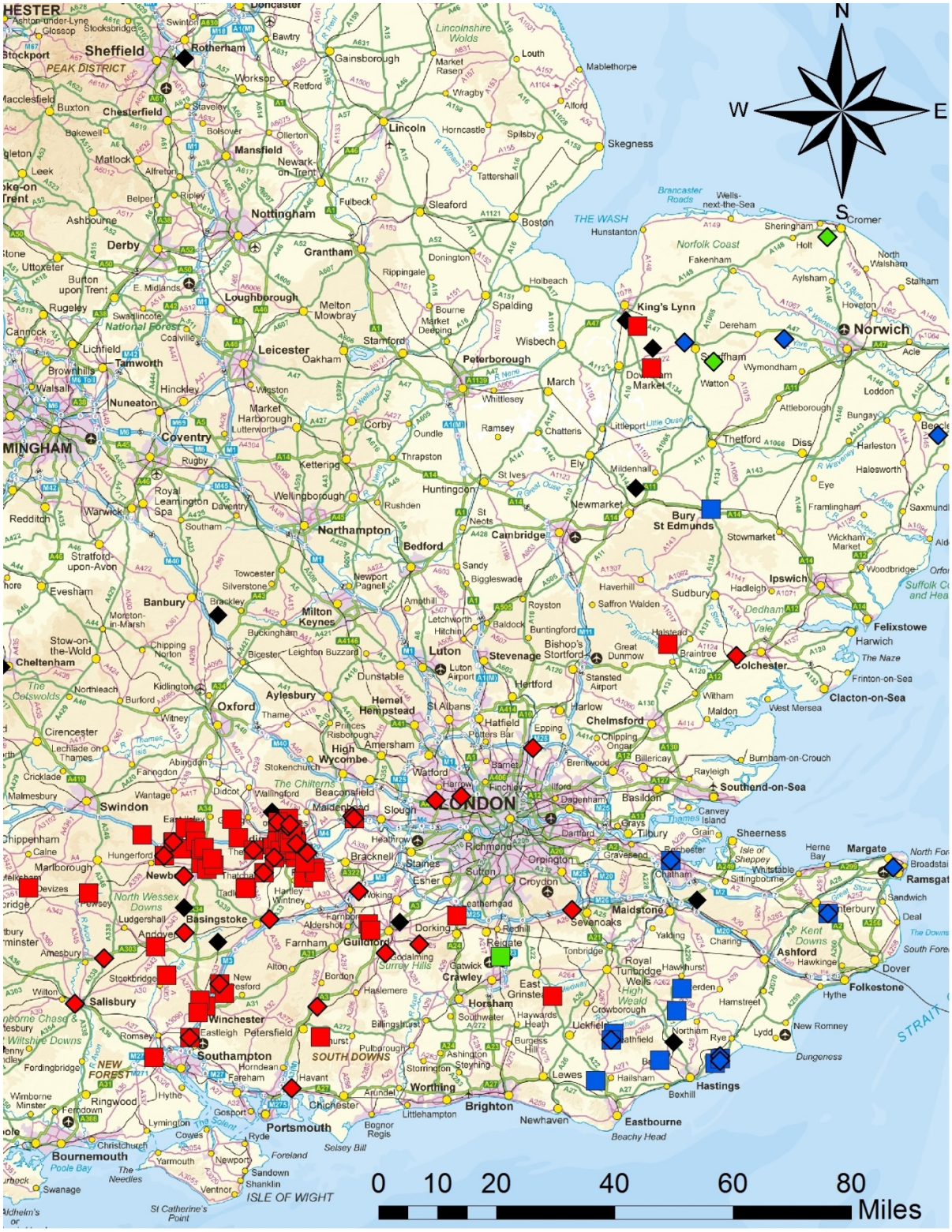

Figure 3.1: VKORC1 exon 3 mutations in south-east England. Squares represent homozygous animals; diamonds represent heterozygous. Blue = Y139F; Red = L120Q; Green = Y139C; Black Diamonds = susceptible (wild-type). Location of Y139F-positive samples taken by Prescott *et al.* (2010) is included for context.

Figure 3.2: VKORC1 exon 3 mutations in Wales, north England and Scotland. Squares represent homozygous animals; diamonds represent heterozygous. Green = Y139C; Yellow = L128Q; Pink = Y139S; Black Diamonds = susceptible (wild-type); Yellow and Red = L128Q and L120Q co-mutation.

Of the 304 samples, 269 (88.49%) were found to be positive for at least one VKORC1 exon 3 mutation. Of the 269 samples that were found to be positive for VKORC1 exon 3 mutations, 186 (69.15%) were homozygous for that mutation (see Table 3.1). In the south-east of England foci of the L120Q, Y139C and Y139F mutations were found to be widespread (see Table 3.2).

3.3.2 Exons 1 and 2

Of the 178 samples taken post-April 2012 that were successfully tested for VKORC1 exon 3 mutations, 92 were successfully tested for four SNPs in exon 1 and 86 were successfully tested for four SNPs in exon 2 (see Table 3.3). Among the mutations investigated in exon 1, no SNPs were found in any of the 92 samples, despite three of them having previously been found in the UK (Rost *et al.*, 2009). Only two SNPs were found in exon 2 across all samples. Nineteen samples were positive for the silent mutation I82I. A previous study in the south-east of England showed that in a sample group of 10 wild rats, the exon 3 mutation Y139F was positively linked with the exon 2 mutation I82I (Prescott *et al.*, 2010). However, of the 19 samples herein that were positive for I82I, only four were associated with Y139F. Six were associated with L120Q, two were associated with Y139C, and seven weren't associated with any of the main five exon 3 mutations. Three rats were homozygous for the I82I mutation; one with the Y139F mutation, and two which were wild-type at exon 3. Five samples (including two with I82I), were found to have a new mutation at position 99, wherein cysteine was substituted for phenylalanine (C99I). All 5 of these samples were heterozygous for this mutation, and were associated in four cases with the L120Q resistance and in one case with Y139F resistance. Thus of 86 samples, 22 were positive for SNPs of exon 2.

Table 3.3: SNPs of exons 1 and 2. SNPs marked with an asterisk (*) are original to this study.

SNP	Total Number found	Mutation type	Exon
A26T	0	Amino Acid Substitution	1
R33P	0	Amino Acid Substitution	1
R35P	0	Amino Acid Substitution	1
Y39N	0	Amino Acid Substitution	1
E67K	0	Amino Acid Substitution	2
I82I	17	Silent Mutation	2
I82L	0	Amino Acid Substitution	2
L94L	0	Silent Mutation	2
C99I*	3	Amino Acid Substitution	2
I82I and C99I*	2	Silent Mutation and Amino Acid Substitution	2

3.4 Discussion and conclusions

3.4.1 Spread of resistance foci and homozygosity

The results shown in Figure 3.1 are not encouraging for stakeholders in the south-east of England, where the bulk of the study has been focused. Of the 304 samples analysed for exon 3, 269 (88.49%) possessed a VKORC1 mutation; of the 86 samples analysed for exon 2, 22 (25.58%) possessed a VKORC1 mutation; and of the 92 samples analysed for exon 1, there were no VKORC1 mutations. The lack of association between the exon 2 I82I and exon 3 Y139F mutations (both were primarily found in the other's absence) indicates that there is unlikely to be any association between resistance-conferring mutations of exon 3 and other mutations in the VKORC1 coding exons. Only 11 of the 269 samples (4.09%) positive for a VKORC1 exon 3 resistance conferring mutation had only L128Q or Y139S, and none of these came from the main study area (see Table 3.1). The three stronger resistance conferring mutations (L120Q, Y139C and Y139F) were discovered to be located in areas they had never been found before 2017 (see Table 3.2).

3.4.2 History and causes of resistance

The presence of the stronger resistance-conferring mutations throughout the study area most likely indicates the use of ineffective rodenticides sufficient to allow the selection of resistance to occur across the south-east of England. This is in spite of the known pleiotropic costs of resistance in Norway rats from Hampshire (Greaves and Cullen-Ayres, 1988). Evidence for and against pleiotropic costs of the L120Q mutation is mixed (see Gill, Kerins and MacNicoll, 1992), but the presence of these mutations wherever they are looked for in south-east England is an indication that ineffective anticoagulant rodenticides have been used there, sufficiently to allow the mutations to become established in Norway rat populations. There is also a high degree of homozygosity across these resistant populations, providing clear evidence that resistance is being, and for years has repeatedly been selected for, via application of ineffective anticoagulant rodenticides such as FGARs, bromadiolone and potentially difenacoum. The selection for resistant animals is compounded by the use of menadione as a supplement in animal feed on farms. Normally, in the absence of the application of anticoagulant rodenticides, some strains of resistant rats will have a reduced fitness compared to susceptible animals, because of their higher dietary requirement for vitamin K (Hermodson, Suttie and Link, 1969; Greaves and Ayres, 1973; Bishop, Hartley and Partridge, 1977; Markussen *et al.*, 2003; Jacob *et al.*, 2012). However, due to the widespread use of menadione-supplemented feed on farms, this pleiotropic effect of the VKORC1 resistance mutations is nullified, thereby maintaining the prevalence – or at least presence – of resistance in Norway rat populations. Therefore, when ineffective rodenticides are applied, their failure to gain complete control of the population is already assured. Due to restrictions on use of certain anticoagulant active ingredients in the UK (European Community, 1998) prior to the introduction of the CRRU rodenticide stewardship scheme, RRAG advocated avoiding the use of difenacoum or bromadiolone to control rat populations

known to possess any of the three mutations found in south-east England (RRAG, 2012). The Campaign for Responsible Rodenticide Use (CRRU) has developed a stewardship scheme for the Health and Safety Executive (HSE) which is currently being put into place, under which stronger SGARs (brodifacoum; difethialone; flocoumafen) thought to be efficacious against resistant rats will be used by persons trained in their safe and effective use (Buckle *et al.*, 2017). Similar schemes are being put in place in France, Germany and Spain and are recommended by the EU. The success of UK stewardship scheme is vital to the future of rodent control in the UK; the results of this study are evidence that the previous model of legislation for rodenticides was unsustainable and at some point could have led to serious public health, economic and environmental fallout.

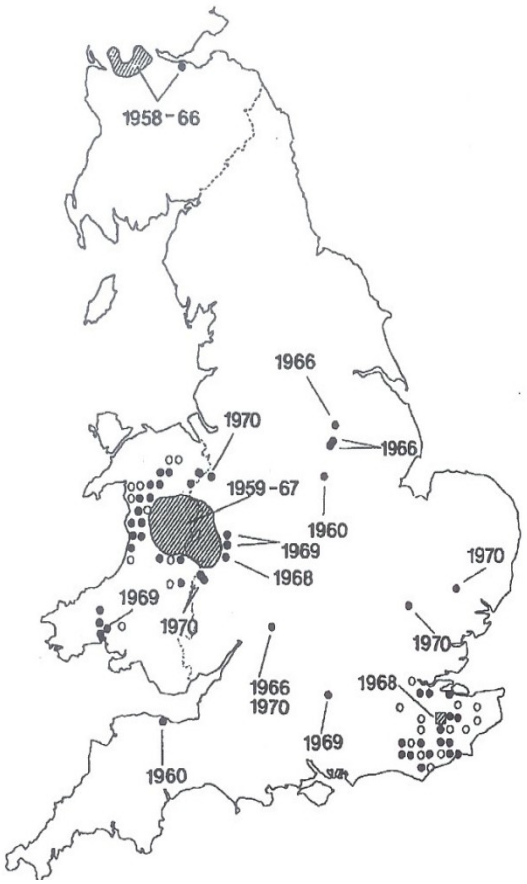

Figure 3.3: Anticoagulant resistance in Norway rats in the UK in 1970. Filled symbols = confirmed resistance. From Greaves and Rennison (1973).

The size and locations of resistance foci, along with the surprising presence of numerous mutations in close proximity, may elucidate the origins of some of the UK's Norway rat resistance. No other country has a comparable variety and number of resistance foci, but aside from the Welsh mutation, all other VKORC1 exon 3 resistance conferring mutations have been identified in Europe and elsewhere. This could be attributed to the UK's island status and associated maritime tradition. Shipping to and from areas of continental Europe containing resistance foci may have spread the mutations far quicker and further than rodenticide-induced selection. What is not clear is whether the UK acted as the source or as a sink for resistant rats; potentially it could have done both. The data from this study also shed light on previous discoveries of resistance before the molecular methods that would enable their identification were available. For instance, resistance was present almost throughout Kent by 1970 (Greaves and Rennison, 1973; see Figure 3.3). It is possible that rats in this resistance focus have always possessed the Y139F mutation, but this cannot be confirmed. Given the now well-known resistance-conferring properties of this mutation (Grandemange *et al.*, 2009), had the molecular techniques used herein been available for use in the 1970's, it is possible that sustained use of ineffective rodenticides could have been avoided, along with the associated previously-discussed impacts (see Sections 1.4.3 and 4.1.2.3). The same is likely true of the south Essex area near Ipswich, where both L120Q and Y139F mutations have been found in rats not far from areas of historical resistance (Greaves and Rennison, 1973).

3.4.3 Methodological constraints

As described above, obtaining DNA samples for analysis was the greatest hurdle faced in this study. Despite a variety of media appearances, adverts in trade publications and appeals from

senior industry figures, very few samples were provided when compared with similar studies from across Europe. For this reason, faecal samples were used as a source of DNA for analysis, but due to the difficulty of retaining their viability in storage and the high likelihood of their being contaminated, there was a low success rate in their analysis. In addition, it bears stating that this study is useful only for delimiting resistance foci, not for exploring the depth of resistance at each site. For this reason, it is acceptable that some of the results may have been skewed by the preference of the pest control industry to only get in touch with researchers once they had encountered what appeared to be practical resistance. The need to avoid false negatives (i.e. samples appearing to show a site as resistance free when in actuality it isn't) is greater than the need to obtain a truly random set of samples.

3.4.4 Future work

The continuation of this work is vital to the future understanding of resistance in the UK, as well as the fight against it. Once these data are published and seen by stakeholders around the country, it is likely that interest in the project will increase and more tail samples will be made available for analysis. More samples from unexplored areas will only strengthen the dataset, and allow much greater analysis of the data therein. This in turn will aid the pest control industry and property owners in their attempts to control infestations of Norway rats. Experiments are required that will fully elucidate the effects that each of the mutations investigated herein have on efficacy of control attempts. Thereby, future editions of maps produced for this study will inform PCOs and other stakeholders not just of where resistance foci exist, but how best to deal with them.

Chapter 4: Determining resistance factors for three strains of Norway rat in the laboratory

4.1 Introduction

4.1.1 History of laboratory resistance tests

Very soon after the initial discovery of anticoagulant resistance, rats were captured in the wild and brought into the laboratory to be subjected to tests intended to elucidate the impact that resistance would have on future attempts at practical control in the field. Breeding experiments utilising the offspring of these wild progenitors revealed that resistance is controlled by a single, dominant autosomal gene mapped to orthologous linkage groups in both Norway rats (*Rw*; chromosome 1) and house mice (*Raw*; chromosome 7) (Greaves and Ayres, 1969; Wallace and MacSwiney, 1976). Just as laboratory feeding tests were used to confirm efficacy of potential anticoagulant rodenticides prior to their marketing and subsequent use in the field (Bentley, Hammond and Taylor, 1955; Bentley and Rowe, 1956; Bentley and Larthe, 1959; Redfern, Gill and Hadler, 1976) so they were used to confirm suspected instances of resistance (Boyle, 1960; Cuthbert, 1963; Lund, 1964) or to confirm their efficacy against various resistant strains (Redfern and Gill, 1978; Gill, 1992). As the scale of resistance in both Norway rats and house mice became clear (Dodsworth, 1961; Bentley, 1968), laboratory tests on these rodents became less reactive and more proactive, focusing on level of tolerance that resistance mutations conferred against the various anticoagulants. These tests involved offering warfarin baits or warfarin-infused foodstuffs to rats at given rates, and monitoring their survivability in what appeared to be largely ad-hoc experiments. Individuals that died contributed to the generation of lethal feeding periods ($LFP_{01\text{-}99}$) rather than lethal doses ($LD_{01\text{-}99}$), because it was not possible to determine the

exact volume of warfarin each individual had consumed. As described in Section 2.2.1, although a standard test methodology was established (six days' feeding on 0.005% warfarin bait) that appeared to identify rats resistant to warfarin (Drummond and Bentley, 1965; Bentley, 1968; Drummond and Wilson, 1968), a method that was quicker and less questionable in its humaneness (because it did not rely on mortality of test animals) was quickly posited (Greaves and Ayres, 1967). BCR resistance tests were expanded to include a variety of anticoagulant active ingredients, establishing discriminating doses for FGARs and SGARs against susceptible rats, and subjecting laboratory-resistant animals of known origin and strain to the same test. Using this information, assuming researchers had identified a particular strain correctly (i.e. by its nominal geographic designation), these resistance tests could be applied to wild rats, and the wild population in question designated as resistant or susceptible based on whether or not individuals were "responders" (i.e. to be significantly affected by the anticoagulant administered). This designation was based upon the percentage coagulation activity (PCA) of test animals. Calibration curves based on dilutions of normal plasma in saline are used to convert measured coagulation times in seconds to PCA in order to ascertain the level of coagulation capability that blood samples have retained. Examples of these tests include chlorophacinone and diphacinone against "Welsh" and "Hampshire" resistant strains (Prescott and Buckle, 2000); warfarin against "Welsh" rats (Greaves and Ayres, 1967; Martin *et al.*, 1979; MacNicoll and Gill, 1993a), "Scottish" and "Hampshire" rats (MacNicoll and Gill, 1993a); and bromadiolone against "Welsh", "Scottish", "Hampshire" and "Berkshire" rats (Gill *et al.*, 1994). Difenacoum tests were carried out against "Welsh", "Scottish" and "Hampshire" rats (Gill *et al.*, 1993), but because these aimed to replicate the results of LFP tests as well as monitor PCA (that is, cause mortality in susceptible rats whilst monitoring PCA over several days, rather than classifying rats as responders or non-responders in a one-time test) discriminating doses are much higher than

expected, and of little use. They also lacked the bonuses of reduced time, cost and humaneness issues. There was little consistency between these tests. SGAR tests utilised a 96-hour period between dosing and blood sampling, whereas a 24-hour period was established when testing FGARs. Furthermore, in SGAR tests rats were considered responders if their PCA was less than 10%; rats in tests involving FGARs were classified as responders if their PCA fell below 17% (see also Baert *et al.*, 2012). The level and standard of efficacy necessary to discriminate between susceptible and resistant rats was not consistent between studies. Only in the tests involving bromadiolone, diphacinone and chlorophacinone was the point of discrimination (ED_{99} or the upper 95% fiducial limit thereof) established prior to testing by Probit analysis (EPPO, 1999b). Furthermore, utilising a different method of analysis (e.g. generating a Probit log dose-response rather than a Probit dose-response) will create varying estimates of ED_{99}. The methods available to generate dose response data are primarily designed to estimate the ED_{50}; as effective dose responses approach 99% (or 1%) the fiducial limits associated with the predicted effective dose grow wider, compared with the size of the predicted effective dose. This effect is compounded when these methods are used in tests against vertebrate animals, numbers of which are reduced for humaneness reasons wherever possible. So even when standardised, tests to determine ED_{99}s are not reliable. In addition to the inherent problems with generating and utilising ED_{99}s, the application of these tests was also limited because their main use was simply to ascertain whether rats of a given resistant strain could tolerate a dose of a given anticoagulant that would kill a given number of susceptible animals. These data cannot be compared without making assumptions based on extrapolation and guesswork. The tests were all specific to each strain, and other than the nominal geographic naming tendencies in the UK, there was no guarantee that results from a particular study could be applied to practical pest control in other areas of the UK, or elsewhere. In addition, in the field there are frequently rats

heterozygous for the resistance mutations in question, which respond differently to anticoagulant and vitamin K-deficient stimuli, than do susceptible or homozygous-resistant rats (see Section 1.4.2).

