Resistance to anticoagulant rodenticides in House mice that convey the *VKORC1* mutation Y139C

Thesis for Degree of Doctor of Philosophy

School of Biological Sciences

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December 2018

Abstract

Anticoagulant resistance was discovered in UK House mice almost 60 years ago, soon after the introduction of the first-generation anticoagulant rodenticides (FGARs). Resistant individuals were found to survive field strength anticoagulant baits, and in response, more potent second-generation anticoagulant rodenticides (SGARs) were developed. Practical resistance to some SGARs is now raising major concerns.

This thesis focuses on the development and implementation of methodologies to investigate resistance in mice.

- Susceptibility baseline data were produced for four FGARs using albino susceptible House mice.
- Resistance baselines were produced using House mice homozygous for the *VKORC1* mutation Y139C, against four FGARs and against five SGARs.
- Resistance baselines were produced using House mice heterozygous for the *VKORC1* mutation Y139C against five SGARs.

For homozygous resistant mice, resistance factors (RFs) were estimated for four FGARs and five SGARs; and for the SGAR bromadiolone and all four FGARs, the magnitude of the RF was sufficient to suggest practical resistance (with a high likelihood of treatment failure). For heterozygous resistant mice, only the SGAR bromadiolone had an RF that would suggest some degree of practical resistance, with all other SGARs expected to be efficacious.

From the above, the continued use of ineffective anticoagulants would be expected to actively select for anticoagulant resistance, and thus exacerbate the control problem. In the UK, resistant populations of House mice carrying the *VKORC1* mutations Y139C and L128S are widespread in animals sampled to date, and the high incidence of homozygous resistance would suggest over reliance on ineffective anticoagulants as a major cause.

Data presented here is now available globally, online via the RRAC website, where it can be used to make decisions on rodenticide selection for mouse populations that carry the Y139 mutation, and to quantify the magnitude of FGAR resistance against other *VKORC1* mutations.

Acknowledgements

Firstly, I would like to acknowledge Colin Prescott for giving me the opportunity to undertake this PhD, and for his wealth of knowledge and guidance along the way as my supervisor. I would also like to thank my co-supervisor Phil Baker for his guidance during the early chapters.

I would also like to thank David Rymer, my former colleague with his assistance in the early genetic studies; his help was invaluable in collating the samples and analysing them. I am extremely grateful to Emily Coan and Clare Jones of the Vertebrate Pest Unit, University of Reading for analysing the later genetic samples and generously allowing me to use their results.

I must thank Pam Rummings, former Technical Manager of the Bioresource Unit at University of Reading and Wayne Knight, senior technician of the Bioresource Unit for their assistance and training in some of my practical work; and also, all the staff of the Bioresource Unit who cared for my large colony of House mice (we were all surprised by the numbers produced). More recently special thanks must go all the Bioresource Unit staff and Dr Karen Henderson, Director of Technical Services who have allowed me to complete my thesis while taking on the role of Technical Manager of the Bioresource Unit.

I would like to acknowledge Alex Cornish of Syngenta AG and Stefan Endepols of RRAC for their support in conducting some of this research.

Thanks also to Dr Kerry-Ann Rostron and Professor Angela Clerk for being my proof readers. I think they both learnt something new as it was a completely new research subject to them both.

Finally, thank you to all my friends and family who have supported me through these last six years, including my dogs Barney, Meg, Jess and Flash who have kept me company during the long days of writing. They have all been there in some way to keep me going, and made sure in the later stages that I kept a good work-life balance.

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

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December 2018

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Chapter 1 - General Introduction

1.1 Rodents as pests

There are almost 2000 species of rodent ranging in size from the Capybara, weighing around 50kg (Macdonald *et al*, 2015) to the Pygmy mouse weighing around 8g (Meehan, 1984). These species include several that are seen as pets such as hamsters, gerbils and guinea pigs, squirrels, as well as rats and mice which people often refer to as pests. Rats and mice are considered pests by humans as they are reservoirs and vectors of disease (Lefebvre *et al*, 2017), cause financial loss by destroying crops and materials, attract predators (Tomlinson *et* al, 2017) and they are despised by most people (Meehan, 1984). In some countries, rodents are ranked as one of the top three most important pests. The International Union for Conservation of Nature rank *Mus musculus domesticus* (House mouse) as one of the top 100 most invasive species (Goulois *et al*, 2017), and in many developing countries, they compete directly with humans for food (Singleton *et* al, 1999). Rodents are successful pests due to their reproductive rates, the fact that they are omnivorous and their adaptations to gnawing (Meehan, 1984).

Of the 2000 rodent species known to man, *Rattus norvegicus* (Norway rat), *Rattus rattus* (Black rat) and *Mus musculus* (House mouse) are described as commensal rodents and seen as pests and carriers of disease (see Table 1.1 for comparison). Commensal means "sharing ones table", but MacDonald and Fenn (1994) regard the term kleptoparasitic, where one animal takes the food from another, as more appropriate as commensal implies no damage to the host (MacDonald & Fenn, 1994) which is clearly not the case. Commensal rodents are opportunistic and have managed to exploit man-made environments to their own advantage which causes conflict with humans. They may inhabit structures, chew cables and fixtures,

scavenge food as well as urinate and defecate on surfaces. These three major commensal species are found worldwide, although in some areas other species are of more importance. In Asia and the Philippines, the species *Rattus argentiventer* (Rice Field rat), *Rattus tiomanicus* (Oil Palm rat) and *Rattus tanezumi* (Oriental House rat) are of major importance due to the damage they cause to the highly valuable local crops of maize, rice, oil palm, wheat and millet (Wood, 1994).

Norway rats (the common brown rat), *Rattus norvegicus* are believed to have originated in the former USSR and spread across the world aboard ships (Lund, 2015). Norway rats thrive in any environment inhabited by humans where they can find water, food and shelter (Lund, 2015). Black rats, *Rattus rattus* were the most common rat species found worldwide until the spread of Norway rats in the 1700s (Lund, 2015). The species was common in western Europe until the 1960s but is nowadays more commonly found in the tropics and subtropics (Lund, 2015).