With the initial identification of the resistance-conferring mutations (Li *et al.*, 2004; Rost *et al.*, 2004) and their confirmed locations (e.g. Pelz et al., 2005), data from all future resistance tests – and any previous tests wherein viable tissue of the test subjects had been retained, or the strain in question was still extant with no further introductions of foreign animals – could potentially contribute to the sum of all researchers' knowledge, regardless of the animals' provenance. Until now, most research has focused on identification and delimitation of resistance foci. Given the extent of the L120Q focus in the south-east of England (see Section 3.3.1), there is a clear requirement to quantify the level of tolerance (resistance factor: RF) to anticoagulants that the various L120Q strains confer.

4.1.2 International normalised ratio (INR)

The standardised method was established by Prescott et al. (2007) when a range of baseline effective doses of the second generation anticoagulant rodenticides were established for susceptible laboratory animals. Although there may be a range of differences between laboratory and wild rats, using laboratory rats allowed for a homogenous study group of known provenance. There are multiple thromboplastin reagents used for the assessment of clotting activity, with varying sensitivities, which would result in different Prothrombin Time (PT) results when used to assess the same sample. Therefore, a method of standardisation is required. In human haematology, the various methods and reagents have been afforded a calibration reference system (Denson, 1998) based on a WHO standard. The International Sensitivity Index (ISI) measures the sensitivity of each methodology, applying a ratio of

sensitivity (the INR) of the method and reagent compared with the reference material. For instance, a reagent with an ISI of 2 would obtain a PT for a human blood sample exactly twice the time of that obtained by the reference material. The reagent used herein (Diagen freeze-dried rabbit brain thromboplastin; RBT) has an ISI of 1.4. Norway rat PCA calibration curves produced using the reagent showed that a PCA of 17% corresponded with Diagen RBT INR of 5. As suggested by Prescott et al. (2007), and in accordance with instructions supplied by Diagen, safe human coagulation has an INR range of 2 – 4.5. Therefore, a PT of 47.5 seconds (which corresponds to INR 5) was identified as the discriminating point, above which rats were classified as "responders" to the applied anticoagulant dose.

4.1.3 Resistant Norway rat strains housed at the University of Reading

The history of resistant laboratory strains established in the UK is discussed in detail in Section 2.2.2.1. There are currently three strains of anticoagulant-resistant rats housed at the University of Reading. A previous strain of laboratory resistant rats, derived from animals caught in Hampshire, was present until recently; this "Hampshire" strain was lost due to excessive inbreeding prior to the start of this work. Some resistance factors were established for this strain prior to their loss (Prescott et al., 2007). The central-southern England L120Q focus appears to be largely comprised of homozygotes (see Section 3.1), making the uniformly homozygous rats housed at the University of Reading a suitable model.

4.1.3.1 Welsh strain

Henceforth referred to as Welsh rats, this strain is descended from a group of resistant rats from Wales (Bentley, 1968) which were captured and taken to the Pest Infestation Control Laboratories, situated at Tolworth. There they were challenged with anticoagulants in order

to be selected for homozygosity and back-crossed onto susceptible laboratory rats as described in Section 2.2.2.1. The Tolworth laboratories were run under the auspices of the Ministry of Agriculture and Fisheries (MAF), and consequently had access to the MAF's own susceptible strain of rats, the Tolworth Albino Susceptible (TAS) rats, and it was onto these rats that the wild resistant rats were back-crossed. They are homozygous for the resistance mutation Y139S. These rats are grey-black; some individuals attain silver-white hairs on their backs. It is thought that their colouration is related to their resistance status (Prescott, pers. Comm.; Greaves and Ayres, 1969). The Welsh resistance focus has been repeatedly shown to be susceptible to the SGARs, so their inclusion in this study is for the purpose of identifying the least toxic compound which can be safely – and effectively – used for their control, and also as a comparison with the other strains housed at Reading.

4.1.3.2 Reading Berkshire strain

Henceforth referred to as Reading Berkshire rats, this strain is descended from a group of resistant rats from north Berkshire where rats were discovered in 1992 to have a mutation that conferred technical resistance to Brodifacoum (Gill, Kerins and MacNicoll, 1992) and practical resistance to Bromadiolone (Quy *et al.*, 1995). Rats captured at this site were taken to the University of Reading where they were back-crossed onto susceptible CD albino Norway rats obtained from Charles River Ltd, and the resulting progeny challenged with anticoagulants in order to be selected for homozygosity as described in Section 2.2.2.1 (Hussain, 1998). They are homozygous for the resistance mutation L120Q. These rats are white with black hoods and varying sizes and patterns of black patches running down the ventral and dorsal surface.

4.1.3.3 CSL Berkshire strain

Henceforth referred to as CSL Berkshire rats, this strain's wild ancestors originated from the same site as those of the Reading Berkshire strain. Rats trapped at this site were taken to the Central Science Laboratory (CSL) facility in Yorkshire and back-crossed onto individuals from a susceptible strain of rats selected for homozygosity, as described in Section 2.2.2.1. Like the Welsh rats, TAS rats were used as the laboratory susceptible rats with which the wild resistant rats were crossed. Thereafter they were, for six generations, repeatedly subjected to anticoagulant feeding tests devised to achieve partial mortality amongst test subjects. Only rats which survived these tests were allowed to breed, resulting in a "Selected Line" of highly resistant animals. Rats of this strain are therefore characterised by a much greater tolerance to anticoagulants than Reading Berkshire rats, despite possessing the same resistance mutation (for which they are homozygous), and appearing largely identical in colouration. There may be other unknown differences between the two strains as a result of their being descended from different strains of susceptible rats. The strain was later gifted to Sorex in its entirety, and was then in turn gifted to the University of Reading.

4.2 Methods

4.2.1 BCR tests

BCR tests were conducted using Amelung KC4 micro semi-automatic haemostasis equipment (Diagnostica Stago UK Ltd, Theale, United Kingdom). Four cuvettes were used for each plasma sample. Diagen freeze dried rabbit brain thromboplastin (Diagnostic Reagents Ltd, Thame, United Kingdom) was reconstituted with 5 ml purified water and left for 10 minutes, as per manufacturer's instructions.

With each cuvette:

1. a ball bearing was placed in each cuvette
2. 50 µl rabbit brain thromboplastin added
3. 50 µl plasma was added, and the cuvettes were allowed to heat in the Amelung for two minutes
4. 25 µl calcium chloride (Diagnostic Reagents Ltd) solution was added using an automated pipette which starts the Amelung timer
5. On clot formation the ball bearing was prevented from moving and the timer was stopped
6. The clotting time (PT) was recorded

The calcium chloride initiated the clotting cascade by forming complexes with the activated clotting factors which could be hosted by phospholipid membranes, which in turn activate further clotting factors in the coagulation cascade, resulting in the activation of prothrombin to thrombin (See Section 1.3.5.2). For each plasma sample, the replicate values were recorded, and if three values were within a 10-second window, the average of these was taken as the final PT, and was converted to INR using tables provided with the Thromboplastin Reagent. If three values were not within a 10-second window, the process was repeated.

4.2.1.2 Vitamin K

As described in Section 2.2.2.2, vitamin K was not co-administered to rats used in this study: the vitamin K available in the diet supplied was greater than the 0.5 mg/kg bodyweight identified as the minimum requirement to prevent vitamin K deficiency (Hussain, 1998).

4.2.2 Dosing methodology

The stock solutions of 10mg/ml and 1 mg/ml, when administered to rats at a standard rate of 0.5 ml per 100 g of bodyweight, will deliver a dose of 50 mg/kg bodyweight and 5 mg/kg bodyweight of active ingredient respectively. Prior to gavage, stock solutions were diluted by weight with PEG200 to the required concentrations, as described in Section 2.2.3. Rats to be dosed were weighed to within 1 g. After dosing, each rat was given a unique tail mark and test code for identification purposes. Blood sampling was conducted 24 hours after dosing. The 24-hour interval was chosen over 96 hours in order that the data could be compared with the susceptible data generated by Prescott *et al.*, (2007). Rats were sedated under terminal anaesthesia with isoflurane (Merial, Harlow, United Kingdom), and 0.9 ml of blood withdrawn via cardiac puncture into 0.1 ml of 3.2% tri-sodium citrate (to prevent clotting). The latter was made by dissolving 3.2 g of pure tri-sodium citrate crystals (Sanofi UK, Guildford, United Kingdom) in 100 ml purified water. The blood samples were centrifuged at 5300 rpm for six minutes, resulting in plasma being separated from all other blood contents. Plasma was removed using a pipette and either processed immediately, or frozen at -21^{o}C for future processing.

4.2.3 Second generation anticoagulant rodenticides

All five SGARs were utilised in this study. It was intended that resistance factors be obtained for Welsh, Reading and CSL Berkshire rats against brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen. PEG200 (polyethylene glycol, molecular weight 200) and triethanolamine were supplied by Sigma Aldrich, and were used to dissolve and dilute the pure SGARs brodifacoum, bromadiolone, difenacoum and flocoumafen to stock solutions of either 10 mg/ml (bromadiolone and difenacoum) or 1 mg/ml (brodifacoum and flocoumafen)

as described in Section 2.2.3. Difethialone was supplied as a 1 mg/ml dilution in PEG200 by Liphatech (Liphatech, Bonnel, 47480 Pont du Casse, France).

4.2.4 Study rats

Between October 2009 and May 2017, rats were dosed with SGARs and their blood clotting activity was determined as detailed in Section 4.2.1. For each resistant strain of Norway rats, sexes were dosed separately, and active ingredient was administered over a range of doses in order to generate dose response data that can be analysed by Probit analysis (see Section 4.2.5). Rats were typically dosed when they became available, resulting in highly variable group sizes for each round of dosing. SGAR-strain-gender groups ranged in size between 27 individuals and 67 individuals. Dose rates were based on established ED_{50} data for susceptible rats (Prescott et al., 2007) or on previous data generated within the study from active ingredients with a similar level toxicity. As described in Section 2.2.3, a total of 78 rats underwent BCR testing without anticoagulant dosing. Results from these tests were analysed using Linear Regression Models in R i386 3.2.3 (R Core Team, 2015) in order to discover the impact of rodent bodyweight and sex on resting PT. Rats used in the main study typically had a minimum bodyweight of 150 g, and a maximum bodyweight of 400 g, for ease of handling. However, due to external issues and the late availability of rats retained for breeding this maximum bodyweight was occasionally exceeded. Husbandry of the study animals is described in Section 2.2.2.2 and Section 2.2.2.3.

4.2.5 Data analysis

As described in Section 2.2.5, data from SGAR-strain-gender groups were analysed using the PROBIT procedure in SAS (Statistical Analysis System, Marlow, United Kingdom), in order to obtain 95% fiducial limits for effective dose percentiles (ED_{01}-ED_{99}). When fiducial limits were obtained, data from both sexes of a SGAR-strain group were compared and analysed by the GENMOD procedure in SAS. This established whether the response lines for males and females of the same strain were statistically separate, parallel or coincident, and based on these results, the data for the two sexes were re-analysed using the PROBIT procedure, to provide Probit dose response data with fiducial limits. In situations where fiducial limits were large, clotting times from additional animals were determined, and the resulting data set was re-analysed. These effective dose percentiles were compared with those of susceptible animals (Prescott et al., 2007, in which the same methodology was used thereby affording accurate resistance factors) in order to generate resistance factors for each SGAR-strain-gender group.

4.3 Results

4.3.1 Effect of weight on PT

Linear Regression Models were used to compare the resting PT of male and female CSLxReading Berkshire rats in R i386 3.2.3 (R Core Team, 2015). No significant regression equations were found for bodyweight ($F(1, 77) = 0.3814$, $p = 0.5387$; R^2 of 0.0049), sex ($F(1, 77) = 0.0994$, $p = 0.7534$; R^2 of 0.0013), sex and bodyweight ($F(2, 76) = 0.3065$, $p = 0.7369$; R^2 of 0.008) or an interaction of the two factors ($F(3, 75) = 0.3092$, $p = 0.8186$; R^2 of 0.0122). Therefore the PT of non-anticoagulant compromised rats was not affected in any

significant way by bodyweight, and it was assumed that rats of any size could be used during the BCR tests with SGARs.

4.3.2 Probit response between sexes and between strains

Male and female CSL Berkshire rats dosed with bromadiolone exhibited separate Probit response lines; during the Genmod procedure the Probit response lines deviated significantly from a parallel response, and were not analysed further. Male and female CSL Berkshire rats dosed with all other SGARs exhibited parallel Probit response lines; during the Genmod procedure the Probit response lines did not deviate significantly from a parallel response, but when analysed further they deviated significantly from a coincident response.

Male and female Reading Berkshire rats dosed with brodifacoum exhibited separate Probit response lines, as described above. Male and female Reading Berkshire rats dosed with difenacoum exhibited parallel Probit response lines, as described above.

Male and female Welsh rats dosed with bromadiolone and difenacoum exhibited separate Probit response lines, as described above. Male and female Welsh rats dosed with brodifacoum exhibited parallel Probit response lines, as described above. A summary of these results is provided in Table 4.1.

Male Reading and susceptible rats dosed with brodifacoum and difenacoum exhibited separate Probit response lines, as described above. Female Reading and susceptible rats dosed with brodifacoum, bromadiolone and difenacoum exhibited separate Probit response lines, as described above. Male Welsh and susceptible rats dosed with bromadiolone and difenacoum exhibited separate Probit response lines, as described above. Male Welsh and susceptible rats dosed with brodifacoum exhibited co-incident response lines; during the Genmod procedure

the Probit response lines did not deviate significantly from a parallel response, and when analysed further they did not deviate significantly from a coincident response. Female Welsh and susceptible rats dosed with brodifacoum and bromadiolone exhibited separate Probit response lines, as described above, and female Welsh and susceptible rats dosed with difenacoum exhibited co-incident response lines, as described above. A summary of these results is provided in Table 4.2.

4.3.3 Probit dose-response percentiles and resistance factors

By comparing the Summary Probit dose-response data generated in this study with those made available by Prescott et al. (2007), resistance factors have been calculated at the ED_{40}, ED_{50}, ED_{60} and ED_{99} level for all SGARs and resistant Norway rat strains tested herein. Summary Probit dose-response data and derived resistance factors are presented for CSL Berkshire rats in Table 4.3, for Reading Berkshire rats in Table 4.4 and for Welsh rats in Table 4.5.

Table 4.1. Comparison of the Probit response between male and female rats for various SGARs against various strains of resistant Norway rats.

Resistant rat strain	SGAR	Deviance (Separate)	Deviance (Parallel)	Deviance (Coincident)	Chi Square (Parallel - Separate)	p-value	Chi Square (Coincident - Parallel)	p-value	Result
CSL Berkshire	Bromadiolone	1.0063	7.1859	13.8067	6.1796	< 0.05	-	-	Separate
CSL Berkshire	Difenacoum	7.4219	7.621	31.8721	0.1901	0.6555	24.2511	< 0.0001	Parallel
CSL Berkshire	Brodifacoum	3.3415	4.4792	27.4704	1.1377	0.2861	22.9912	< 0.0001	Parallel
CSL Berkshire	Flocoumafen	4.4359	4.5443	19.6707	0.1084	0.742	15.1264	< 0.0001	Parallel
CSL Berkshire	Difethialone	0.5726	1.9534	9.8267	1.3808	0.24	7.8733	< 0.05	Parallel
Reading Berkshire	Difenacoum	9.4394	12.6925	35.2156	3.2531	0.0712	22.5231	< 0.0001	Parallel
Reading Berkshire	Brodifacoum	3.2398	8.7854	22.8874	5.5456	< 0.05	-	-	Separate
Welsh	Bromadiolone	3.6957	14.0363	34.3458	10.3406	< 0.05	-	-	Separate
Welsh	Difenacoum	9.1407	13.6472	21.5876	4.5065	< 0.05	-	-	Separate
Welsh	Brodifacoum	3.696	4.9011	24.0659	1.2051	0.2723	19.1648	< 0.0001	Parallel

Table 4.2. Comparison of the Probit response between resistant and susceptible rats for various SGARs against males and females.

Resistant rat strain	SGAR	Deviance (Separate)	Deviance (Parallel)	Deviance (Coincident)	Chi Square (Parallel - Separate)	p-value	Chi Square (Coincident - Parallel)	p-value	Result
Reading Berkshire female	Bromadiolone	16.2938	38.7705	73.0947	22.4767	< 0.0001	-	-	Separate
Reading Berkshire female	Difenacoum	6.6565	17.8135	25.5349	11.157	< 0.05	-	-	Separate
Reading Berkshire male	Difenacoum	2.802	16.5801	20.1458	13.7781	< 0.05	-	-	Separate
Reading Berkshire female	Brodifacoum	5.0272	18.6561	31.2904	13.6289	< 0.05	-	-	Separate
Reading Berkshire male	Brodifacoum	2.448	11.1122	13.8977	8.6642	< 0.05	-	-	Separate
Reading Berkshire female	Flocoumafen	4.2592	11.1408	26.3128	6.8816	< 0.05	-	-	Separate
Welsh female	Bromadiolone	35.5877	58.6029	59.0535	23.0152	< 0.0001	-	-	Separate
Welsh male	Bromadiolone	0.8061	15.1577	18.3702	14.3516	< 0.05	-	-	Separate
Welsh female	Difenacoum	0.0024	0.8313	0.8314	0.8289	0.3626	0.0001	0.992	Coincident
Welsh male	Difenacoum	9.1574	16.8313	29.0137	7.6739	< 0.05	-	-	Separate
Welsh female	Brodifacoum	6.7137	16.9046	22.959	10.1901	< 0.05	-	-	Separate
Welsh male	Brodifacoum	1.2177	2.8823	6.024	1.6646	0.197	3.1417	0.0763	Coincident

Table 4.3. Effective doses and resistance factors of all SGARs at various percentiles against male and female CSL Berkshire rats.

		Effective Dose [mg/kg] (lower – upper fiducial limits) **Resistance Factor compared with susceptible rats**				
Effective Dose	**Sex**	**Brodifacoum**	**Bromadiolone**	**Difenacoum**	**Difethialone**	**Flocoumafen**
40%	Male	0.56 (0.41 – 0.66) 2.67	4.61 (3.02 – 4.83) 10.02	3.02 (2.48 – 3.68) 4.72	0.87 (0.75 – 0.98) 2.12	0.71 (0.6 – 0.79) 2.63
50%	Male	0.6 (0.47 – 0.71) 2.73	4.67 (3.48 – 4.93) 9.94	3.28 (2.73 – 4.09) 5.05	0.9 (0.79 – 1.02) 2.09	0.74 (0.64 – 0.82) 2.64
60%	Male	0.64 (0.52 – 0.77) 2.91	4.74 (3.95 – 5.09) 9.88	3.58 (2.98 – 4.57) 5.42	0.93 (0.82 – 1.07) 2.11	0.77 (0.67 – 0.86) 2.66
99%	Male	1.16 (0.92 – 2.18) 4.3	5.32 (5.00 – 14.84) 9.5	7.17 (5.35 – 13.31) 9.08	1.23 (1.07 – 1.73) 2.05	1.08 (0.95 – 1.41) 3.0
40%	Female	1.37 (1.06 – 1.57) 6.23	6.57 (4.25 – 8.63) 10.77	9.17 (7.57 – 11.1) 11.91	1.09 (0.97 – 1.21) 2.54	0.98 (0.85 – 1.11) 3.06
50%	Female	1.47 (1.2 – 1.68) 6.68	7.47 (5.13 – 9.79) 12.05	9.98 (8.34 – 12.33) 12.8	1.13 (1.01 – 1.26) 2.31	1.03 (0.9 – 1.16) 3.12
60%	Female	1.58 (1.34 – 1.83) 7.18	8.49 (6.12 – 11.24) 13.48	10.87 (9.12 – 13.8) 13.59	1.17 (1.06 – 1.32) 2.29	1.07 (0.94 – 1.22) 3.15
99%	Female	2.86 (2.29 – 5.35) 12.43	24.21 (16.78 – 53.54) 33.16	21.8 (16.33 – 40.19) 22.95	1.54 (1.35 – 2.18) 2.23	1.51 (1.31 – 2.05) 3.6

Table 4.4. Effective doses and resistance factors of brodifacoum, bromadiolone and difenacoum at various percentiles against male and female Reading Berkshire rats.

		Effective Dose [mg/kg] (lower – upper fiducial limits) Resistance Factor compared with susceptible rats			
Effective Dose	**Sex**	**Brodifacoum**	**Bromadiolone**	**Difenacoum**	**Flocoumafen**
40%	Male	0.13 (0.08 – 0.2) 0.62	-	0.3 (0.16 – 0.5) 0.47	-
50%	Male	0.16 (0.1 – 0.25) 0.73	-	0.39 (0.21 – 0.65) 0.6	-
60%	Male	0.19 (0.13 – 0.32) 0.86	-	0.5 (0.29 – 0.84) 0.76	-
99%	Male	0.77 (0.41 – 4.54) 2.85	-	3.8 (1.96 – 12.01) 4.81	-
40%	Female	0.49 (0.37 – 0.59) 2.23	1.51 (1.09 – 1.91) 2.48	1.78 (1.09 – 2.69) 2.31	0.42 (0.2 – 0.64 1.27
50%	Female	0.52 (0.41 – 0.63) 2.36	1.77 (1.34 – 2.25) 2.86	2.28 (1.45 – 3.5) 2.89	0.51 (0.28 – 0.76) 1.5
60%	Female	0.55 (0.45 – 0.69) 2.5	2.08 (1.63 – 2.7) 3.3	2.93 (1.93 – 4.66) 3.62	0.57 (0.37 – 0.97) 1.63
99%	Female	0.89 (0.71 – 2.01) 3.87	7.72 (5.04 – 18.85) 10.58	22.4 (11.53 – 76.15) 23.33	1.41 (0.88 – 28.73) 3.28

Table 4.5. Effective doses and resistance factors of brodifacoum, bromadiolone and difenacoum at various percentiles against male and female Welsh rats.

		Effective Dose [mg/kg] (lower – upper fiducial limits) **Resistance Factor compared with susceptible rats**		
Effective Dose	**Sex**	**Brodifacoum**	**Bromadiolone**	**Difenacoum**
40%	Male	0.19 (0.18 – 0.21) 0.91	0.16 (0.06 – 0.34) 0.35	0.37 (0.06 – 0.48) 0.59
50%	Male	0.2 (0.19 – 0.22) 0.91	0.21 (0.09 – 0.48) 0.45	0.41 (0.11 – 0.53) 0.63
60%	Male	0.21 (0.19 – 0.23) 0.96	0.28 (0.13 – 0.7) 0.58	0.46 (0.2 – 0.6) 0.7
99%	Male	0.27 (0.25 – 0.34) 1.0	3.06 (1.08 – 45.22) 5.46	1.13 (0.76 – 52.28) 1.43
40%	Female	0.25 (0.23 – 0.27) 1.14	2.5 (2.0 – 2.87) 4.17	0.76 (0.61 – 0.84) 0.99
50%	Female	0.25 (0.24 – 0.28) 1.14	2.64 (2.21 – 3.09) 4.33	0.78 (0.66 – 0.87) 1.0
60%	Female	0.26 (0.24 – 0.29) 1.18	2.78 (2.4 – 3.38) 4.48	0.81 (0.71 – 0.92) 0.99
99%	Female	0.34 (0.31 – 0.44) 1.48	4.34 (3.51 – 9.54) 5.95	1.02 (0.9 – 2.01) 1.07

4.4 Discussion

4.4.1 Resistance factors and relative anticoagulant efficacy

In every combination of resistant rat strain and active ingredient, the ED_{50} was higher for females than for males. The same is true for ED_{40}, ED_{60} and ED_{99} (with the lone exception being that Welsh males have a higher ED99 for difenacoum than Welsh females). This echoes the susceptible baseline data for SGARs and FGARs (Prescott *et al.*, 2007), and indicates that regardless of resistance status, female Norway rats have a greater tolerance to anticoagulants than males. This runs contrary to previous evidence that, in rats susceptible to warfarin, females are significantly more sensitive to anticoagulant poisoning than males (Back, Steger and Glassman, 1978). In addition, resistance factors for females at each effective dose were noticeably greater than those of males (the lone exception again being the ED_{99} for difenacoum against Welsh rats). Because Probit dose-responses of ED_{99} estimates are inherently inaccurate, with the widest fiducial limits – especially in studies where numbers of test subjects are kept to a minimum – any discrepancies highlighted by ED_{99}s should be ignored. The effective dose percentiles for female resistant rats are expected to be greater than those of males due to the females' greater anticoagulant tolerance, but the increased resistance factors indicates that resistance mutations in female rats have a greater relative effect on individuals' PT, suggesting that the character of anticoagulant resistance imparted by VKORC1 mutations is different between male and female rats. In addition to the most obvious result of this – that female resistant rats are likely to be harder to control in the field than males – higher resistance for female rats will also have the effect of enabling populations to recover in numbers at a more rapid rate than if male and female had similar ED_{50}s. This is because only a single male rat is required to mate with multiple female rats, thereby producing multiple litters.

The Probit dose-response data for the CSL Berkshire strain of resistant rat show clear groupings of the various SGARs in both sexes, with bromadiolone and difenacoum having far greater effective doses at all percentiles than brodifacoum, and flocoumafen and difethialone. This pattern is repeated in the Probit dose-response data for the Reading Berkshire rats, where the effective doses for brodifacoum and flocoumafen against females are lower at all percentiles than for bromadiolone and difenacoum, and effective doses of brodifacoum against males are lower than those of difenacoum. In the case of male Welsh rats, brodifacoum, bromadiolone and difenacoum appear to form a single grouping with uniformly low values for all derived effective doses. The Probit dose-response data for female Welsh rats show bromadiolone forming a separate group of higher effective doses than difenacoum and brodifacoum; brodifacoum effective doses are slightly lower than those of difenacoum.

While the differences between the ED_{50} for the SGARs against susceptible rats are negligible, these data firmly establish that bromadiolone is less toxic to resistant Norway rats than difenacoum. In turn, difenacoum is less toxic to resistant Norway rats than the resistance-breaking anticoagulants brodifacoum, difethialone and flocoumafen. The results herein show conclusively that less brodifacoum is required than bromadiolone and difenacoum to have an anticoagulant effect on Norway rats positive for the L120Q mutation. When comparing the Probit dose-response data for the bromadiolone and difenacoum BCR tests with CSL Berkshire rats, the ED_{50}s for female rats are high (7.47 and 9.98 mg/kg, respectively), as are resistance factors (12.05 and 12.8, respectively). The ED_{50}s are lower for male rats (4.67 and 3.28 mg/kg, respectively). The resistance factors for bromadiolone and difenacoum against male CSL Berkshire rats vary considerably (9.94 and 5.05, respectively), suggesting that difenacoum resistance in males may only be of the technical variety. Nonetheless, the severity of the resistance against both bromadiolone and difenacoum exhibited by female CSL Berkshire rats indicates that the L120Q mutation can confer

practical resistance to these SGARs, and they should not be used for the control of resistant rats in south-east England (RRAG, 2012). When comparing the Probit dose-response data for difethialone, flocoumafen and brodifacoum against CSL Berkshire rats, the differences between ED_{50}s for male rats are small (0.9, 0.74 and 0.6 mg/kg respectively) as are their resistance factors (2.03, 2.64 and 2.79 respectively). Meanwhile female rats have slightly higher ED_{50} for brodifacoum (1.47 mg/kg) than for difethialone and flocoumafen (1.13 and 1.03 mg/kg respectively); in comparison to these relatively similar values, the resistance factors of female CSL Berkshire rats for brodifacoum (6.68) are far greater than those for difethialone and flocoumafen (2.31 and 3.12, respectively) . The implication is that in the case of female rats with the L120Q mutation, brodifacoum is a less efficacious resistance breaker than both difethialone and flocoumafen. The raw data from the susceptible baseline study (Prescott *et al.*, 2007) elucidate this issue: according to those data, the ED_{50} for brodifacoum against susceptible rats is less than half the ED_{50} for difethialone, and is less than 75% that value for flocoumafen. The whole range of effective dose percentiles for brodifacoum against susceptible female rats (ED_{01} – ED_{99}) is within the range of 0.211 mg/kg and 0.233 mg/kg (Prescott *et al.*, unpublished data). This very narrow range of doses indicates a very high level of efficacy for brodifacoum against susceptible Norway rats, but also cast brodifacoum in an artificially negative light when tested against rats possessing anticoagulant resistance. This effect is replicated in the results for Welsh rats and Reading Berkshire rats, as all resistance factors are based upon the same susceptible baselines (Prescott *et al.*, 2007). Brodifacoum has been shown to consistently cause lethal effects at concentrations as low as 0.4 mg/kg in rats thought to be positive for the Y139S mutation and as low as 0.6 mg/kg in susceptible laboratory rats (Redfern, Gill and Hadler, 1976). It has also been shown to completely control the majority of populations within the Y139S resistance focus within 18 days at a concentration of 0.0005% (Rennison and Dubock, 1978).

Finally, it has been shown to be efficacious in the south-east of England, in areas where bromadiolone and difenacoum had failed to control Norway rat populations (Greaves, Shepherd and Quy, 1982; Meyer, 2009). The inflated resistance factors for brodifacoum cast doubt on the notion that it could be used to effectively control a wild population of Norway rats with a level of resistance comparable to the CSL Berkshire rats tested herein, suggesting practical resistance could be encountered in the field. It is more likely, given the low brodifacoum doses required to elicit anticoagulant responses in CSL Berkshire rats, that wild rats with the L120Q mutation would only possess technical resistance to brodifacoum, along with difethialone and flocoumafen.

The ED_{50} for brodifacoum against male Reading Berkshire rats is less than half that of difenacoum (0.16 and 0.39 mg/kg, respectively), but the associated resistance factors are similar due to the effect of the susceptible brodifacoum data described above (0.73 and 0.6, respectively). Resistance factors below 1 suggest that male Reading Berkshire rats are more susceptible to SGARs than are male wild-type rats. Against female Reading Berkshire rats, brodifacoum and flocoumafen elicit very similar ED_{50}s (0.52 and 0.51 mg/kg, respectively), much lower than those of bromadiolone and difenacoum (1.77 and 2.28 mg/kg, respectively). Again, due to the effect of the susceptible brodifacoum data, the associated brodifacoum resistance factor (2.36) is noticeably higher than that of flocoumafen (1.5), but still lower than those of bromadiolone and difenacoum (2.86 and 2.89, respectively). ED_{50}s and resistance factors for all SGARs against Reading Berkshire rats are low; these rats possess only technical resistance.

When comparing the Probit dose-response data for brodifacoum, bromadiolone and difenacoum against Welsh rats, the differences between ED_{50}s for male rats are small and notably low (0.2, 0.21 and 0.41 mg/kg respectively), resulting in resistance factors below 1 (0.91, 0.45 and 0.63 respectively); as with Reading Berkshire rats, male Welsh rats appear

more susceptible to SGARs than do wild-type rats. For female Welsh rats, the ED_{50}s show greater variety (0.25, 2.64 and 0.78 mg/kg, respectively), whereas the resistance factor for bromadiolone is much higher than those for brodifacoum and difenacoum (4.33, 1.14 and 1.0 respectively). These results show clearly that in Welsh rats, resistance to SGARs at the ED50 level is either entirely, or almost entirely, undetectable. The exception is resistance to bromadiolone in females; with ED50 of 2.64 mg/kg and a resistance factor of 4.33, this is likely to be technical resistance with little practical effect in the field.

Resistance factors were higher at the ED_{99} than ED_{50} for all strains and all SGARs (the exception being difethialone against CSL Berkshire rats), suggesting that in a resistant population, a small proportion of rats will be highly resistant, and that use of ineffective rodenticides will select for these rats in the same manner in which a Selected Line is created in the laboratory. Typically, the weaker the SGAR, the greater the difference between the resistance factors at the various effective doses. The Probit-response lines are steeper for resistant rats than for susceptible rats, with the steepest lines generated for the least effective SGARs; the less effective the anticoagulant, the more noticeable the effect of the resistance, and therefore the greater the likelihood that treatment failure would occur in a practical setting. The unreliability of ED_{99}s produced by Probit analysis cast doubt on the accuracy of the idea that resistance factors are actually higher for ED_{99}s than ED_{50}s; still, due to the low ED_{99}s and associated fiducial limits across resistance breakers, it is recommended that the practical impact of resistance-breaking SGARs be further explored in the field.

4.4.2 Response between strains

4.4.2.1 Y139S

The Probit response lines of Welsh female and wild-type female rats dosed with difenacoum were co-incident, as were the Probit response lines of Welsh male and wild-type male rats dosed with brodifacoum (wild-type data from Prescott *et al.*, 2007). Because all other Probit response lines of Welsh rats compared with wild-type rats dosed with the same anticoagulants were statistically separate, it appears that in the majority of cases, rats possessing the Y139S mutation in the wild would be significantly harder to control via the use of anticoagulants than would susceptible animals. Scrutiny of the resistance factors listed, however, shows that in the case of males, Welsh rats appear to be more susceptible to SGARs than are susceptible rats: at the ED_{40}, ED_{50} and ED_{60} level resistance factors were below 1 (at the ED_{99} level all resistance factors were above or equal to 1). Moreover, resistance factors for female Welsh rats against bromadiolone were below 6 at all effective doses, and below 1.5 against difenacoum and brodifacoum, confirming the findings of Redfern and Gill (1980), that bromadiolone is fully effective against Welsh-resistant rats. Indeed, with susceptible rats capable of consuming enough 0.005% bromadiolone bait in a single day to achieve complete mortality (Marsh, 1977; Redfern and Gill, 1980), a resistance factor below 6 should not be an insurmountable obstacle to a well carried-out field trial. Successful trials in the Y139S resistance focus have already been carried out using bromadiolone at this strength (Richards, 1981). The comparatively low resistance factors for difenacoum and brodifacoum reflect previous experiments showing little to no impact of the Y139S mutation on the efficacy of brodifacoum (Redfern, Gill and Hadler, 1976) and difenacoum (Hadler, Redfern and Rowe, 1975) in the laboratory, or in the field (Rennison and Dubock, 1978; Rennison and Hadler, 1975, respectively).