Rodents carry a range of zoonoses; including *leptospirosa* (Weil's disease) and *salmonella*, that can be passed on to humans through direct contamination of food with urine and faeces or by contaminating surfaces that food may come into contact with (Meerburg *et al,* 2008). Up to 30% of Norway rats have leptospire bacteria which they pass in their urine (Syngenta, 2004). They can also transmit diseases to livestock, and biosecurity is very difficult to maintain in the presence of rodents (Syngenta, 2004). The potential economic loss to farmers and manufacturers is due to damage of growing crops and the contamination and spoilage of products with hair, faeces and urine (Bayer, 2007). It is estimated that rodents contaminate three times more stored food than they eat (Bayer, 2007). Food and crops are most valuable when they are in storage due to the combined costs of crop protection, harvesting, transport, processing and packing so any loss due to rodents is of great financial significance, and most

losses occur in countries where food shortages are common (Syngenta, 2004). Rodents are associated with dirty environments and disease and the need to control their numbers has led to the development of many measures that cause suffering (Meerburg *et al*, 2008).

Table 1.1: Comparison of Norway rats, Black rats and House mice (from Lund, 1994)

	Norway rats	Black rats	House mice
Habitat	Farms, refuse tips, in fields, earth banks, around farm buildings, sewers, undisturbed storage areas, wall cavities, move indoors in autumn and winter.	Tropics and subtropics, rainforests, swamps, coastal areas and ports.	Buildings, behind walls, skirting boards, under floors, ceiling voids. Can live outdoors and indoors.
Diet	Omnivorous and need water.	Fruits, seeds, grain, and need water.	Graminivorous, get moisture from food.
Behaviour	Primarily nocturnal. Can climb, jump and swim. Often kill mice. Hierarchy dominate food sources. Territories are defended. Leave 'runs' to and from food sources. If food readily available seasonality related to habitat is not found. Know their territory well and wary of new objects (neophobia).	Primarily nocturnal. More agile than Norway rats, good climbers. Nests high up in buildings or in trees.	Primarily nocturnal. Erratic, sporadic feeders, good climbers, constant gnawing. Territorial, usually one male and several females.
Zoonotic diseases	*Leptospirosis, Salmonella, Toxoplasmosis,* Weil's disease, murine typhus, trichinosis.	Bubonic plague, *Leptospirosis, Salmonella*	*Salmonella, Leptospirosis.*
Damage caused	Damage food in storage and contaminate it. Damage crops. Damage buildings due to burrowing.	Damage to food in storage, damage in orchards.	Damage electric cables, water and gas pipes, and packaging due to chewing. Contamination of food.
Why they are a problem	Economic, disease, structural damage.	Economic, disease, structural damage.	Economic, disease, structural damage. More of a problem because they live indoors.
Control	Rodenticide baits, trapping	Rodenticide baits, trapping	Rodenticide baits, trapping
Proofing and hygiene options	Eliminate harbourage, clear up spilt food. Block holes and gaps into buildings. Store food in proofed structures.	Clear up spillages, store food in proofed structures.	Block entry holes to buildings. Store foods in mouse-proof containers, clear up spillages, remove rubbish.

1.2 The evolution of the House mouse (*Mus domesticus)*

The House mouse is the most widespread of all the rodent species, it is found in all geographical and climatic areas on all six continents (Meehan, 1984). They can live in any available space such as in cavity walls, under floors and behind skirting boards. House mice breed seasonally in temperate regions but in favourable conditions they can breed continuously (Lund, 2015). In some areas they are commensal and in others they live independently of man (Lund, 2015). Commensalism has led to the domestication of the House mouse initially as fancy mice and then as a laboratory animal (Boursot *et al*, 1993) and they are now the most widely used experimental animal (Lund, 2015).

It was originally considered that there was only one species of House mouse, *Mus musculus,* but it has since been discovered that there are several species or semi-species of House mouse and they were reclassified in 1981 (MacNicoll, 1995), *Mus domesticus* (the European House mouse) being the species found in the UK and western Europe (Lund, 1994). All albino laboratory mice and fancy mice are thought to originate from *M. domesticus* (Buckle & Prescott, 2012; RRAG, 2012). It is believed that the various species of House mice evolved from the species *M. musculus,* which originated from Central Asia and is widely considered to be the true House mouse (Meehan, 1984). The House mouse is widely referred to as a commensal rodent, because they are frequently found "sharing the table" with humans [the Latin for table is 'mensa']. In Asia, the House mouse was found living in granaries eating the stored cereals which man had grown and was storing (Meehan, 1984).

The House mouse spread from Central Asia via trade and passive transport by humans due to its ecological association with man (Boursot *et al,* 1993). To date there are seven species of House mouse identified throughout the world (Lund, 2015), however due to the evolution of

new molecular techniques it is highly probable that new species will be found. *M. musculus* and *M. domesticus* are the more commensal species of House mouse, whereas *M. spretus*, *M. macedonicus* and *M. hortulanus* are the wild-ranged 'aboriginal species', as they are typically found in dry areas such as savannahs, steppes and deserts and normally live independently of man (Auffray *et* al, 1990; Berry & Scriven, 2005). House mice can live in dry, arid conditions, surviving with minimal water because they are able to utilise the water created by metabolism. However, if food contains little water, reproduction is reduced and only increases again when there is access to water (Lund, 2015).