4.4.2.2 L120Q

All Reading Berkshire rats exhibited separate Probit response lines from those of susceptible rats of the same sex, dosed with the same SGAR. For all male Reading Berkshire rats, resistance factors at the ED_{40}, ED_{50} and ED_{60} level were below 1, but at ED_{99} were above 1. These differences in blood clotting response when dosed with SGARs are enough to explain the statistical separation between the datasets. Resistance factors were greater than 1 for all female Reading Berkshire rats, and were greater than equivalent resistance factors for female Welsh rats at all effective doses of all SGARs; male Reading rat resistance factors were lower at the ED_{50} level for brodifacoum and difenacoum than those of male Welsh rats. It has long been established that the resistance present in central-southern England (L120Q) has a greater negative impact on anticoagulant rodenticide effectiveness than does the resistance centred on the Anglo-Welsh border (Y139S): this is confirmation that completely eradicating a population of rats with the L120Q mutation will be more difficult than doing the same to a population of rats with the Y139S mutation (due to female survival). That is not to say that rats with the weaker form of the L120Q resistance ("Reading Berkshire") cannot be controlled: as stated above, this strain of resistant rats possesses only technical resistance to SGARs. This lends credence to studies that, upon re-evaluating field trial data from the 1980s and 1990s, suggested that Hampshire-Berkshire resistance may not have always been the primary cause of reported treatment failure during that period bait (Quy *et al.*, 1992; Quy, Shepherd and Inglis, 1992). During initial evaluations of brodifacoum (Redfern, Gill and Hadler, 1976) baits comprised of as little as 0.0005% brodifacoum achieved 100% mortality after two days' feeding by non-resistant rats, with lethal doses as low as 0.6 $mg.kg^{-1}$. Given that SGAR baits in the UK will be required to have a strength of less than 0.003% in order to be available for registration, as recently suggested by the European Chemicals Agency (ECHA), no problems – caused by anticoagulant resistance – eradicating rats with the

Reading Berkshire form of the L120Q mutation are foreseen. Despite anecdotal evidence that bromadiolone and difenacoum are still efficacious in areas of known L120Q resistance and the low resistance factors reported for Reading Berkshire rats, the results of previous laboratory tests (Gill *et al.*, 1993; Quy *et al.*, 1995) and the potential for the creation of resistant lines such as the CSL Berkshire rats, suggest that usage of these baits in the L120Q focus will be harmful to non-target wildlife and select for resistance within Norway rat populations.

In comparison to the Reading Berkshire form of the L120Q mutation, the CSL Berkshire form is highly resistant to all comparable SGARs (Tables 4.1 and 4.2). Male and female CSL Berkshire rats had higher ED_{40}s, ED_{50}s, ED_{60}s and ED_{99}s than male and female Reading Berkshire rats for brodifacoum, bromadiolone and difenacoum, and therefore higher resistance factors at all levels. Several years after the two strains were initially separated, the CSL Berkshire strain of rats still possesses a much greater tolerance to anticoagulants than Reading Berkshire rats. Therefore, it is imperative that the use of difenacoum and bromadiolone in the L120Q focus of central-southern England is avoided. It is currently impossible to determine if a population has undergone artificial selection due to inappropriate use, or misuse, of ineffective SGARs without conducting extensive BCR tests: in order to avoid using ineffective SGARs against more highly resistant rats in the field, only use of resistance breaking SGARs (brodifacoum; difethialone; flocoumafen) is advocated. Reading Berkshire rats appear to be more susceptible to bromadiolone and difenacoum than rats of the Hampshire line (Prescott et al., 2007). This adds further weight to the argument that bromadiolone and difenacoum cannot be safely used to control rats in the large L120Q focus of central-southern England; advice for rodenticide users in Berkshire can be safely applied to Hampshire, and indeed the whole of the L120Q focus as well.

It is curious to note that with CSL Berkshire rats, bromadiolone is more efficacious than difenacoum against females (i.e. bromadiolone elicits "responder" statuses at lower doses than does difenacoum), and difenacoum is more efficacious than bromadiolone against males; in the BCR tests with Welsh rats, the reverse is true. Regardless, this does not change the fact that the use of both bromadiolone and difenacoum against Norway rats should be avoided in areas of L120Q resistance, and should not be problematic in areas of Y139S resistance.

4.4.3 Liabilities of the methodology

Despite Linear Regression Models showing that Norway rat resting PT is not affected by bodyweight, the true effect of bodyweight on the response of rats dosed with anticoagulant rodenticides is not known. The same Linear Regression Models showed conclusively that sex can only explain 0.13% of resting PT variation, but as shown above, male and female rats of the same resistant strain show parallel or separate responses to the various SGARs, but never co-incident responses. It is therefore possible that, when a rat is dosed with or consumes anticoagulants, its weight may have a significant effect upon the resulting PT, and therefore survival. Exploring this issue would require utilising many more rats in a BCR study using a repeated dose of a single anticoagulant in order to elucidate the effect of a factor which will be impossible to control for in the field.

The original intention for this work was to generate Probit dose-response lines for all five SGARs against the three resistant rat strains housed at the University of Reading. Doing so relies upon a sufficient supply of resistant rats to test. During the course of this experiment, the breeding behaviour of the three strains tested has been variable, resulting in periods of rat shortage, when the number of BCR tests that could be carried out was limited,

therefore prolonging the generation of Probit dose-response lines. Causes of reduced supply of rats include refusal of breeding pairs to mate; repeated infanticide; and sex ratios heavily biased towards females (as noted by Bishop, Hartley and Partridge, 1977). In order to complete all Probit dose-response lines, it is recommended that further work be carried out to complete the bromadiolone, difethialone and flocoumafen dosing work with the Welsh and Reading Berkshire rats housed at the University of Reading.

It is important to reiterate that although the Probit analyses herein generated a range of effective doses (and associated resistance factors) from the 1% to 99% levels, the most accurate and reliable results are those associated with ED_{50}s. It is tempting to read too far into the implications of the extreme ends of the fiducial limits of ED_{99}s and ED_{01}s regarding the extreme ends of the resistance conferred by VKORC1 mutations and the likely impact in the field. This should be avoided however, with the focus of this and any other Probit-related discussion remaining on and around the ED_{50} values. This focus on ED_{50} values may limit the value of these results when attempting to predict the ability of SGARs to gain full control in field applications, in areas where resistance has been repeatedly selected for over many years. The results herein may be applied successfully to the majority of the population, but some individuals may possess resistance factors far exceeding those listed at ED_{50} level.

Furthermore, there are several aspects of the methodology which have inherent inaccuracies. Rats up to 200 g could be dosed used 1 ml syringes, which are accurate to within 0.01 ml, but rats above this weight were dosed with 2.5 ml syringes, which are only accurate to within 0.1 ml. In addition, the balances used to weigh out dosing solutions were only accurate to within 0.01 g. Finally it is possible that the original stock solutions of the SGARs supplied variously by RSSL and Liphatech (see above and Section 2.2.3) could not be made up to precisely 10 or 1 mg/ml. Therefore there was potential for inaccuracy at all

levels of dosing preparation, further emphasising the need to restrain analysis to the more reliable ED_{50}s and associated resistance factors.

As with all laboratory experiments, these results may not predict with 100% accuracy the situations likely to be encountered in attempts to control Norway rats in the wild with the anticoagulants tested. Many anticoagulant applications in this experiment elicited "responder" status from rats that, based upon known lethal doses of these active ingredients (see, for example, Redfern, Gill and Hadler, 1976; Gill, Kerins and MacNicoll, 1992; Quy *et al.*, 1995), may not have died if left in observation. It is possible to compare derived resistance factors at the ED_{50} level with previously published resistance factors at the LD_{50} level, where the data for both sets of experiments exist. LD_{50} resistance factors for rats with the Y139S resistance mutation are higher than the derived ED_{50} resistance factors herein for bromadiolone (2.7 – 6.9 and 0.45 – 4.33, respectively) and difenacoum (1.1 – 1.3 and 0.63 – 1.0, respectively), and comparable for brodifacoum (1.0 – 1.1 and 0.91 – 1.14, respectively) (Greaves and Cullen-Ayres, 1988). It is noticeable that for these results, the difference between LD_{50} and ED_{50} resistance factors falls in size as efficacy of the SGAR increases. If this pattern continued between strains and was influenced by the level of resistance conferred by each mutation, it would further emphasise the need to avoid ineffective anticoagulants. Unfortunately, this is not the case; previous results for the Hampshire strain of L120Q resistance (Greaves and Cullen-Ayres, 1988; Prescott *et al.*, 2007) show that LD_{50} resistance factors are substantially lower than ED50 resistance factors for bromadiolone (1.5 – 2.9 and 3.0 – 6.8, respectively), but comparable for difenacoum (3.9 – 4.1 and 2.2 – 5.0, respectively). It is therefore uncertain how reliably ED_{50} resistance factors can predict the efficacy of various SGAR doses between strains. For these reasons, the results herein (and potential future results from studies involving rats possessing the L128Q, Y139C or Y139F mutations) require contextual confirmation in the field.

Chapter 5: Assessing the efficacy of three SGARs in the L120Q focus of south-east England

5.1. Introduction

Resistance to anticoagulant rodenticides in Norway rats has been a growing problem in Europe for some time, especially in the UK, where it was first discovered (Boyle, 1960). More resistance-conferring mutations are found in the UK than anywhere else in the world (RRAG, 2012) and the majority of rats studied in the UK are positive for mutations known to confer practical resistance to commonly used SGARs (Section 3.3.1). The L120Q resistance focus of central-southern England, in particular, has yet to be entirely delimited and the mutation's effect on control in the field is uncertain. Resistance had been discovered in central-southern England by the 1970s (Greaves and Rennison, 1973) and the resistant rat populations there were found to be able to tolerate SGARs known to be efficacious against established resistance foci in Powys, Wales (Quy, Shepherd and Inglis, 1992). Numerous studies in the 1980s and 1990s showed mixed results when testing bromadiolone and difenacoum against these strains of resistant rats in laboratory trials and in the field (Redfern and Gill, 1978; Richards, 1981; Greaves, Shepherd and Gill, 1982; Quy, Shepherd and Inglis, 1992; Cowan *et al.*, 1995), with bromadiolone and difenacoum both appearing to be the more effective active ingredients. These inconsistent results suggested that there may have been multiple resistance phenotypes in central-southern England. This was confirmed in the early 1990s when over 800 kg of 50 ppm bromadiolone bait was consumed by rats at a farm in north Berkshire, without achievement of control (Quy *et al*, 1995). The two strains of resistance are known as Hampshire and Berkshire resistance after the locations where they were first found. However, molecular techniques (Rost *et al*, 2004; Pelz *et al*, 2005) have

confirmed that both strains are positive for the amino acid polymorphism L120Q, one of many anticoagulant resistance-conferring mutations of the Norway rat VKOR gene found across the UK. A comparison of data on Reading Berkshire rats from Section 4.4.2.2 and the limited tests carried out by Prescott *et al.* (2007) with Hampshire rats suggests that Hampshire-resistant rats do in fact possess a greater tolerance to bromadiolone and difenacoum than the Reading Berkshire resistant strain. It is now thought that historically, control of L120Q-resistant rats in Berkshire may have been achieved using bromadiolone and difenacoum when the rats in question did not avoid the bait due to neophobia or presence of stored alternative grain, and reinvasion did not occur (Quy *et al.*, 1992; Quy, Shepherd and Inglis, 1992). Despite the nominal difference between the two groups of resistant rats, both have now shown demonstrable resistance to bromadiolone and difenacoum, whilst being susceptible to the resistance-breaking SGAR brodifacoum. A series of field trials in Hampshire showed difenacoum to be of little use against the resistant rats there, with bromadiolone performing little better; control was achieved not only at brodifacoum trial sites, but also at sites at which difenacoum and bromadiolone had recently failed (Greaves, Shepherd and Quy, 1982). Rats from a farm in north Berkshire survived multiple bromadiolone treatments, and captured survivors then survived the 5.0 mg/kg body weight difenacoum resistance test posited by Gill *et al.* (1993) before 101 of 116 succumbed to a brodifacoum feeding test (Gill and MacNicoll, 1991; Quy *et al.*, 1995). More recently an infestation in Hampshire consumed 213 kg of bromadiolone and difenacoum bait over the course of two years without control being achieved. In this instance a carefully monitored use of brodifacoum bait was applied under an emergency extension of Health and Safety Executive (HSE) approval, requiring just 3.4 kg of bait before the infestation was eradicated in 18 days (Meyer, 2009). Other studies have shown brodifacoum to be effective in controlling rats in both the Y139S (Rennison and Dubock, 1978) and L120Q foci (Greaves,

Shepherd and Quy, 1982). Infestations of Norway rats positive for the Y139C resistance mutation in the Westphalia region of Germany have been successfully controlled using as little as 1.45 kg 0.005% brodifacoum bait (Buckle, Klemann and Prescott, 2012), whereas treatments of other Y139C-positive infestations in the region using up to 28.2 kg 0.005% difenacoum bait (Buckle *et al.*, 2013) and 43.4 kg 0.005% bromadiolone bait (Endepols *et al.*, 2012) have failed to achieve control. Despite evidence that brodifacoum is one of the most effective anticoagulants currently available, technical resistance has been found in the UK (Greaves, Shepherd and Quy, 1982; Gill, Kerins and MacNicoll, 1992). It is important that the resistance breaking properties of all the anticoagulants are fully explored; recent legislature has banned the use of almost all non-anticoagulant rodenticides in the EU, leaving stakeholders with almost no practical recourse for the control of large rat infestations should the anticoagulants no longer be efficacious. Until recently, use of anticoagulants for control of outdoor rat populations in the UK was limited solely to bromadiolone and difenacoum. This resulted in multiple areas where the presence of practical resistance meant that, in the absence of acute rodenticides (see Section 1.3.3), no legal control methods could be recommended (RRAG, 2012). The recent introduction of the anticoagulant rodenticide stewardship scheme (see Section 1.4.4) in the UK, supported by CRRU, has made it possible for competent users to utilise resistance-breaking anticoagulant rodenticides in and around buildings (Buckle *et al.*, 2017). Given the risk to non-target animals that the use of ineffective rodenticides pose (Daniells, Prescott and Buckle, 2011), it is now necessary to determine which anticoagulant rodenticides are safe to use in the L120Q focus of central-southern England. This will result in effective and targeted applications of anticoagulant rodenticides which enable users to control populations of resistant Norway rats, while reducing unnecessary risk to non-target species.

5.2. Methods

5.2.1 Trial sites

A total of 6 field trials were undertaken from 2009 – 2016. These trials were conducted on behalf of Syngenta AG, a commercial client and were compliant with guidelines set out by the European and Mediterranean Plant Protection Organisation (EPPO, 1999a) and where necessary with guidelines set out by the CRRU stewardship scheme (Buckle *et al.*, 2017). In the latter case this required two of the workers, employees of the University of Reading, to undertake the necessary exams showing professional competence. Workers included the primary researcher and three members of the Vertebrate Pests Unit of the University of Reading. Two trials were carried out for each of three SGARs: bromadiolone, difenacoum and brodifacoum. All rodenticide baits were supplied by Syngenta AG. All 6 sites were located within a 520 km^2 area, which was entirely within what is now known to be the large L120Q focus of southern England (see Section 3.1). All sites were surveyed and maps indicating suitable bait locations were drawn prior to each treatment. Before the pre-treatment census phases, tracking patches were laid out around the site (see Table 5.1); these remained in place throughout the trials, except where stated. Three types of bait point were used during the trials: bait trays under natural cover, bait boxes and tyre baiters. These are described in detail in Section 2.3.4. Number of bait point types for all trials are described in detail in Tables 5.2 and 5.3. Number of bait points used, and apparent level of control based on census bait consumption have already been mentioned for Trials A – D elsewhere (Daniells, 2011). Some of the field trials considered here are of a commercially sensitive nature and results are therefore reported with respect to the volume of active ingredient consumed by rats in the environment, rather than volume of bait eaten.

5.2.1.1 Trial A

Trial A was carried out between 13th February and 21st April 2009 at a farm in western Hampshire. A proprietary 0.005% bromadiolone pellet bait (Contrac™) was used in this field trial. The site was a poultry farm consisting of 13 large sheds for the housing of ~15,000 chickens, and several augers for the storage and movement of feed. The sheds were arrayed in an L shape, with a line of four sheds extending to the south-west, and the remaining nine in a line extending to the south-east. During daylight hours, chickens had access to outdoor areas associated with each shed. Rats had easy access to spilled chicken feed in these areas and under augers, and had found harbourage underneath all of the houses and throughout a hedgerow bordering the south-west sheds. To the south, enclosed on two sides by the poultry sheds, was an arable field. The site was bordered on all sides by farmland used for pasture or crop growth. The site had an area of 2.3 ha. During the pre and post-treatment censuses, 150 g of whole wheat was placed in each bait point, and replenished when necessary. When complete takes of the 150 g were recorded (see Section 2.3.4), the amount of whole wheat placed at those bait points was increased to 300 g. When complete takes of 300 g were recorded, the amount of whole wheat placed at those bait points was increased to 450 g. Bait points and tracking patches were laid out ten days before the start of the pre-treatment census, to allow rodents to become accustomed to them. During the treatment phase, bait consumption was recorded on Monday and Thursday every week. Bromadiolone bait application is described in Daniells (2011). After five weeks both tracking patch activity and bait consumption had plateaued, so the treatment phase of the field trial ceased in favour of the post-treatment lag phase and census.

5.2.1.2 Trial B

Trial B was carried out between 13th February and 8th May 2009 at a farm in western Hampshire. A proprietary 0.005% difenacoum pellet bait (Ratak™) was used in this field trial. The site was a dairy farm consisting of four large connected sheds housing ~ 4000 dairy cattle, mounted on a concrete apron throughout. Adjacent to the row of sheds on the north-western side was a smaller calving shed, and to the north-east were silage clamps and a compactor, and beyond those were two slurry tanks set into the ground. Rats had easy access to an alternative food source in the form of cattle feed which was available at all times on the floor of the sheds. Rats were able to find harbourage underneath the concrete apron upon which the sheds were mounted, as well as in a swathe of waste ground adjacent to the silage clamps, and in burrows bordering the calving shed. The site was bordered on all sides by improved grassland for the grazing of cattle. The only buildings in the vicinity were two farm sites 100 m to the south and 100 m to the east over open grassland. A small managed woodland (2 ha) was located 150 m to the north. Bait points and tracking patches were laid out ten days before the start of the pre-treatment census, to allow rodents to become accustomed to them. The site had an area of 1.4 ha. During the pre and post-treatment censuses, 150 g of whole wheat was placed in each bait point, and replenished when necessary. When complete takes of 150 g (see Section 2.3.4) or near-complete takes were recorded, the amount of whole wheat placed at those bait points was increased to 300 g. When complete takes of 300 g were recorded, the amount of whole wheat placed at those bait points was increased to 450 g. When complete takes of 450 g were recorded, the amount of whole wheat placed at those bait was points was increased to 600 g. For trial B, the duration pre-treatment census was increased to six days due to a lack of consumption by rats on site; the extra two days allowed for a maximum daily bait consumption that more accurately reflected the perceived size of the infestation. Because of this, the post-treatment census was

increased to six days as well. Difenacoum bait application is described in Daniells (2011). Despite running concurrently with trial A, the placement of rodenticide bait in trial B lasted two weeks longer, and the recording of tracking patches during the treatment phase lasted a week longer. This was because these measures of Norway rat activity had not plateaued or ceased entirely at the five-week point (see Section 2.3.8).

5.2.1.3 Trial C

Trial C was carried out between 26th October 2009 and 24th January 2010 at a farm in West Berkshire. As in Trial A, the proprietary 0.005% bromadiolone pellet bait (Contrac™) was used. The site was a mixed stock farm with multiple large barns for housing animals and storing machinery and feed. There were ~500 cattle, ~300 sheep and ~100 pigs and piglets. Low numbers of chickens and geese were left to roam freely throughout the site. Two barns contained only machinery and tools, and offered little in the way of food to rats. Another housed light aircraft. One barn for the seasonal housing of cattle or sheep was built on hard standing and rarely contained animal feed or cover for rats. A smaller barn housed sheep when they were not grazing in fields to the north or in a penning area on the east of the site. The floor of this barn was covered in straw, affording cover to rats. Cattle were usually housed in a shed to the west, separated from the other animal-housing buildings by barns containing machinery. Cattle feed was readily available, but there was no cover or housing for rats. Two smaller barns were used solely for storage of grain and feed in the open, and rats could easily be observed in and around these sources of food. The main part of the infestation was centred in and around a pig barn and an adjacent drainage system. In the pig barn, feed and harbourage were readily available at ground level at all times. Some waste ground interspersed around and between these buildings contained seemingly active burrows. All buildings and barns had hollow and easily accessed walls with evidence of rat activity, except for the cattle shed and the barn housing light aircraft. Finally, several grain augers

were located between the sheep barn and the seasonal cattle and sheep barn. Immediately surrounding the farm on all sides was grassland for the grazing of cattle and sheep, while to the north-east was a large arable crop field. Further out were more fields for the grazing of livestock and arable crops, and a single patch of woodland, 180 metres west of the farm site. The site had an area of 1.6 ha. Bait points and tracking patches were laid out ten days before the start of the pre-treatment census, to allow rodents to become accustomed to them. During the pre and post-treatment censuses, 150 g of whole wheat was placed in each bait point. Replenishment of bait points, and increases to the total volume of whole wheat used followed the same methods as in Site A. During the treatment phase, bait consumption was recorded on Monday and Thursday every week where possible, but due to external factors this was not always the case, and on several occasions Site C was visited and bait consumption and tracking patch activity recorded on other days. Bromadiolone bait application is described in Daniells (2011). After five weeks, weekly bromadiolone consumption was still falling, but observable rat activity on the site appeared to be increasing, and the decision was made to cease bait application and begin the lag phase and post-treatment census. After a three-day lag phase, the post-treatment census started, carried out in identical fashion to the pre-treatment census. However, due to extremely poor weather, during which roads around the site were impassable and tracking patches and bait points largely inaccessible due to snowfall, the census was abandoned. The census was restarted after 14 days and carried out successfully.

5.2.1.4 Trial D

Trial D was carried out between 26th October 2009 and 24th January 2010 at a farm in West Berkshire. As in trial B, the bait used was the proprietary 0.005% difenacoum pellet bait Ratak™. The site was a small pig farm with a series of pigsties surrounded several barns of varying sizes. There were ~80 piglets associated with individual sows, and low numbers of

poultry and goats were housed in a courtyard opposite the pigsties. Rarely, the gate to this courtyard was open and the stock therein left free to roam. One large barn (approximately 15m x 30m) housed machinery and offered no harbourage or food for rats. One small barn was used for grain storage; grain was openly available here in a large pile. One end of this barn contained disused wooden pallets and fencing material, providing harbourage for rats. A final barn in the north-east of the site, which was heavily dilapidated and unsafe undoubtedly provided cover, harbourage and water to the rats, but for the most part was inaccessible to researchers. The centre of the farm was dominated by two rows of concrete pigsties which opened onto a central courtyard. Immediately south of the pigsties was a covered hay store, with bales stacked several metres in the air. The main part of the infestation was centred in this area, with food readily available from the pigsties and adjacent grain storage barn, and safe harbourage amongst the hay bales and the nearby small sheds and buildings in various states of disuse and dilapidation. Bordering the farm to the south-east was a small field for grazing of the farm's < 10 cattle, beyond which was a separate site with very large storage sheds which could have housed rats. To the south and west was the river Lambourn and woodland, and to the north were arable crop fields. The site had an area of 0.38 ha. Bait points and tracking patches were laid out five days before the start of the pre-treatment census, to allow rodents to become accustomed to them. During the pre and post-treatment censuses, 150 g of whole wheat was placed in each bait point. Replenishment and increases to total whole wheat applied followed the same methodology as at Site A, up to a maximum of 600 g, depending on nearby visible rat activity. During the treatment phase, bait consumption was recorded on Monday and Thursday every week where possible, but due to external factors this was not always the case. Difenacoum bait application is described in Daniells (2011). During the first week of the treatment phase one bait point was removed because it could not be sheltered effectively during poor weather conditions, and after the

fourth week of the treatment phase another was removed for the same reason. In the fifth week bait consumption increased, so the decision was made to cease bait application and begin the lag phase and post-treatment census. As with trial C, the post-treatment census was abandoned due to severe weather conditions, but then restarted and successfully carried out fourteen days later.

5.2.1.5 Trial E

Trial E was carried out between 22nd April 2016 and 29th July 2016 at a farm near Reading in Berkshire. An experimental brodifacoum formulation containing less than 30 ppm of active ingredient was used in this field trial. The site was a large dairy farm containing ~550 cattle, with a large office building in the centre of the site, and a large shed for the intensive feeding and milking of cattle to the north-east, and a smaller shed for experimental dairy treatments to the south-west. Further farming infrastructure, including slurry tanks, grain silos, storage sheds and rearing barns, was spread around the northern half of the site. Due to the very large size of the farm, the area selected for the field trial was the surroundings of the experimental shed at the south-west of the site, including an adjacent field and woodland border and an area of rough scrub housing a disused caravan, old tyres and a disused slurry tank. Immediately to the west of the experimental cattle shed was a series of concrete hoardings containing various experimental silage formulations. Active rat burrows were present throughout the rough scrub, and signs of rat activity were observed in adjacent areas. This area of the site was separated from the north-eastern half of the site by the central office building and a large swathe of open ground, indicating that the population of rats around the experimental shed was separate to the population centred on the intensive rearing barn. Surrounding countryside to the west, south and east consisted of cornfields and grassland for the grazing of cattle. Further to the south was a largely derelict farm site offering harbourage but no food to rats. The countryside to the north was woodland wherein 2000 pheasants were

reared annually for shooting. A single field to the north contained 12 sheep and 7 llamas, and pheasants were present wherever there was woodland cover. The whole site had an area of 3.0 ha, but the area used for the field trial had an area of 0.5 ha. Bait points and tracking patches were laid out three days before the start of the pre-treatment census, to allow rodents to become accustomed to them. During the pre-treatment census, 200 g of whole wheat was placed in each bait point, and replenished when necessary. When complete takes (see Section 2.3.4) or near-complete takes were recorded, the amount of whole wheat placed at those bait points was increased to 400 g. During the post-treatment census, 100 g of whole wheat was placed in each bait point, and replenished when necessary. During the treatment phase, bait consumption was recorded every Monday, Wednesday and Friday. Bait points were replenished when there was evidence of rat consumption, or if degradation due to environmental conditions was visually identified. During the second week of the treatment phase, five bait points were added to the original 50, due to high bait takes. These remained in place throughout the remainder of the treatment phase. After four weeks squirrels were recorded (via camera traps) entering bait boxes and removing brodifacoum bait. Therefore on trial day 45 (site visit 13 of the treatment phase) bait was removed from 5 bait points and replaced with cut peanuts. The cut peanuts were ignored by the squirrels, which were confirmed to be entering a further 10 bait points in search of rodenticide formulation which again was removed and replaced with cut peanuts. When this did not prevent squirrels attempting further depredations of rodenticide bait, all bait was removed and the whole treatment was temporarily halted. After two weeks of trapping squirrels (using the same methodology as described for Norway rats, see Section 2.1.3.1) a total of 6 squirrels were removed from the site. Subsequently there was no more evidence of squirrel activity, and the treatment was re-started. Ten weeks after the start of the treatment phase, tracking patch activity had ceased entirely, and bait consumption had plateaued at a very low level, so the

decision was made to cease bait application and begin the post-treatment lag phase and census.

5.2.1.6 Trial F

Trial F was carried out between 10th October 2016 and 23rd December 2016 at a farm in northern Hampshire. An experimental brodifacoum formulation containing less than 30 ppm of active ingredient was used in this field trial. The site was a small mixed stock farm containing ~100 sheep and ~20 cattle. The farmhouse was at the centre of the site, with a large barn used for housing sheep in the winter to the north-east. Attached to this barn was a large covered area for storage of hay bales and animal feed, as well as a small pen housing cattle, including calves. South-east of the house was a garden with fenced and unfenced areas. Approximately 20 geese and chickens lived in the fenced area, with a further 13 chickens housed in a pen outside of the garden fence. Poultry were usually free to roam throughout the day. The rest of the trial site was made up of scrub and waste ground containing scrap metal, old wooden pallets and disused fencing. The site was bordered on its eastern side by a country road, and to the south-west by a series of crop fields extending for a linear distance of between ~250 m and ~600 m. A matrix of semi-natural woodland and improved grassland extended >1 km beyond the road. Active rat burrows were visible in large portions of the waste ground, and the concrete surface of the indoor barns were thickly covered with rat droppings. Old straw (from the previous winter) offered harbourage to rats, as did the stacked hay bales in the covered areas. The floor of the barn was scattered with wheat husks, and animal feed was openly available to rats in poultry feeders and in open storage sacks near the cattle pen. The site had an area of 0.7 ha. Bait points and tracking patches were laid out three days before the start of the pre-treatment census, to allow rodents to become accustomed to them. Due to external circumstances, the farm's sheep were brought in to the main barn area three weeks earlier than the tenants had previously stated,

necessitating the movement or removal of 26 bait points during the treatment phase. A further two bait points were moved during the trial phase when a small sheltering structure was removed from the farm site. During the pre-treatment census, 200 g of whole wheat was placed in each bait point, and replenished when necessary. When complete takes or near-complete takes were recorded (see Section 2.3.4), the amount of whole wheat placed at those bait points was increased by 100 g, up to a maximum of 400 g. During the post-treatment census, 100 g of whole wheat was placed in each bait point, and replenished when necessary. During the treatment phase, bait consumption was recorded every Monday, Wednesday and Friday. Bait points were replenished when bait take was recorded or if degradation due to environmental conditions was visually identified. Eight weeks after the start of the treatment phase, tracking patch activity and bait consumption had ceased entirely, so the decision was made to cease bait application and begin the post-treatment lag phase and census.

5.2.2 Genetic analysis

Rats from the sites of trials A, B, C and D were trapped both before and after the field trials. Live capture traps were set out and left open for 6 days, pre-baited every 2 days to allow rats to become accustomed to them. On the 7th day, the traps were baited and set overnight, and on the 8th day the traps were brought in, along with any trapped rats. Rats were dispatched using a Home Office schedule 1 method and and their tail tips removed and stored individually in 70 – 80% industrial methylated spirits at -18°C until analysis. Fresh droppings were taken from the sites of trials E and F, as were tails from fresh carcasses found on site during the course of field trials E and F. Droppings were selected and stored as described in Section 2.1.3.2; tails were stored in the same manner as those taken from trials A, B, C and D. DNA from all samples was extracted as described in Section 2.1.4. Samples from trials A,

B, C and D were analysed as described by Rost *et al*. (2004), and samples from trials E and F were analysed as described in Section 2.1.4.

5.2.3 Carcass searching

During the second and third phases of Trials E and F, searches for carcasses of Norway rats and other vertebrates were carried out once a week. These were carried out as described in Section 2.3.6. Any carcasses found were stored individually at -18°C.

Table 5.1. Numbers of bait points and tracking patches for each field trial

Trial	Census Bait points	Trial Bait points	Trial / Census Tracking patches
A	78	77	54
B	58	57	45
C	94	97	46
D	82	90	39
E	50	55	33
F	50	90	32

Table 5.2. Number of bait point types during census phases

Trial	Wooden Bait Box	Plastic Bait Box	Tyre Baiter	Bait Tray Under Cover
A	64	0	0	14
B	27	5	10	16
C	38	0	10	49
D	28	0	9	63
E	35	0	15	5
F	44	0	6	0

Table 5.3. Number of bait point types during trial phases

Trial	Wooden Bait Box	Plastic Bait Box	Tyre Baiter	Bait Tray Under Cover
A	63	0	0	14
B	26	5	10	16
C	35	0	10	49
D	20	0	9	63
E	35	0	10	5
F	55	0	12	23

5.2.4 Burrow baiting

During treatment phases that are achieving apparent success, it is sometimes the case that there is clear evidence of rat activity but very little bait take. When this situation arose during the field trials described, a thorough search of the trial site was made in order to determine the locations of Norway rat burrows. All burrows were baited with up to 20 g of treatment bait and entrances blocked with straw, disturbance of which would indicate subsequent rat activity. Burrows were subsequently monitored for signs of activity including straw disturbance. Bait was, where possible, removed from all burrows before the post-treatment lag phase. Because this could not be guaranteed, and the volume of baits applied to burrows consumed could not be satisfactorily measured, these values were not included in the total bait application figures in Table 5.12.

5.3. Results

5.3.1 Genetic analysis

Prior to the commencement of field trials A, B, C and D, tissue samples were obtained and successfully analysed from 13 rats from Site A, 18 rats from Site B, 19 rats from Site C and 27 rats from Site D; as described (Daniells, 2011) all samples were positive for the L120Q mutation, with a high incidence of homozygosity (see Table 5.4). Following the end of field trials, a further 94 rats were trapped across the sites of these field trials, with 89 producing useable results. At Site A, three rats were caught and DNA successfully analysed. At Site B, 34 rats were caught and their DNA successfully analysed; at Site C, 32 rats were caught and their DNA successfully analysed. At Site D, 20 rats were caught and DNA successfully analysed. At Site E, 13 fresh droppings were taken prior to the commencement of the field

trial and the DNA therein analysed. Only one of these samples was successfully analysed. Tails were taken from eight fresh rat carcasses found during the treatment phase, and tissue samples analysed; six were analysed successfully. At Site F, tails were taken from ten fresh rat carcasses found during the treatment phase, and tissue samples analysed; nine were analysed successfully (see Table 5.5).

Table 5.4. Resistance status of rats before and after trials at sites A-D

Trial Site	Genotype Ratios (L120Q Homozygous: L120Q Heterozygous: Wild-type)	
	Before Trial	After Trial
A	12:1:0	2:1:0
B	17:1:0	33:1:0
C	17:2:0	26:5:1
D	24:3:0	12:5:3

Table 5.5. Resistance status of rats before and during trials at sites E and F

Trial Site	Genotype Ratios (L120Q Homozygous: L120Q Heterozygous: Wild-type)
E	5:1:1
F	4:5:0

5.3.2 Bait consumption and tracking patch activity

5.3.2.1 Trial A

Bait take at Site A was high during the first week, with consumption of 501.3 mg of bromadiolone recorded over week one of the treatment phase (see Table 5.6). The bait initially appeared to be very efficacious, with recorded consumption of bromadiolone dropping to 165.25 mg during the second week of the treatment phase, and 75.05 mg during the third week of treatment. This precipitous drop was followed by a plateau in bromadiolone consumption, with a small increase during week four and a small decrease during week five. Similarly, recorded tracking scores at Site A peaked with a total score of 49 on the second recording day, falling sharply to 8 on the third recording day. Thereafter, tracking scores

showed minor peaks and troughs, but never rose above a total value of 21 for the remainder of the treatment phase (see Appendix 3). Daily totals of census bait taken and tracking scores were recorded during the pre- and post-treatment census periods, so that the maximum daily scores of each census period could be compared (see Figure 5.1). Using the values in Table 5.12 and the formula described in Section 2.3.9, the percentage efficacy of bromadiolone bait at Site A was calculated to be 66.4% according to census bait takes, and 37.2% according to tracking scores.

Table 5.6. Trial A: Weekly consumption of bromadiolone active ingredient by Norway rats

Trial Week (Days)	Bromadiolone (mg)
1 (16-22)	501.30
2 (23-29)	165.25
3 (30-36)	75.05
4 (37-43)	79.15
5 (44-50)	63.60

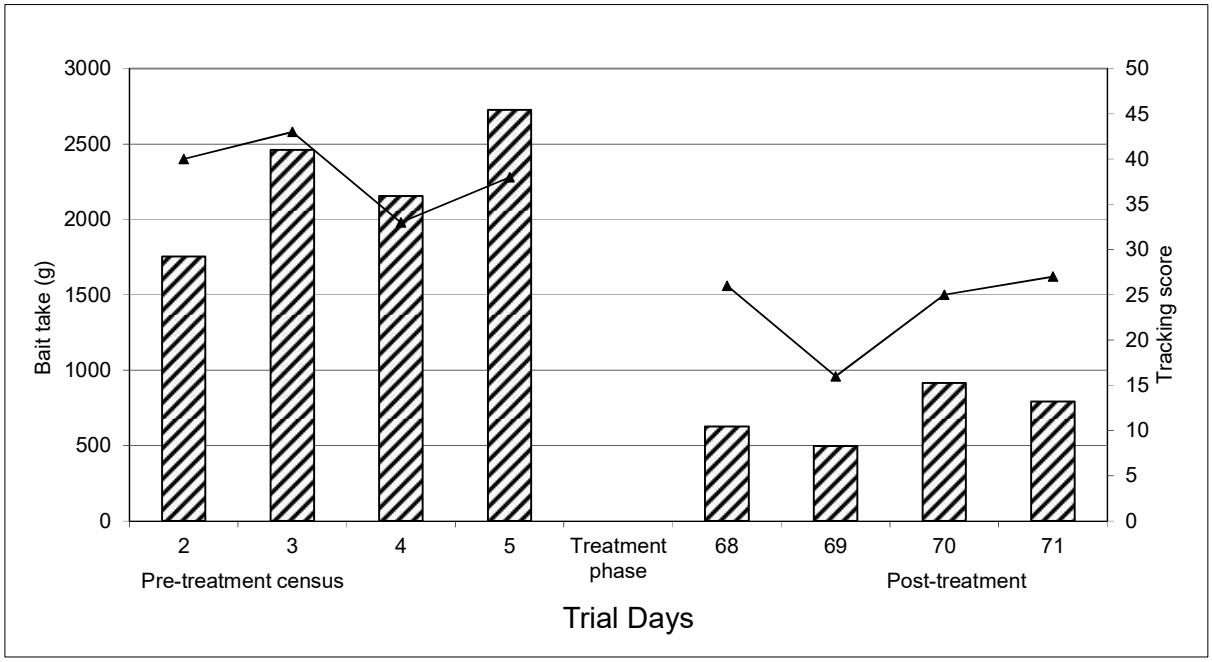

Figure 5.1. Trial A: Pre- and post-trial census bait takes (hatched vertical bars) and tracking scores (lines) for a field trial of 0.005% bromadiolone bait.