The following species of House mice are found throughout the world (Figure 1.1):

1. *M. musculus*, the Linnaeus' House mouse, is found in Sweden, eastern Denmark, parts of Germany and Serbia and is believed to have evolved in Southern Russia from *Mus spicilegus* (Meehan, 1984). It is closely related to *Mus wagneri* which is found in central and northern Asia where the House mouse originated (Lund, 2015).
2. *M. domesticus*, the European House mouse, is found throughout Europe but also in the Americas, Australia and Africa. In Europe it is found in the UK, Netherlands, Belgium, France, Spain and western Denmark. It is larger than *M. musculus* and is more readily found indoors but can be found in crop fields in the absence of other small animals (Lund, 2015). *M. domesticus* was carried on ships from Spain and Portugal to Latin America with the first explorers, and then to North America on French, Dutch and British ships (Meehan, 1984). Genetic analysis of House mice in Australia has determined that they are most likely ancestors of House mice that arrived on British ships in the 1700s (Gabriel *et al*, 2011). Unlike *M. domesticus* in Europe, the Australian mice live predominantly outdoors in feral populations of about 150 mice per hectare (Pocock *et al*, 2005) but they can readily adapt to favourable

environmental conditions, and in some years can form plagues of up to 2500 to 2750 mice per hectare causing substantial environmental and economic problems (Singleton & Redhead, 1990). This trait is not seen in any other populations of *M. domesticus* in the world (Gabriel *et al*, 2011). Favourable environmental conditions during the breeding period in the year prior to a plague and the lack of competition from other granivores both contribute to the breeding success of *M. domesticus* in Australia compared to other *M. domesticus* populations throughout the world (Singleton & Redhead, 1990). *M. domesticus* is not as successful living out of doors across the rest of the world due to competition from local rodent species and it has not become a serious pest of crops outside Australia (Lund, 2015). *M. domesticus* is the most successful of all the mouse species having colonized every continent and different environments.

3. *M. spretus*, Lataste's mouse, is found in southern France and Spain in the Mediterranean region. It lives alongside *M. domesticus* over its entire range (Boursot *et* al, 1993; Lund, 2015).
4. *M. macedonicus* (*M. abbotti* or *M. spretus*), the Eastern Mediterranean short-tailed mouse, lives outdoors in the region from Macedonia to Turkey and from the Balkans to Cyprus (Boursot *et al*, 1993; Lund, 2015). It lives alongside *M. domesticus* apart from in the Balkans area of its range (Boursot *et al*, 1993).
5. *M. hortulanus* (*M. specilegus*) has a range similar to *M. musculus* covering Austria, Romania and to Ukraine (Boursot *et al,* 1993). It lives in granaries or grain fields whereas *M. musculus* is usually found in farm buildings (Lund, 2015).
6. *M. molossinus* is closely related to *M. musculus* and is found living indoors and outdoors in Japan (Lund, 2015).
7. *M. castaneus* (*M. musculus castaneus*), the Asian House mouse, lives in cities and towns in an area stretching from India to the Pacific islands (Lund, 2015).

Figure 1.1: Distribution of the various species of House mice (Lund, 2015).

House mouse species are also reported to successfully breed between species (and thus are able to share genetic information) while retaining their distinctiveness (Berry & Scriven, 2005). This adaptability to different environments and the ability to share genetics between species has led to the House mouse being one of the most successful and widespread mammals in the world.

1.3 Methods used to control rodents

Due to the huge problem of rodents as commensal pests a wide variety of rodent control products are available. These control products/methods include traps, poisons, hunting, shooting, fumigation, deliberately causing diseases and electrical repellers (Meerburg *et al*, 2008). These control products/methods can be classified as: non-poisonous methods, bait

poisons or fumigant poisons (Meerburg *et al,* 2008). Ideally, rodent control should avoid contamination of the stored product or environment and the poisoning of non-targets (Smith & Greaves, 1986). Different methods of rodent control vary in how humane they may be viewed, the amount of distress caused to the rodent, the length of time the rodent takes to die, whether the rodent suffers any discomfort or symptoms before death and the possibility of poisoning non-target species (Mason & Littin, 2003).

Rodent infestations may also be controlled and physically prevented by making the environment unattractive to the rodent population. To survive, rodents require food, water and shelter and if any of these are denied, an infestation can be less problematic than if all three are available. On farms, food should be stored in bins which rodents cannot chew, any spilt food should be cleared away, rubbish should be removed and vegetation around buildings cleared as these provide cover for rats. These control measures are sustainable and prevent the selection of rodenticide resistant rodents (Buckle & Prescott, 2012). However, it is highly unlikely that a farm environment will be maintained in such a way as to make it completely unattractive to rodents. Storing food in rodent-proof containers and clearing away any spillages reduces the chance of rodents remaining in an environment (Natural England, 2012). It is essential to prevent rodents entering buildings and becoming established and any gaps that rodents can enter a building should be blocked (e.g. around utility pipes). In practice, it is very difficult to proof against mice as they are able to enter buildings through gaps as small as 1cm diameter, similar to those found in ventilation blocks.

Despite taking preventative measures infestations occur and further control measures are required. The main control measures available are trapping and poison baiting. Trapping can be an effective control measure if there are only a few rodents involved. The traps must be

sited in locations the rodents move along, so it is essential to survey the area to identify rat runs. Mice move along the base of walls so traps can be placed in these locations (Natural England, 2012). Trapping can be time consuming as the traps must be inspected at least daily, especially if cage traps are used. This is in order to be humane; with kill traps it is necessary to ensure that animals are actually killed, with live capture traps it is important to dispatch animals quickly and ensure that any non-target species that have been captured are released as soon as possible. When large numbers of rodents need to be controlled poison baiting is most effective, especially when manpower is limited.

1.4 Rodenticides

Rodenticides are the most popular and cost-effective measure used for rodent control as they provide a means of controlling a large number of rodents (Hadler & Buckle, 1992). They are commonly administered as poison baits but some can also be liquids, gases or dusts (Buckle, 1994). Poison baits have several humanitarian issues. They are ingested while rodents are foraging, so adult females may ingest these baits and be killed leaving any dependent pups in the nest to succumb to starvation and dehydration leading to death. Additionally, non-target animals may be accidentally poisoned, scavengers or predators that eat dead or dying rodents may be poisoned , and the intensity and length of suffering depends on the mode of action and dose of the bait consumed (Mason & Littin, 2003).