5.3.2.2 Trial B

Difenacoum bait take at Site B steadily increased until the third week of the treatment phase, when weekly consumption was more than double that of the first week (see Table 5.7). By the fifth week of the treatment phase, weekly consumption of difenacoum was still more than double that of the first week but had been slowly decreasing since the third week, so the decision was made to increase the trial length in order to allow the bait more time to achieve control of the rat population. However, weekly bait consumption increased to its peak thereafter, so this additional treatment period only lasted 2 weeks. Tracking data were constant during the first two weeks (see Appendix 3), afterwards increasing with deep fluctuation for the remainder of the treatment phase. Tracking data were not recorded on the 13th and 14th recorded days (the second week of additional treatment). Daily totals of census bait taken and tracking scores were recorded during the post treatment census period, so that the maximum daily score of each could be compared with the equivalent values from the pre-treatment census period (see Figure 5.2). Using the values in Table 5.12 and the formula described in Section 2.3.9, the percentage efficacy of difenacoum bait at Site B was calculated to be 15.7% according to census bait takes, and -1.8% according to tracking scores. This negative score arose because the maximum tracking score of the post-treatment census was greater than that of the pre-treatment census, indicating increased Norway rat activity over the course of the trial.

Table 5.7. Trial B: Weekly consumption of difenacoum active ingredient by Norway rats

Trial Week (days)	**Difenacoum (mg)**
1 (16-22)	102.75
2 (23-29)	152.9
3 (30-36)	242.6
4 (37-43)	222.35
5 (44-50)	211.25
6 (51-57)	242.9
7 (58-64)	253.6

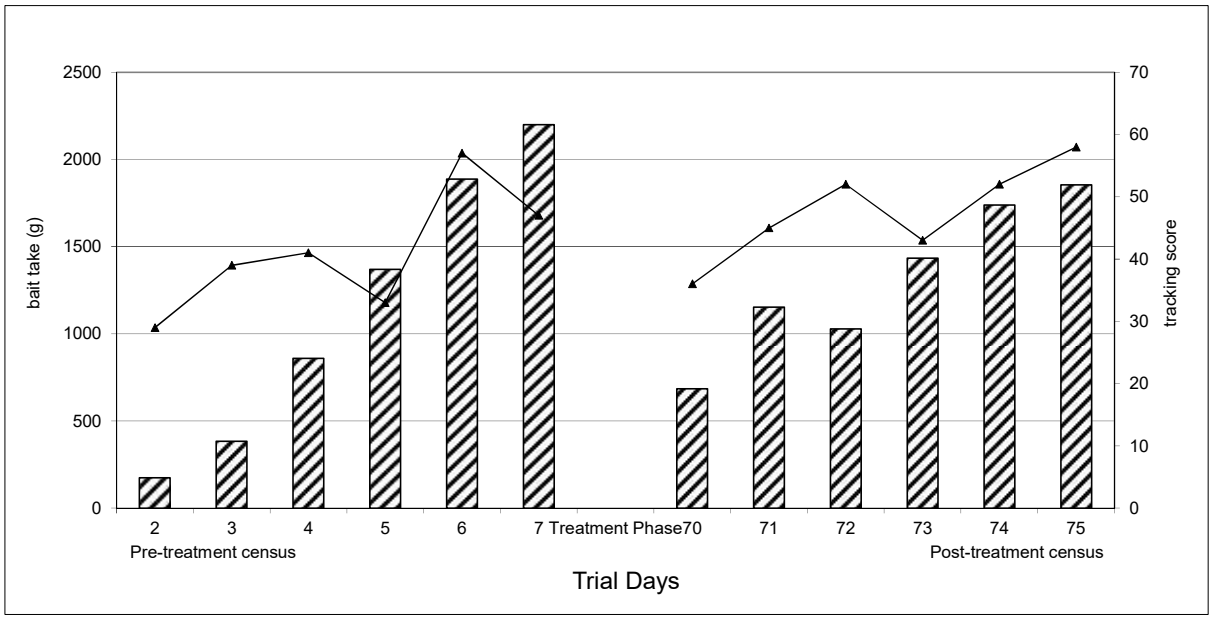

Figure 5.2. Trial B: Pre- and post-trial census bait takes (hatched vertical bars) and tracking scores (lines) for a field trial of 0.005% difenacoum bait.

5.3.2.3 Trial C

Bromadiolone intake by Norway rats started high at Site C and decreased irregularly during each week of the treatment phase, with the biggest reduction in bromadiolone consumption being recorded from the fourth to fifth weeks (see Table 5.8). Tracking patch activity fell consistently from the second week, but began to rise in the fifth week; for this reason the treatment phase was ended. Daily totals of census bait taken and tracking scores were recorded during the post treatment census period, so that the maximum daily score of each could be compared with the equivalent values from the pre-treatment census period (see Figure 5.3). Using the values in Table 5.12 and the formula described in Section 2.3.9, the percentage efficacy of bromadiolone bait at Site C was calculated to be -2.4% according to both census bait takes and tracking scores; both measures of treatment efficacy suggested that the population of Norway rats at Site C was higher after the treatment phase than it was before.

Table 5.8. Trial C: Weekly consumption of bromadiolone active ingredient by Norway rats

Trial Week (Days)	Bromadiolone (mg)
1 (19-25)	596.20
2 (26-33)	493.30
3 (34-39)	413.35
4 (40-46)	365.25
5 (47-53)	232.65

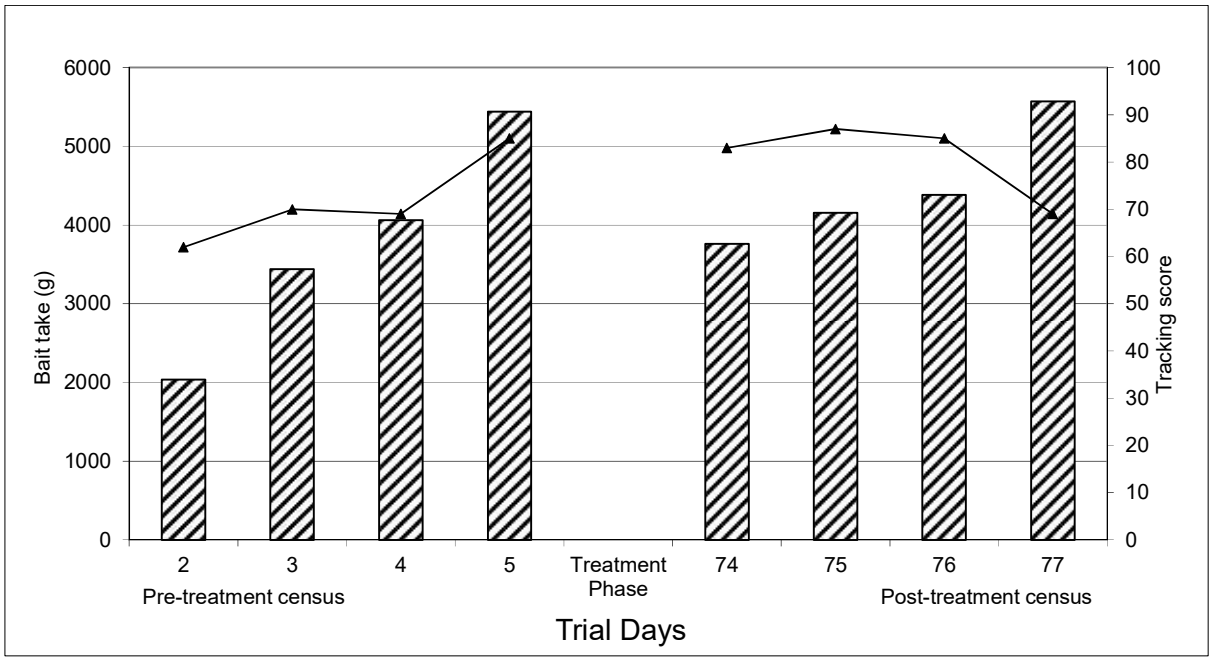

Figure 5.3. Trial C: See Figure 5.1 for description.

5.3.2.4 Trial D

Weekly difenacoum consumption was at its peak during the first week of the treatment phase at Site D (see Table 5.9). This fell sharply during the second week, before increasing during the third week and repeating this pattern during the fourth and fifth weeks. Tracking patch activity reached its peak on the first recorded day, and fell from there, with three minor peaks (see Appendix 3). Daily totals of census bait taken and tracking scores were recorded during the pre- and post-treatment census periods, so that the maximum daily scores of each census period could be compared (see Figure 5.4). Using the values in Table 5.12 and the formula described in Section 2.3.9, the percentage efficacy of difenacoum bait at Site D was

calculated to be 60.6% according to census bait takes, and 57.4% according to tracking scores.

Table 5.9. Trial D: Weekly consumption of difenacoum active ingredient by Norway rats

Trial Week (Days)	Difenacoum (mg)
1 (18-24)	895.60
2 (25-31)	539.65
3 (32-38)	583.70
4 (39-45)	468.45
5 (46-52)	495.20

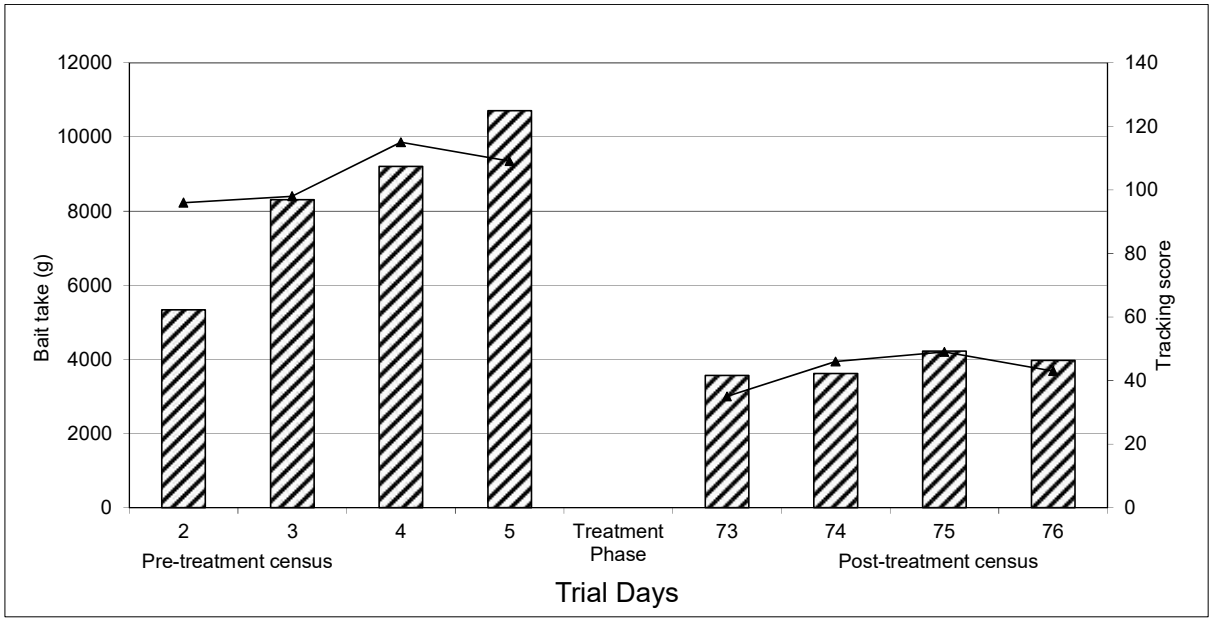

Figure 5.4. Trial D: See Figure 5.2 for description.

5.3.2.5 Trial E

Brodifacoum consumption was at its highest during the first two weeks of Trial E, before it fell rapidly during the third and fourth weeks. By the fifth week it was at 1.33% of its week one peak, and plateaued from there (see Table 5.10). After five weeks the trial was temporarily halted due to the presence of squirrels on site, which were observed entering bait boxes, as described above. Once the squirrels were removed, brodifacoum bait application was renewed, with very little brodifacoum consumption recorded during the remainder of the

treatment phase. Due to lack of recorded bait consumption, but the evident continued presence of rats on site, 11 rat burrows were baited, as described in Section 5.2.4. One of these 11 burrows showed activity thereafter; the entrances of the rest remained blocked with straw for the duration. Tracking patch activity was at its peak during the first recorded day, after which it fell slowly; during the second week it plummeted, plateauing in the third and fourth weeks (see Appendix 3). When the treatment was restarted after squirrels were removed, tracking patch activity fell slowly; a total of 0 was recorded for a week before the treatment phase ended. Daily totals of census bait taken and tracking scores were recorded during the post treatment census period, so that the maximum daily score of each could be compared with the equivalent values from the pre-treatment census period (see Figure 5.5). Using the values in Table 5.12 and the formula described in Section 2.3.9, the percentage efficacy of brodifacoum block bait at Site E was calculated to be 99.6% according to census bait takes, and 100% according to tracking scores.

Table 5.10. Trial E: Weekly consumption of brodifacoum active ingredient by Norway rats

Trial Week (Days)	**Brodifacoum (mg)**
1 (16-22)	46.87
2 (23-29)	41.65
3 (30-36)	9.52
4 (37-43)	2.05
5 (44-50)	0.62
Trial paused (51-64)	-
8 (65-71)	1.04
9 (72-78)	1.93
10 (79-85)	1.86
11 (86-87)	0.48

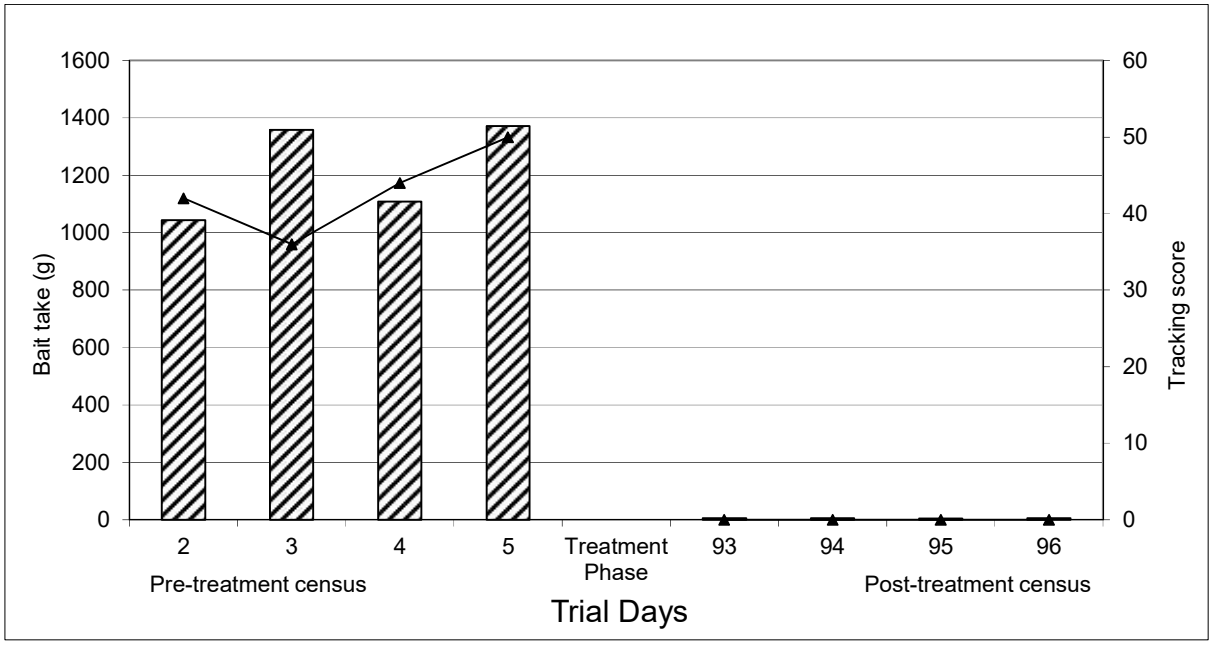

Figure 5.5. Trial E: Pre- and post-trial census bait takes (hatched vertical bars) and tracking scores (lines) for a field trial of < 0.003% brodifacoum bait.

5.3.2.6 Trial F

Brodifacoum consumption reached its peak in the first week of the treatment phase at Site F, and had fallen by two thirds by the second week (see Table 5.11). After the third week of treatment, brodifacoum intake by Norway rats fluctuated, before its nadir in the final week of the treatment phase. Due to concern at this fluctuation, and the forced movement or removal of 28 bait points between the 6th and 12th site visits, 23 rat burrows were baited from the 13th site visit, as described in Section 5.2.4. On the 15th site visit 10 fresh burrows were discovered and baited. Over the next three site visits a further eight fresh burrows were discovered and baited. No burrows showed any activity after baiting. Like Trial E, tracking patch activity peaked immediately (see Appendix 3). Thereafter it fell at a steady rate (with minor peaks) for the duration. No activity was recorded on the final day of the treatment phase. Daily totals of census bait taken and tracking scores were recorded during the post treatment census period, so that the maximum daily score of each could be compared with the equivalent values from the pre-treatment census period (see Figure 5.6). Using the values in

Table 5.12 and the formula described in Section 2.3.9, the percentage efficacy of brodifacoum bait at Site F was calculated to be 98.9% according to census bait takes, and 96.9% according to tracking scores.

Table 5.11. Trial F: Weekly consumption of brodifacoum active ingredient by Norway rats

Trial Week (Days)	Brodifacoum (mg)
1 (13-19)	119.26
2 (20-26)	39.70
3 (27-33)	14.15
4 (34-40)	12.97
5 (41-47)	24.70
6 (48-54)	11.04
7 (55-59)	1.15

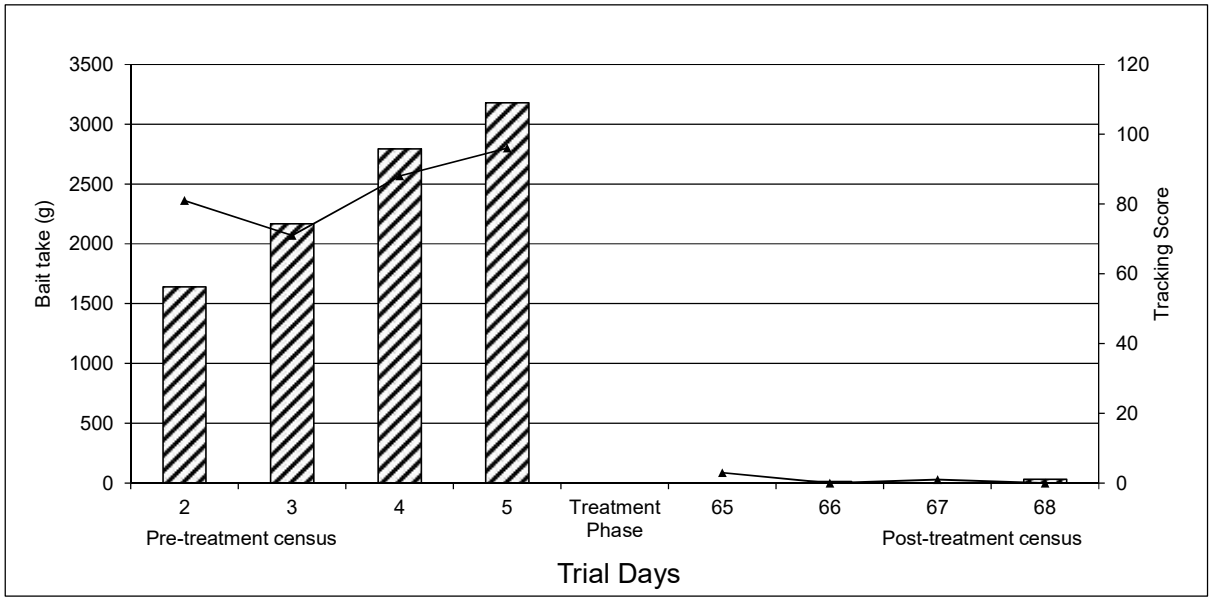

Figure 5.6. Trial F: See Figure 5.5 for description.

Table 5.12. Volume of active ingredient consumption attributed to rats at each trial site and associated population reduction. Population estimates based upon rats consuming 10% bodyweight per night, and the average rat weighing 200 g (Meehan, 1984; Buckle, Endepols and Prescott, 2007)

Trial	Trial Bait	Estimated Norway rat starting population	Max daily pre-treatment census bait consumption (g)	Max daily post-treatment census bait consumption (g)	Population reduction based on census bait consumption (%)	Max daily pre-treatment tracking patch activity (total score)	Max daily post-treatment tracking patch activity (total score)	Population reduction based on tracking patch activity (%)	Total active ingredient consumption (mg)
A	Bromadiolone	123	2460	916	66.4	43	27	37.2	884.35
B	Difenacoum	110	2200	1855	15.7	57	58	-1.8	1428.35
C	Bromadiolone	272	5440	5570	-2.4	85	87	-2.4	2100.75
D	Difenacoum	535	10708	4221	60.6	115	49	57.4	2982.6
E	Brodifacoum	68	1372	6	99.6	50	0	100	106.03
F	Brodifacoum	159	3180	32	98.9	96	3	96.9	222.96

5.4. Discussion

5.4.1 Efficacy of active ingredients

The bromadiolone and difenacoum baits utilised in the trials all failed to control the populations of Norway rats at sites A, B, C and D, according to EU regulations, which require recorded population reductions of > 90% during trials for product authorisation (Daniells, 2011). However, the results at Site C (Table 5.8) show a clear reduction in weekly bromadiolone consumption, which may not have been clear during the treatment phase (when results were recorded twice-weekly; bait consumption had plateaued for the final three site visits, and tracking patch activity had increased). Total bromadiolone consumption at Site C was 2100.75 mg with an estimated starting population of 272. A model used to predict SGAR residues for Trials A – D showed that rats from Trial C would reach whole body residues of 4.0 mg/kg bromadiolone within a week of initial bait application (Daniells, 2011). Rats captured at Sites A and C had average whole-body residues of 3 mg/kg bromadiolone, and average liver residues of 15 mg/kg bromadiolone (Daniells, 2011). Data from France show lethal effects to foxes with liver bromadiolone residues as low as 0.8 mg/kg and barn owls with liver residues as low as 0.2 mg/kg (Berny *et al.*, 1997). Regardless of whether or not control might have been achieved, for five weeks predators of rats were exposed to potentially lethal volumes of bromadiolone (Daniells, 2011) in rats which, given the reduction in consumption, were likely to be slowly dying and therefore easier to catch (Cox and Smith, 1992; Brakes and Smith, 2005). If control had been achieved, it is questionable whether or not the cost in non-target wildlife and the use of in excess of 42 kg of bromadiolone bait would have been acceptable.

The brodifacoum baits applied at sites E and F both achieved > 90% control. This occurred despite the brodifacoum baits used having recorded brodifacoum concentrations of

below 0.003%, whereas the difenacoum and bromadiolone baits contained 0.005% active ingredient. Recently, the ECHA proposed that nine anticoagulant rodenticides, including all SGARs, be classified as toxic to reproduction in baits with concentrations higher than 0.003%, thereby preventing their authorisation for use by the general public. These proposals were adapted to the CLP Regulations (EC no. 1272/2008 on classification, labelling and packaging), making them legally binding in the UK (HSE, 2014). It is important, therefore, that anticoagulant rodenticides be efficacious at concentrations below 0.003% against both susceptible and resistant populations of Norway rats. All DNA samples taken from the six trial sites prior to or during the field trials came from rats positive for the L120Q mutation, except for one wild-type sample at Site E, and all sites were located within the central-southern England L120Q focus described earlier (see Section 3.3.1). These results show, conclusively, that difenacoum and bromadiolone baits cannot be consistently applied in an efficacious and legal manner to sites where Norway rat populations are shown to be positive for the L120Q mutation; the mutation confers resistance sufficient to allow animals to survive consumption of bromadiolone- and difenacoum-containing baits, but not brodifacoum-containing baits. As well as failing to achieve control in Trials A, B, C and D, the amount of bromadiolone and difenacoum applied in the field trials far exceeded that of brodifacoum (see Table 5.6). Despite the known greater toxic effects of brodifacoum (Prescott *et al*., 2007; see Section 4.3.1), the volume of bromadiolone and difenacoum that would have to be used to successfully control resistant Norway rats increases the risk not only of primary poisoning to non-target wildlife, domesticated animals and humans, but also of secondary poisoning to predators eating the surviving rats which have not yet eliminated the active ingredients from their liver tissue. Analytical models suggest that use of more potent poisons will reduce the risk of non-target poisoning for this reason (Daniells, Prescott and Buckle, 2011). Relative to the initial population sizes at all sites, a far lower volume of brodifacoum was consumed by

Norway rats than difenacoum and bromadiolone (See table 5.12), whilst achieving a far greater level of control. The starting population density at site D was the highest amongst the trial sites by a considerable margin (see Table 5.12; these values based upon Norway rats consuming 10% of bodyweight per night (Meehan, 1984), with the average adult Norway rat weighing 200 g); the vast difference between Site D and all other trial sites suggests that population density was not a factor in the effectiveness of the brodifacoum baits. Site D had the smallest area of any of the trial sites, and achieved the greatest level of control among the non-brodifacoum trials. Given the relatively small size of the brodifacoum trial sites, it might be the case that the efficacy of the brodifacoum baits was influenced by the small site areas, relative to the areas of Sites A, B and C.

5.4.2 Disparity between measures of population reduction

The results of Trial A indicate that the bromadiolone bait caused either 37.2% or 66.4% reduction in Norway rat numbers on site (measured by changes in tracking patch activity and census bait consumption, respectively). The lower figure obtained for tracking patch activity indicates that rats were indeed present in high numbers on site, but that many were either neophobic or behaviourally resistant to bait points at Site A. Bromadiolone consumption during the third week of the treatment phase was only 14.97% of its peak in the first week, suggesting that the surviving rats were resistant to the bait, and eating it in low amounts due to loss of appetite, or many of them were avoiding it altogether. The raw data (see Appendix 3 and Table 5.6) show that both measures of rat activity had fallen by the last week compared to peaks in the first week. Therefore it is probable that the figure obtained from census bait consumption is correct, and the bromadiolone bait caused a 66.4% reduction in rat numbers, and the high levels of recording tracking patch activity during the post-treatment census are

due to upheaval within rat social systems, as less dominant animals moved in, causing an apparent increase in numbers. Alternatively, if the tracking patch figure is correct, it indicates that the site suffered reinvasion from rats that were neophobic towards the census bait points. It can be inferred from the results of Trial B that, because difenacoum bait consumption reached its peak in the last week of the trial, overall numbers of Norway rats at Site B were the same (1.8% increase) by the end of the treatment phase as they were at the beginning; the site was at carrying capacity, and the minor reduction in census bait take was a result of the high neophobia present in the population. The remaining trials show sufficient similarity between tracking patch activity and census bait take results that it can be assumed that they are an accurate demonstration of the decrease (or increase) in rat populations at those sites.

5.4.3 Factors affecting trial outcomes

5.4.3.1 Reinvasion

When viewed as weekly consumption of bromadiolone, the results of site C show a clear decline in rat activity during the course of the five-week treatment phase. This suggests that the treatment phase may have been inappropriately truncated (see above). It is improbable that the enforced three-week post-treatment lag period in Trial C had no influence on the results, given the much reduced bromadiolone consumption at the end of the treatment phase. Unfortunately, the few data obtained on the one day of the first attempt at a post-treatment census cannot be used to infer any conclusions, as it is beyond doubt that the snowfall which prevented the researchers accessing bait points and tracking patches also obfuscated the data by preventing access by Norway rats. The extended lag period caused by the weather conditions at the time provided additional time in which the remaining population at Site C could have recovered via high-fecundity breeding and immigration from nearby populations.

The final results of the field trial at Site C suggest that the bromadiolone bait achieved no control. Two tracking patches (from a total of 46) were removed after the pre-trial census, which may have artificially reduced the maximum daily tracking score of the post-treatment census. 32 Norway rats were successfully trapped and their L120Q status investigated after the treatment phase of field trial II. The reduced L120Q homozygosity of the population after treatment compared with before treatment (81.3% and 90.0%, respectively) and the presence of at least one wild-type rat suggest that the cause of the apparent population increase in Site C is immigration of non-resistant or less resistant rats from nearby populations. Although there is some evidence for pleiotropic costs of resistance (Smith, Townsend and Smith, 1991; Heiberg, Leirs and Siegismund, 2006; Jacob *et al.*, 2012), it has been shown that the costs associated with resistance are not sufficiently deleterious to cause a population wide decline in resistance in the absence of anticoagulant selection pressure (Heiberg, Leirs and Siegismund, 2003). Methods are available which, using DNA extracted from rat tissue samples, can help to elucidate the level of migration to a given geographic area are available, and recommended for situations such as this, but it should be acknowledged that they rely on the ability to obtain multiple tissue samples from a notoriously neophobic species (Desvars-Larrive *et al.*, 2017). The final results which suggest a very similar population size to the beginning of the trial may indicate that during the pre-treatment census, Site C had reached its carrying capacity for Norway rats, and the population had reached this level again during the extended post-treatment lag phase.

Due to the trial site's location within a large working farm, it is likely that Site E suffered from a minor reinvasion of rats during the two week period taken to ensure the absence of squirrels. It is possible that these were rats inhabiting some disused pipes in a nearby field and had not technically reinvaded the trial site completely; indeed this is likely, because in the time that burrow baiting was carried out, only one of eleven burrows was

disturbed (and therefore active). Once squirrel bait take was removed from the dataset, it became clear that brodifacoum had nearly achieved complete control by the fourth week of treatment. Despite issues with squirrels and apparent immigration of rats from nearby satellite populations, the brodifacoum bait appeared to achieve 99.6% – 100% control, exceeding regulatory requirements. These results are supported by camera traps set up at Site E which recorded no Norway rat movements during the post-treatment census. The absence of tracking patch activity during the post-treatment census lends further credence to the supposition that most rat activity after Site E was declared free of squirrels was due to rats which only entered the trial area to forage, and therefore would have been less inclined to use the runs around which the tracking patches were located.

The reduced prevalence of the L120Q mutation at Site D may have been a function of mild reinvasion during the enforced lag phase, but it does not refute the plateau in difenacoum consumption towards the end of the treatment phase (see Table 5.9), and therefore is unlikely to have been a direct cause of treatment failure.

At Site F, following a period of mild fluctuation in recorded bait take, a sharp drop in bait take was recorded on the fourth week of the treatment phase, followed by a sudden relatively large increase and steady decline. The fluctuation prior to this is likely to be caused by upheaval within the rat population at Site F, where the raw data indicate a large number of rats had died very quickly – frequent changes in locations of bait take and activity, and the volume thereof, suggest that rats were moving in to more desirable locations after the dominant rats there died. The sudden removal of several bait points, due to the aforementioned unexpected movement of sheep indoors, may have contributed to fluctuation in bait consumption and masked the effects of brodifacoum poisoning on the population. Without constant monitoring of the genetic status of the population, it is difficult to determine whether sudden changes in rat activity (denoted by bait consumption and tracking patch

activity) are due to reinvasion or social upheaval. In order to avoid consistently removing rats for tissue samples (which may have further effects on the trial outcome), faecal samples may be required to monitor population genetics; faecal samples have been associated with a low success rate (see Section 3.4.3).

Despite sites B, D and E being more vulnerable to reinvasion (due to presence of nearby buildings capable of housing rats), Site C was the only site in which the final results were clearly affected by reinvasion. It is likely that Site C would have suffered reinvasion during the extended lag phase regardless of which rodenticide baits were used.

5.4.3.2 Refusal to take bait

The Norway rat population at Site B had, as described above, easy access to ample alternative food, and harbourage directly underneath this food source. Because their needs were so easily taken care of, the rats at Site B were slow to consume the census and treatment baits. As such the census periods were extended to allow a more accurate estimate of population size and changes; in both census periods bait consumption rose continuously over the six days, and peak difenacoum consumption did not occur until the last (seventh) week of the treatment phase. It is possible that the rats at Site B, with their high levels of neophobia and susceptibility to disturbance, would have been behaviourally resistant to brodifacoum to a limited degree, and increased their rate of brodifacoum consumption much more slowly than did the rats at sites E and F.

At Site F, the large central barn for the seasonal housing of sheep was largely covered in whole wheat from prior storage – if whole wheat was more palatable than the treatment bait, it was readily available to rats in the barn area. This, in addition to the animal feed left open in poultry feeders and storage sacks around the farm may have extended the treatment phase beyond the time it may have taken on a site with good housekeeping.

Previous research has established that rats homozygous for VKORC1 resistance mutations are often smaller than susceptible rats (Smith, Townsend and Smith, 1991; Smith *et al.*, 1993), and furthermore that social dominance in rats is linked to size (Smith, Smith and Sibly, 1991), and that dominant rats access food first, preventing subdominant animals from doing so (Sibly and Nott, 1993). Given the presence of established alternative food sources at all sites, it is possible that dominant rats, displaying a level of neophobia towards the anticoagulant bait, would have avoided consumption thereof for some time. This would have the effect of forcing only resistant rats to consume the treatment bait, assuming that any susceptible rats display size-based dominance. Given the L120Q prevalence at all sites (see Tables 5.4 and 5.5), the number of susceptible rats that never accessed any treatment bait would have been very low. Food competition, therefore, may have contributed to the scale of treatment failure at Sites A, B, C and D, but is unlikely to have been the difference between success (i.e. 90% population reduction) and failure.

5.4.3.3 Resistance

Although the rats are Site B were undoubtedly neophobic, the volume of difenacoum consumed over the course of seven weeks strongly indicates that the presence of L120Q in the population – with most rats being homozygous for the mutation – conferred a level of resistance sufficient enable most rats that did eat the difenacoum bait to survive. Even considering high resistance factors against difenacoum for rats derived from wild L120Q-positive rats (see Section 4.3.3), 1428.35mg is an exceptional volume of difenacoum for 110 rats to consume without any detectable control. Therefore, assuming that the population estimate wasn't severely inaccurate, the most likely explanation for this level of anticoagulant survival is that, spread over 7 weeks, the concentration of difenacoum per rat at any one time was low enough to allow the most resistant animals (which formed the majority of the population) to recover following elimination of the active ingredient and before absorption of

the potentially-lethal bait-meal. The very high volume of alternative bait available on site would have provided sufficient food that rats did not need to subsist entirely on the difenacoum bait, aiding in their survival over several weeks. A reduction in difenacoum consumption occurred after the third week; it is possible that this represents a minor drop in the Norway rat population as those individuals heterozygotic for L120Q in the population died. Numbers of Norway rats present had already recovered and reached pre-treatment levels by the time of the post-treatment census.

Although the population at Site C suffered a dramatic drop in numbers over the course of the treatment phase, the volume of bromadiolone consumed during the course of the trial was excessive (see Table 5.12); it is likely that there was a level of tolerance to bromadiolone among the resistant population, even supposing constant immigration from surrounding areas.

Trial D saw the greatest volume of anticoagulant active ingredient (difenacoum) consumed during the course of the six field trials (see Table 5.12); the plateau in bait consumption after the third week shows that the remaining animals consuming difenacoum bait were sufficiently resistant to the bait to allow them to consume it with no repercussions beyond possible temporary appetite loss (which are not demonstrated by these data). The relatively low rate of L120Q homozygosity at Site D after cessation of the field trial suggests that there had been immigration by less- or non-resistant rats during the treatment phase. It is possible that the bait consumption plateau towards the end of the treatment phase was not affected by this immigration because the newcomers were less likely to enter bait points than the resistant rats which were familiar with them. This is a more likely explanation of the results of molecular testing at Site D than L120Q-heterozygous and wild-type rats being behaviourally resistant to anticoagulant baits.