Rodenticides are either acute or chronic in their mode of action. Acute rodenticides cause the rodent to become ill quickly, often within hours of administration, which can have the negative effect of causing the animals to develop 'conditioned bait aversion' (bait shyness), preventing the consumption of a lethal dose (Lund, 1972; Buckle, 1994). Rodents are

intelligent pests and can link cause (the bait) with effect (the induced symptoms) therefore sub-lethally dosed animals may develop symptoms of poisoning and link the cause with effect and stop eating the bait. It may be possible to overcome bait shyness by pre-baiting the location (Prescott, 2011). A non-toxic food is used as a pre-bait (ideally using the same formulation base as the rodenticide that will be used) and once the rodents become accustomed to feeding at the bait point the food is replaced with the rodenticide. Rodents, especially rats, are also neophobic and are shy of new objects in their environment. This can cause a delay in them consuming bait and can also cause an increase in the number of individuals receiving a sub-lethal dose of acute rodenticides (Prescott, 2011). The introduction of anticoagulant rodenticides overcame the problem of 'conditioned bait aversion' due to the delay between consuming the anticoagulant bait and the onset of symptoms by which time rodents have consumed a lethal dose.

1.4.1 Acute rodenticides

Zinc phosphide and sodium fluoroacetate (commonly known as 1080) are both examples of acute rodenticides. Zinc phosphide is a commonly used acute rodenticide and is available in ready-to-use formulations in the USA (Buckle & Eason, 2015). Zinc phosphide works by reacting with the acid in the stomach of rodents and producing phosphine gas. The gas then enters the bloodstream damaging internal organs and causing heart failure (Buckle & Eason, 2015). The highly toxic compound 1080 (sodium fluoroacetate) is also used to control rabbits, possums and wallabies in Australia. It works by blocking the tricarboxylic acid cycle leading to a build-up of citric acid which causes convulsions and circulatory failure (Buckle & Eason, 2015). Other important acute rodenticides in the control of House mice are alphacholorose and cholecalciferol. Alphacholorose is a narcotic which works by slowing metabolic processes including respiration, heart rate and brain activity. This leads to hypothermia and eventual

death (Buckle, 1994). Cholecalciferol is a subacute rodenticide, a lethal dose may be eaten in the first 24 hours but death may not occur as rapidly as with other acute rodenticides. Cholecalciferol works by causing the absorption of calcium in the intestines leading to hypercalcaemia (Buckle, 1994). There are no antidotes for acute rodenticides, and due to their speed of action, it would be very difficult to administer an antidote in time, so secondary non-target species may be lethally poisoned (Meerburg *et al,* 2008).

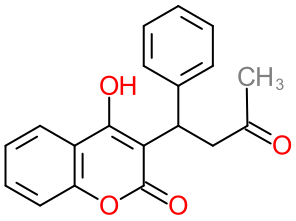
1.4.2 Anticoagulant rodenticides

Anticoagulant rodenticides such as warfarin, coumatetralyl and bromadiolone are chronic rodenticides and were first developed in the early 1950s (RRAG, 2010), and are now the most common means of rat and mouse control in the USA and UK (Mason & Littin, 2003). Tomlinson *et al* (2017) reported that almost 40% of farms surveyed in a UK study used anticoagulant rodenticides all year round to control rodents, and the majority of these farms were mixed farms which had a lot of food stores and harbourage for rodents.

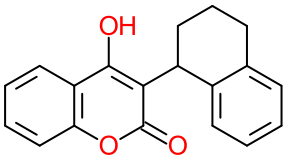
Dicumarol, the precursor to warfarin, was first identified in the USA in the 1930s as the cause of haemorrhagic fever in cattle. Clover contains coumarin, the precursor of dicumarol, and coumarin is converted to dicumarol by fermentation when clover fodder is not stored properly making the clover toxic (Lefebvre *et al*, 2017). Dicumarol and its synthetic derivatives were researched as potential treatments for human thrombosis, warfarin was identified as the most active and its potential as a rodenticide was recognised in the 1940s (Buckle, 1994). Anticoagulants are slow acting so rodents do not associate cause with effect which allows rodents to ingest a lethal dose before the first symptoms of illness (Bentley, 1972). The minimum time to death is two to three days and on average, death occurs within five to seven

days (HSE, 2004). Symptoms of anticoagulant toxicity such as laboured respiratory activity, inactivity and anorexia may be observed for several days before death occurs (Tomlinson *et* al, 2017). Anticoagulants have a cumulative mode of action in that they are not toxic enough to cause mortality after a single dose and there needs to be continuous access for successful rodent control (Buckle, 1994). Complete control relies on all aspects of rodenticide treatment being conducted correctly: baits need to be placed correctly, rodents must feed on the baits daily for several weeks and the baits need to be visited and replenished frequently (RRAG, 2010).

Anticoagulants are known as either first-generation anticoagulant rodenticides (FGARs) or second-generation anticoagulant rodenticides (SGARs) depending when they were developed (Fisher, 2005). The second-generation anticoagulants are also known as superwarfarins. Warfarin, coumatetralyl, diphacinone and chlorophacinone are examples of FGARs whereas difenacoum, bromadiolone, brodifacoum, flocoumafen and difethialone are SGARs. First-generation anticoagulants are either 4-hydroxycoumarin derivatives with a coumarin core (Figure 1.2) or indane-dione derivatives with a 1,3-indandione core (Lefebvre *et al*, 2017) (Figure 1.3). Warfarin and coumatetralyl are coumarins, and chlorophacinone and diphacinone are indane-diones and rodents were found to be highly susceptible to them, although the rodents needed to feed on them over several days to achieve lethality (Buckle, 1994). The SGARs are 4-hydroxycoumarin derivatives (Figure 1.4) and have a coumarin core (Lefebvre *et al*, 2017), with the exception of difethialone, which is identical to brodifacoum but with the oxygen atom of the 4-hydroxycoumarin structure replaced by a sulphur atom (Figure 1.5) (Buckle, 1994), this is known as a thiocoumarin core (Lefebvre *et al*, 2017). The SGARs contain three benzene structures in the radical which increases their fat solubility (Lefebvre *et al*, 2017).