All trial sites had copious alternative food sources for rats, which had a clear impact on the acceptance of treatment bait at Site B, and probably had limited effects at the remaining sites. The variety of factors negatively influencing bait take in these trials is an indication that thorough research into factors beyond anticoagulant resistance is required when targeting rat populations, in order to minimise the volume of rodenticides to which non-target species are directly and indirectly exposed, and to minimise the financial cost of purchasing and using unnecessary amounts of rodenticide bait.

5.4.4 Impact of brodifacoum on non-target species

At Sites E and F, recorded bait take was occasionally attributed to non-target rodents such as wood mice (*Apodemus sylvaticus*), bank voles and field voles due to the repeated presence of droppings and kibbles in some bait boxes. These were more frequent in areas away from buildings and centres of rat activity. Grey squirrels (*Sciurus carolinensis*) were also present at Site E in limited numbers, and were highly attracted to the trial bait, frequently causing full takes either due to direct consumption or removal for caching. Any recorded bait loss that could be attributed to non-target animals was not included in the raw data.

Despite bait take being frequently attributed to non-target rodents, very few non-target carcasses were found Site E: an unfledged blue tit *Cyanistes caeruleus* chick which is thought to have fallen from its nest; and two bank voles both of which were found in or near to bait points. The bank voles are thought to have died due to rodenticide exposure. No non-target animal carcasses were found at Site F. The consistency of bait take by non-target rodents suggests that Norway rats were more susceptible to these baits than the non-target mice, voles and squirrels which took a limited volume of bait, although this could indicate that squirrels may have been caching the brodifacoum bait rather than consuming it. Brodifacoum baits

frequently required replacement due to depredations by slugs, which have been shown to represent a viable contamination pathway to non-target species (Alomar *et al.*, 2018). Because of the lack of non-target carcasses found in Sites E and F, it is not known what impact the potentially contaminated slugs had on their predators. The potential impact of Trials A-D on non-targets are thoroughly explored by Daniells (2011), wherein the excess volumes of both consumed and uneaten difenacoum and bromadiolone to which non-target animals were exposed through various routes is acknowledged.

5.4.5 Response to L120Q in south-east England

All trial sites were within the large central-southern England L120Q focus, and all rats but one trapped prior to each trial were positive for the L120Q mutation. Despite the apparent differences between the responses of the Hampshire and Berkshire strains of L120Q, it isn't currently possible to ascertain to which strain an individual rat belongs without actively administering one of the active ingredients and measuring the response (either in a feeding or oral gavage/intraperitoneal injection test). Based upon estimates of the initial populations, rats at sites B and C consumed the greatest volume of difenacoum and bromadiolone, respectively, but the trials at these sites achieved the lowest levels of control. This lends credence to the suggestion that there are sites in south-east England where repeated use of ineffective rodenticides has continually selected for highly resistant animals, in the same way in which the CSL-Berkshire strain of L120Q-resistant rats was created. The volumes of bromadiolone and difenacoum consumed at those trials (Daniells, 2011), with only limited control achieved, suggest that the character of L120Q resistance found there was similar to that of the CSL Berkshire rats tested in Chapter 4. This is especially alarming given the distances between the four sites; Sites A and B are over 30 miles from Sites C and D.

Therefore, the results of all trials considered herein support the proposal that where the L120Q mutation is potentially present (ie anywhere in the area covered by the large central-southern England L120Q focus) the use of difenacoum or bromadiolone is inadvisable (RRAG, 2012; Buckle, 2013a). It has been shown that corn silage, which is increasingly used in continental Europe, is a suitable food source for Norway rats and in some cases can supply the dietary vitamin K required by resistant animals to prevent increased blood clotting times (Jacob and Freise, 2011). The silages seen at Sites B and E were not corn based, but if the demand for corn silage (e.g. for biofuels) increases in the UK, this may become a factor to consider in the prevention of the survival of resistant populations (e.g. Welsh and Hampshire resistances).

Chapter 6: General Discussion

6.1 Overview

Norway rats have acted as pests in the UK for nearly 300 years and, along with house mice, are unlikely to be replaced as the foremost vertebrate pests in most areas of the country. Anticoagulants obviate the neophobic behaviour of rats, and provide sufficient time to provide a viable antidote (Vitamin K) to poisoned non-target humans and domesticated animals. However, the time taken to be sure of completely controlling a rat population can make their use expensive, and care must be taken to minimise the risk to non-target animals, a risk that can never be completely removed. Since the initial discovery of anticoagulant resistance in Norway rats (Boyle, 1960), resistance has been discovered in many areas of Europe. The south-east of England potentially represents the worst known area in terms of number of resistance-conferring mutations, and their impact on the effectiveness of anticoagulants (Gill, Kerins and MacNicoll, 1992; Gill *et al.*, 1993, 1994; Pelz *et al.*, 2005; Prescott *et al.*, 2010; RRAG, 2012; Haniza *et al.*, 2015). Resistance exacerbates the cost of control due to increased baiting periods (and therefore volume of bait applied) or outright failure, and also increases the risk of non-target poisoning (Cox and Smith, 1992; Atterby, Kerins and MacNicoll, 2005; Shore *et al.*, 2015). Habitat management and good housekeeping on farmsteads have been shown to be effective at reducing the impact of Norway rats and resistance (Lambert *et al.*, 2008). However, in the case of large-scale infestations with the potential to cause a great deal of damage in a short time frame, the only viable option for control is the use of anticoagulant rodenticides (see Section 1.3.3). The response to anticoagulant resistance in the UK has largely been reactive up until now, with the only notable attempt to prevent resistance the aborted containment zone in Wales (Drummond, 1966; Pamphilon, 1969). Because of the lack of recent innovation in the

production of rodenticides available for use in the UK (Buckle, 2013a), the response to the extent and severity of resistance should be to understand the likely impact on control, and respond by using anticoagulants in the safest and most effective manner possible. Thus, the experiments herein were designed to facilitate this by revealing the extent and impact of the VKORC1 resistance-conferring mutations in Norway rats in the south-east of England.

6.2 VKORC1 mutations in the south-east of England

The results of the study in Chapter 3 are not encouraging for PCOs and landowners who seek to control rats in the south-east of England. The vast majority of samples tested for mutations were found to be positive for the VKORC1 mutation L120Q, which previous tests have shown to confer practical anticoagulant resistance to rats that possess it. In some cases this is the strongest known form of resistance (Gill, Kerins and MacNicoll, 1992; Quy *et al.*, 1995; RRAG, 2012) with no pleiotropic cost to individual rats or populations (Gill, Kerins and MacNicoll, 1992; Smith *et al.*, 1993). Interestingly, the three strongest VKORC1 mutations (L120Q, Y139C and Y139F) were all found in the south-east of England, in some cases within very close proximity of each other. A previous study investigating gene flow between Norway populations discovered 12 individual rats with both the L120Q and Y139C mutations, and 53 rats with the Y139C mutation in the area covered by the experiment described in Chapter 3 (Haniza *et al.*, 2015). All of the samples from that study were taken between 1990 and 2000, so it was unexpected that the results herein do not reflect this. Additionally, the only samples from the dataset in Chapter 3 that had multiple mutations were from Scotland. Resistance in Germany appears to be limited to the north-west of the country in a 27,000 km^2 area (Pelz, 2007). This resistance area appears to be growing, but different studies disagree on the prevalence of resistance therein (Pelz, 2007; Runge *et al.*, 2013).

Studies from the Netherlands (Meerburg *et al.*, 2014), France (Grandemange *et al.*, 2010) and Belgium (Baert *et al.*, 2012) show very low prevalence of VKORC1 mutations in Norway rat populations (33.04% of samples, 44.57% of sites with the Y139F mutation present, and up to 20.41% of samples, respectively). This contrasts strongly with the results of this study, wherein 73.68% of samples were positive for the L120Q mutation (88.49% were positive for any of the five searched-for VKORC1 resistance conferring mutations; admittedly influenced by the fact that the majority of samples were received by PCOs who suspected they had encountered resistance). There also multiple large resistance foci in the UK, cited in this study area, Wales (Drummond, 1966; Pamphilon, 1969) and Scotland (Brodie, 1976); there have also been individual resistant animals from the north of England (Haniza *et al.*, 2015; Chapter 3).

The greatest issue faced while conducting the practical work for Chapter 3 was obtaining samples, as described in Section 2.1.3.1. This issue has not been described or commented on by the authors cited above, which suggests that they received more support from organisations dedicated to controlling resistance, whose influence could be brought to bear on PCOs and gamekeepers who supplied samples. It is possible though that the difficulty in obtaining samples was a function of the aim of this study: to locate and delimit resistance foci. Therefore, an even spread of samples were required from all over the study area; every site with samples successfully analysed represented a reduction in suitable sites for the future. Obtaining samples grew easier as stakeholders became more engaged, and became aware of the benefit of contributing to the dataset. Companies whose employees sent samples for which results were obtained were increasingly likely to send in more samples, knowing that they might receive an explanation for treatment failures. 48 of the samples successfully analysed here came from a single source (a local council), with whom an agreement had been reached regarding supply of results, if they could be used within this dataset; this could

represent a model for future attempts to undertake this kind of study. Once these results are made available to all stakeholders, it is likely that any attempt to expand this study to the rest of the UK would be made easier.

6.3 BCR Tests

With the discovery that the L120Q mutation is present almost wherever it is looked for in the south-east of England, it was and is necessary to discover the level of resistance conferred to Norway rats that possess it. This study was the first of its kind to generate effective dose data and resistance factors for resistant strains by following on from previous susceptible data (Prescott *et al.*, 2007). The results showed conclusively that resistant female rats are more anticoagulant-tolerant than male resistant rats, regardless of resistance mutation and susceptible strain with which the wild progenitors were bred. This indicates that resistant populations are harder to eliminate than previously thought, because more females will survive control attempts than males, allowing a greater surviving effective population. These results confirm previous studies that have shown that rats with the Y139S mutation possess either no, or limited technical resistance to SGARs (Hadler, Redfern and Rowe, 1975; Redfern, Gill and Hadler, 1976; Redfern and Gill, 1980), and therefore the Welsh resistance focus is unlikely to present problems with control in the future, unless rats with the L120Q, Y139F or Y139C mutations are introduced there. The results for the Reading Berkshire strain (L120Q mutation) show that these rats can be controlled by all SGARs, supporting the belief of many PCOs in Reading that difenacoum and bromadiolone are still effective there (R Kiff, pers. comm.). The missing results for difethialone (and bromadiolone and flocoumafen against males) are unlikely to refute this. Previous results for Hampshire-resistant rats (also possessing the L120Q mutation) show that they possess a much greater tolerance to

bromadiolone and difenacoum (Prescott *et al.*, 2007), which may account for the supposed discrepancy between the two strains' dietary vitamin K requirements (Greaves and Cullen-Ayres, 1988; Gill, Kerins and MacNicoll, 1992).

Results for the CSL Berkshire strain, meanwhile, show that rats of this strain are only susceptible to the resistance-breaking SGARs, with resistance factors and ED_{50}s against difenacoum and bromadiolone that indicate practical resistance. Because of the potential for the L120Q mutation to confer resistance of this level (by repeated selection through the use of bromadiolone and difenacoum), and the uncertainty of which phenotype of L120Q resistance is present in any given site in the large L120Q focus of southern England, it is advised that only resistance-breaking SGARs are used for rat control in this area. ED_{50} and resistance factor data are not currently published for rats with the L128Q, Y139C and Y139F mutations, so there is not yet any evidence to dispute that CSL Berkshire rats possess the greatest resistance factors against any SGAR. Given the results for the Welsh rats in this study, any results for L128Q are unlikely to be of use. Y139C and Y139F strains are currently being investigated by collaborators in Germany; these results will be of interest due not only to their known impact in the laboratory and field trials (Grandemange *et al.*, 2009; Endepols *et al.*, 2012; Buckle *et al.*, 2013), but also because these mutations are present in south-east England.

Some inaccuracies are likely to have resulted from the methodology employed in Chapter 4 due to the difficulty of ensuring that rats received the exact dose prescribed, but this cannot be controlled for. Fiducial limits are wider for rats that are more tolerant to given SGARs, resulting in some exceptionally wide fiducial limits at the ED_{99} level (see Tables 4.3, 4.4 and 4.5). Results at the ED_{50} level are therefore the most useful and trusted, but cannot be used to replace LD_{99} data. The greatest difficulty faced in carrying out the practical work for Chapter 4 was attempting to source enough rats to carry the experiments out on. All three

strains faced breeding difficulties at some point during the study (i.e. refusal to breed despite changing and creating new breeding pairs; repeated infanticide; sex ratios of offspring skewed towards females). This resulted in the study being prolonged by months, if not years, with some Probit lines unfinished.

The uncertainty of the application of these results to the L120Q focus of southern England and the likely discrepancies between effective and lethal doses meant that confirmation of these results in the field was required.

6.4 Field trials

The results of the field trials conducted for this study are clear: regardless of whether they took place in Berkshire or Hampshire, the trials utilising bromadiolone and difenacoum baits failed to achieve control. The only equivocacy is in the steady reduction of bromadiolone consumption during the treatment period in Trial C, which may indicate that control was being achieved before the trial was ended. The resistance status of the rats trapped prior to this Trial (100% L120Q; 90% homozygous), in combination with the steady reduction of bromadiolone consumption, indicates that this form of L120Q might have been controlled by sufficient volumes of bromadiolone. A total of 2100.75 mg of bromadiolone was consumed during the five weeks of the treatment phase at Site C, equivalent to 42.02 kg of 0.005% bait, yet did not achieve complete control of a population of ~272 rats within five weeks. This represents a large volume of bait and/or contaminated rats to which non-target animals were exposed.

Both brodifacoum trials achieved success in attaining >90% control of rats at Sites E and F, but treatment phases took 11 and seven weeks, respectively. This may represent too

great a cost in time and money for some stakeholders, but use of resistance breakers represented the difference between control success and failure, and under the UK rodenticide stewardship scheme, saturation baiting (as took place in Trials A – D; Daniells, 2011) is unlikely to be supported.

The results of these trials reflect the results and conclusions of similar studies in Germany against rats with the Y139C mutation, in which brodifacoum was found to be efficacious (Buckle, Klemann and Prescott, 2012), but bromadiolone (Endepols *et al.*, 2012) and difenacoum (Buckle *et al.*, 2013) were not. Given the results of Chapter 4 regarding rats with the Y139S mutation, and previous studies against rats with the L128Q mutation (Hadler, Redfern and Rowe, 1975; Greaves and Ayres, 1982; Greaves and Cullen-Ayres, 1988), field trials against Welsh and Scottish rats are unlikely to be of importance. Results of initial studies investigating the strength of resistance to SGARs conferred by the Y139F mutation suggest that similar field trials involving this strain would be useful (Grandemange *et al.*, 2009). Since recent legislative changes in the UK, Trials E and F are the first of their kind to offer evidence that resistance breakers used at a concentration of below 0.003% can be fully efficacious against L120Q-resistant rats in both Hampshire and Berkshire (although see Greaves, Shepherd and Quy, 1982). Because all SGARs are classified as toxic to reproduction, and the risk that exposure to these chemicals presents to human pregnancies is only accepted at conentrations below 0.003%, the failure to achieve control using difenacoum and bromadiolone at 0.005% during Trials A – D is confirmation that these SGARs should not be used in the south-east of England, due to their ineffectiveness against the L120Q mutation, which appears to be present throughout this area.

These trials had voles and mice repeatedly entering bait points. Because these rodents are smaller than Norway rats, they can access any bait point that a rat can – this cannot be prevented except by refraining from using SGARs outdoors. This being the case, if SGAR

baiting is to continue, it must be ensured that permanent baiting does not occur, and only proven, effective baits should be used in order to minimise the duration of bait use. All anticoagulant application should also be conducted and monitored by trained, competent users.

6.5 The future of anticoagulant rodenticides as rat control in the United Kingdom

Given the recommendations made above, the advent of the UK rodenticide stewardship scheme is fortuitous and timely. Under the stewardship of CRRU, competent users (designated as such by attending a training course – see CRRU UK, 2016) are afforded the use of resistance breaking SGARs. The results herein indicate that with the use of brodifacoum (or other resistance breakers) Norway rat resistance as it stands in south-east England should not be problematic. Therefore, the success of the scheme relies upon users utilising SGARs in responsible fashion, and continued monitoring of non-target species (Buckle *et al.*, 2017).

Regardless of the stewardship scheme, the expansion to the rest of the UK of the study described in Chapter 3 is advised. It is possible that there are areas of the UK where the use of bromadiolone or difenacoum might still be feasible, if doing so would present less of a risk to non-target animals. Expanding this form of monitoring will give stakeholders the knowledge to prevent selecting for resistance and consequently creating strains with similar resistance statuses to CSL Berkshire rats. Furthermore, identifying and delimiting all resistance foci will enable researchers to monitor how resistance changes temporally; the results herein represent only a snapshot in time, rather than an investigation of how resistance

came to become so prevalent in south-east England. Such information may help with the prevention of the establishment of resistance elsewhere. The completion of the Probit lines left unfinished in Chapter 4 is also required. We do not currently know which form of L120Q resistance is present in any given site within the large focus of central-southern England, and therefore we should attempt to generate these data for all of them.

The L120Q, Y139C and Y139F mutations are all found in south-east England, and are also all found in North-Western Europe. It is possible that these mutations originally come from continental Europe, and rats from this area infested ships bound for England, or vice versa. An investigation of rat populations in the UK suggest they are not yet at equilibrium, having only relatively colonised the country (Haniza *et al.*, 2015). Further investigations of this sort in both the UK and Europe may elucidate the provenance of the various resistance mutations, and aid in future attempts to prevent their spread. It would be hasty to assume that resistance will no longer be a problem in the UK, because effective rodenticides are now available to users. As noted elsewhere, SGARs are currently the only large scale option for the control of Norway rats in this country. It may appear unlikely now, but if their efficacy or availability is ever reduced for any reason, the situation could become untenable. The results of Chapter 3 show conclusively that resistance in south-east England is widespread and prevalent; it is the first attempt to fully delimit and identify every resistance mutation in a geographic space. Chapters 4 and 5 represent the first quantification of resistance factors for resistant strains, and the first confirmation that the L120Q focus identified in Chapter 3 can be successfully controlled in light of changes to the registration and use of SGARs in the UK, despite their apparent similarity to Selected Lines of rats with increased tolerance to anticoagulants. It is important therefore that the above recommendations are taken into account, so that our knowledge can be expanded, and the fight against resistance become proactive for the first time.

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