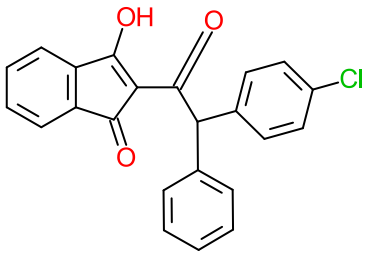


Warfarin

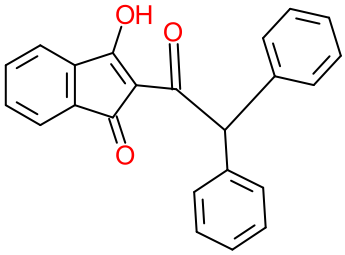


Coumatetralyl

Figure 1.2: Chemical structures of first-generation 4-hydroxycoumarin derivatives warfarin and coumatetralyl.

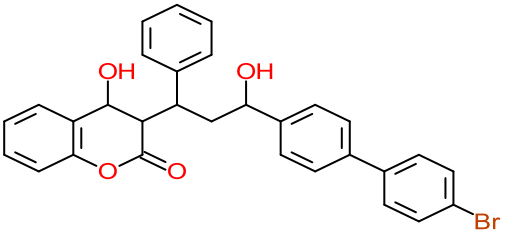


Chlorophacinone

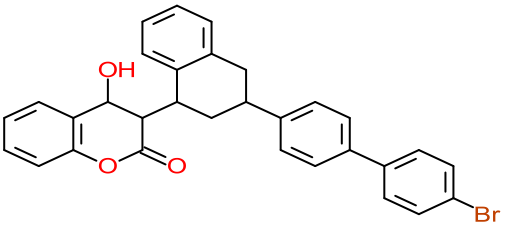


Diphacinone

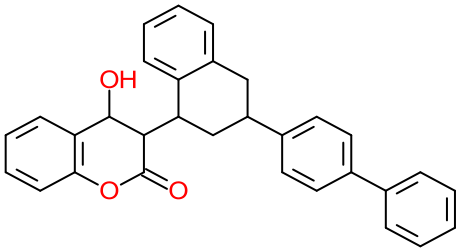
Figure 1.3: Chemical structures of the first-generation indane-dione derivatives chlorophacinone and diphacinone



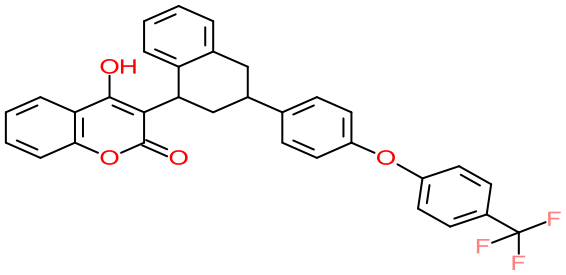
Bromadiolone



Brodifacoum

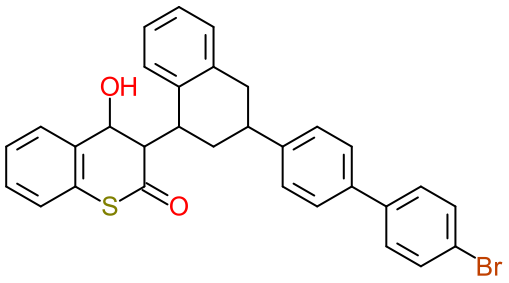


Difenacoum



Flocoumafen

Figure 1.4: Chemical structures of the second-generation 4-hydroxycoumarin derivatives bromadiolone, brodifacoum, difenacoum, flocoumafen.



Difethialone

Figure 1.5: Chemical structures of the second-generation anticoagulant difethialone.

The SGARs are much more potent than the FGARs due to their higher binding affinity in the liver and claims have been made that they achieve lethality following a single feed (Fisher, 2005). This contrasts with FGARS, rodents need to feed for several days on FGARs for toxicity to occur (Smith & Greaves, 1986). Due to anticoagulants being slow-acting, there is a delay before coagulation is compromised (Prescott, 2011). Consequently, bait shyness does not occur and the rodents continue to feed until a lethal dose has been consumed (HSE, 2004). The length of time of any suffering depends on the dose consumed, the active ingredient and the site(s) of haemorrhage (as bleeding does not cause pain but the accumulation of blood in joints is painful), and symptoms can last several days (Mason & Littin, 2003). Another important advantage of all anticoagulants is that there is a complete antidote in vitamin K_1 (phylloquinone) and blood products if administered in time (Bentley, 1972; Mason & Littin, 2003).

1.5 The vitamin K cycle

Vertebrates require the micro-nutrient vitamin K to maintain blood clotting activity. This requirement is normally met by microbial synthesis in the digestive system, dietary intake of vitamin K and by recycling via the vitamin K cycle (Witmer & Burke, 2009). Indeed, one vitamin K molecule may be recycled 100-1000 times (Thijssen, 1995). Blood coagulation factors II (prothrombin), VII, IX and X are all vitamin K dependent (Stafford, 2005,) and without vitamin K recycling they would not be synthesised.

The vitamin K cycle (Figure 1.6) involves the vitamin K-dependent carboxylation of glutamate residues to gamma-carboxyglutamate residues in vitamin K-dependent proteins (Pelz & Prescott, 2015; Tie & Stafford, 2015). The gamma-carboxyglutamate residues are calcium binding and produce active clotting factors (Pelz & Prescott, 2015), which bind to membrane surfaces where damage has occurred (Tie & Stafford, 2015). Dietary vitamin K levels are generally low but deficiency is rare due to the recycling of vitamin K (Lefebvre *et al,* 2016). The vitamin K cycle occurs in the endoplasmic reticulum of hepatic cells where vitamin K is metabolised slowly. Vitamin K is also metabolised in the mitochondrion quickly (Sutcliffe *et al*, 1987). Vitamin K recycling in the endoplasmic reticulum is inhibited by anticoagulants, whilst metabolism in the mitochondria continues, resulting in depletion of vitamin K (Sutcliffe *et al*, 1987). However, large doses of vitamin K can be utilised in the mitochondria and overcome the action of anticoagulants (Meehan, 1984).

The vitamin K cycle is catalysed by the enzymes vitamin K epoxide reductase (*VKORC1*), vitamin K reductase (VKR) and gamma-glutamyl carboxylase (GGCX) (Tie & Stafford, 2015). Vitamin K reductase enzymes are responsible for the reduction steps in the vitamin K cycle. Vitamin K epoxide reductase reduces vitamin K 2,3-epoxide (KO) to vitamin K and VKR reduces

vitamin K to vitamin K hydroquinone (KH_2) (Tie & Stafford, 2015). The reduction of KO to vitamin K has been found to be approximately 50 times faster than the reduction of vitamin K to KH_2 (Tie *et al*, 2011). Vitamin K epoxide reductase is located in the endoplasmic reticulum and is believed to have 3 to 4 alpha-helical transmembrane segments (Ishizuka *et* al, 2008). Gamma-glutamyl carboxylase (GGCX) catalyses carboxylation and depends on oxygen, carbon dioxide and KH_2, the reduced form of vitamin K (Tie & Stafford, 2015; Whitlon *et al*, 1978). The carboxylase, GGCX carboxylates glutamate residues (Glu) of vitamin K dependent proteins (VKDP) to carboxyglutamic acid (Gla) (Lefebvre *et al*, 2016) at the same time as KH_2 is oxidised to KO to provide energy for carboxylation (Tie & Stafford, 2015). The Gla residues bind calcium (Lefebvre *et al*, 2016) which promotes the formation of clotting factors binding on membrane surfaces (Tie & Stafford, 2015), sealing wounds with an insoluble fibrin clot (Bishop, 1981). Gamma-glutamyl carboxylase has multiple active sites that are used during carboxylation. There is a propeptide binding site which binds the VKDP, during the binding of the propeptide GGCX reorientates so the Gla of the VKDP is positioned near the carboxylation site on GGCX (Tie & Stafford, 2015).

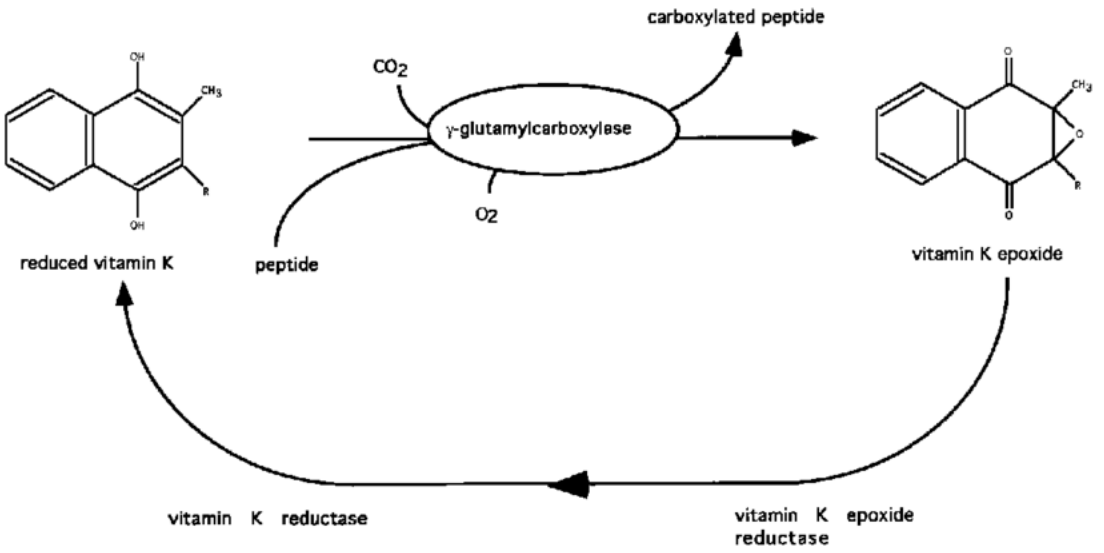


Figure 1.6: The vitamin K cycle. Anticoagulants inhibit the activity of vitamin K reductase enzymes therefore disrupting the recycling of vitamin K (Chu *et al*, 1996).

There are similarities between the chemical structures of the anticoagulants and vitamin K. It is thought that vitamin K binds to and is released from the *VKORC1* rapidly as it is converted from the epoxide to the hydroquinone. The anticoagulants bind more persistently, with a much longer half-life of elimination. Gebauer (2007) demonstrated that deprotonated warfarin binds to the active sites of *VKORC1* in a similar manner to vitamin K. It is further believed that the toxicity of the anticoagulant is directly related to the *VKORC1* half-life of elimination, with second-generation anticoagulants having a longer half-life of elimination than first-generation anticoagulants (Thijssen, 1995; Ishizuka *et al*, 2008). The coagulation time of blood in rats and mice is compromised by anticoagulants within 24 hours (Valchev, 2008), but typically it takes between 3 and 12 days for lethality to occur. The lag in time between exposure to anticoagulant rodenticides and impaired blood clotting being detected is because the half-life of functional clotting factors ranges between 6 and 120 hours and these will continue to support haemostasis until they are depleted (Rattner *et* al, 2014). An important result of this delay is that rodents do not associate their symptoms with the consumption of the anticoagulant bait, and thus conditioned bait aversion does not occur (Buckle, 1994). The dose required to inhibit *VKORC1* varies between anticoagulants, but once *VKORC1* is inhibited, there is no difference in time to death. Moreover, the locations of lethal haemorrhages are the same, because anticoagulants all have a common mode of action (Hadler & Buckle, 1992).

After the administration of dietary vitamin K_1, it is reduced to vitamin K hydroquinone by a low affinity quinone reductase that is located in the cytoplasm and the carboxylation of clotting factors is restored (Rattner *et al,* 2014) (Figure 1.7), this allows blood clotting to be maintained and therefore acts as an antidote in cases of accidental poisoning (Buckle & Eason, 2015). Rodents which have a degree of anticoagulant resistance require more dietary vitamin K than

susceptible rodents as they do not bind vitamin K and anticoagulants with the same affinity due to the structure change of *VKORC1* (Meehan, 1984).

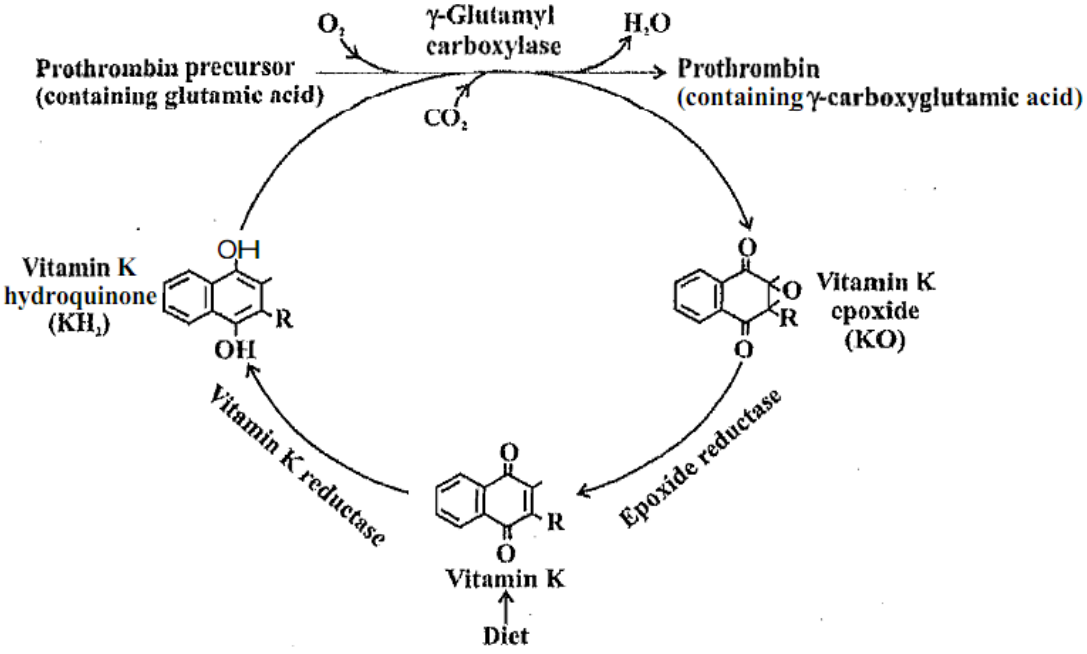


Figure 1.7: Action of dietary vitamin K as a cofactor for the carboxylation of glutamate residues to enable the vitamin K cycle to continue (adapted from ExpertsMind, 2013).

1.6 Anticoagulant resistance

Warfarin was the first anticoagulant to become available in the 1950s, and then (within a few years) diphacinone, coumatetralyl and chlorophacinone were available in the UK (RRAG, 2010). As discussed above, these compounds are (FGARs). Chlorophacinone and diphacinone were found to be good alternatives to warfarin as they were more toxic to Norway rats and had a similar potency to House mice. Coumatetralyl is the most toxic of the FGARs (Prescott *et al,* 2007).

Resistance in Norway rats was first reported in 1958 in Scotland during a routine field trial (Pelz *et al,* 2005; RRAG, 2010), resistant rodents can survive doses that would kill most

susceptible individuals in the population (Fisher, 2005). Survivors from the field trial who had been exposed to diphacinone for 30 days then warfarin for a following 18 days were taken into the laboratory to undergo feeding tests and a survival rate of almost 90% was observed (RRAG, 2010). 'Warfarin resistant' House mice in the UK were first reported in 1961 by Dodsworth (Pelz *et al*, 2005; Rowe & Redfern, 1965); some of these mice were trapped and fed warfarin for 21 days. Survivors of the 21-day feeding test were then fed diphacinone and chlorophacinone and were also found to be resistant to these compounds (Buckle & Prescott, 2012).

The 'Cambridge Cream' House mice are a population of mice found around Cambridge which were discovered after the initial populations of 'warfarin resistant' House mice were identified (Buckle & Prescott, 2012). The unique coat colour of the 'Cambridge Creams' appears to be linked to the gene for warfarin resistance as 'Cambridge Creams' were also found to be warfarin resistant (RRAG, 2012). Genetic investigation identified the locus for warfarin resistance in House mice is located on chromosome 7 and closely linked to coat colour, previous research in Norway rats had shown that the locus for warfarin resistance was linked to the coat colour gene; this may suggest that the mechanism of warfarin resistance is similar in House mice and Norway rats (MacNicoll, 1995). Early resistance work also indicated that female House mice were more resistance than males, suggesting resistance is influenced by sex (Berny *et* al, 2018). Wild House mice trapped around Reading were found to be resistant to warfarin and bromadiolone (Prescott, 1996). There appeared to be a degree a cross-resistance where House mice resistant to first-generation compounds also expressed a degree of resistance to second-generation compounds (Berny *et al*, 2018). It is unsurprising that resistance to anticoagulants occurred due to their extensive use at the exclusion of other rodenticides after their introduction (Bishop, 1981), as there was a selective advantage to rodents that were resistant (Smith & Greaves, 1986).

Early resistance tests in the laboratory made it difficult to distinguish between animals which were poor feeders and those that were truly resistant. Wild caught rodents do not readily feed or feed naturally in a laboratory environment therefore in any feeding tests it was possible that they either did not consume any bait or did not consume the equivalent of a lethal dose. Some rodents which survived feeding tests and were distinguished as resistant, only did so by not consuming enough bait. House mice are also sporadic feeders; they do not feed consistently from a single food source (Meehan, 1984), so it made early feeding tests more likely to be unsuccessful

In 1994 the following definition of practical anticoagulant resistance was proposed by Dr John Greaves.

"Anticoagulant resistance is a major loss of efficacy in practical conditions where the anticoagulant has been applied correctly, the loss of efficacy being due to the presence of a strain of rodent with a heritable and commensurably reduced sensitivity to the anticoagulant" (Greaves, 1994).

This definition means that although anticoagulant rodenticides are applied according to their guidelines, they are not working against resistant strains of rodents and this resistance is a heritable trait that has a practical effect. Resistant individuals are selected by the continued application of anticoagulant rodenticides and the trait spreads through the local population due to the movement of resistant individuals (Bishop, 1981). Susceptible animals are killed off allowing the surviving resistant animals to pass on their resistance genes to their offspring in a Mendelian fashion (Fisher, 2005; Prescott, 2011). Throughout the UK, warfarin resistant

populations of rodents have been documented (Merrburg *et al,* 2008). Bailey and Eason (2000) stated that anticoagulant resistance can develop in a rodent population after 5-10 years of continuous use of anticoagulant rodenticides. Bell and Caldwell (1973) suggested that in resistant strains of rodents, the conversion of inactive vitamin K epoxide to hydroquinone is not blocked by anticoagulants, and therefore the synthesis of blood coagulation factors is not prevented. Since the publication of the paper by Bell and Caldwell further research has found that anticoagulants do block the vitamin K cycle in resistant rodents but they do not bind for long enough to have a lethal effect. Li *et al* (2010) produced a crystal structure of *VKORC1* which identified that Leucine128 and Tyrosine139 are part of the structure which binds either vitamin K or warfarin. Any amino acid substitution at these sites, which is seen in resistant rodents, will have an effect on the binding of vitamin K or inhibitors of *VKORC1* such as anticoagulants.

Table 1.2 outlines the timeline of rodenticide resistance. Norway rats and House mice resistant to first-generation anticoagulants in the UK and USA have been identified since the late 1950s (Lund, 1984). House mice in Holland, Sweden, Belgium, Finland and Canada are also found to be resistant to first-generation anticoagulants (Lund, 1984). Resistance to anticoagulants has only so far been identified where rodenticide treatment using anticoagulants has failed (Lefebvre *et* al, 2016). There is variability in natural susceptibility and slight sex-specific differences, House mice are naturally more tolerant to anticoagulants than Norway rats, and have a naturally low susceptibility to first-generation anticoagulants (Greaves, 1994; Syngenta, 2004). The evolution of resistance has been slightly different in Norway rats and House mice mainly due to their differences in feeding behaviour: Rats mainly eat from one food source so consume a lot of anticoagulant bait whereas mice eat from many food sources therefore diluting the bait down with other food sources (Lefebvre *et al*, 2016). Tolerance is due to genetic variability of populations, some populations are more tolerant as

they have been subjected to sustained anticoagulant rodenticide treatment in comparison to rodenticide naïve populations (Bailey & Eason, 2000). Greaves (1995), and Bailey and Eason (2000) stated that a random genetic event that causes a strong selection of individuals results in resistance but Greaves (1995) stated that resistance has become a practical problem due to human activity, the continued use of ineffective anticoagulant rodenticides.

Table 1.2: The evolution of rodenticide resistance (adapted from RRAG, 2010)

Evolution of rodenticide resistance
Early 1950s
Chronic anticoagulants first developed (warfarin, chlorophacinone, diphacinone, coumatetralyl)
Late 1950s
Resistance to warfarin and other FGARs identified in some populations of Norway rats and House mice.
1958
First documented case of resistance to diphacinone and warfarin recorded in Norway rats in Scotland.
1960s
Massive use of FGARs to try to eradicate Norway rats.
Resistance identified in Norway rats in Anglo/Welsh border, Kent/Sussex border, and Hampshire.
Genetic resistance recognised and studied in House mice but still not well understood. 'Cambridge Cream' strain identified and found to carry leucine128serine mutation (L128S).
Early 1970s
SGARs developed
Difenacoum marketed as effective against resistant populations of Norway rats and House mice
Bromadiolone developed and marketed as effective
Mid 1970s
Norway rats in central southern England resistant to difenacoum and bromadiolone
Resistance also recorded in Scandinavia

1.7 Second-generation anticoagulants

Second-generation anticoagulant rodenticides (SGARs), sometimes referred to as superwarfarins, were first developed in the early 1970s following the evolution of resistance to warfarin (Fisher, 2005). Anticoagulant rodenticides are synthetic compounds which have a similar structure to coumarin, the compound in sweet clover that causes haemorrhagic fever in cattle (Eason & Wickstrom, 2001). They were developed to be more potent than the first-generation compounds and to be effective against resistant populations of rats and mice (RRAG, 2010). Difenacoum and bromadiolone were both marketed as being effective against resistant populations of Norway rats and House mice (RRAG, 2010). As they are licensed in the UK for use both indoors and outdoors, difenacoum and bromadiolone are the most commonly used SGARs in Britain (Shore *et al*, 2003). Brodifacoum and flocoumafen are the most potent of the SGARs and House mice are most susceptible to them (Fisher, 2005) with a dose of 1mg/kg being usually effective against resistant strains of rodents (Eason & Wickstrom, 2001). The SGARs are more potent due to their higher binding affinity to specific binding sites on the enzyme vitamin K-epoxide reductase, which is predominantly located in the liver, kidneys and pancreas (Eason & Wickstrom, 2001; Thijssen, 1995). The SGARs are absorbed through the gastrointestinal tract and accumulate and persist in the organs where vitamin K-epoxide reductase is located (Eason & Wickstrom, 2001). Second-generation anticoagulant rodenticides are not metabolised rapidly and have a long half-life in the liver and kidneys. This poses a risk of secondary poisoning to non-target species that may feed on poisoned rodents (Fisher, 2005; Merrburg *et al,* 2008) and they may be recirculated via the biliary tract (Eason & Wickstrom, 2001).

Prior to 2017 brodifacoum and flocoumafen were only licensed for use indoors or around buildings, after 2017 SGARs could be used outdoors but under strict licencing. In field trials of

brodifacoum and flocoumafen against wild House mice average control levels of 98.8% and 97.2%, respectively were recorded and these compounds have the added advantage that only small quantities are required to achieve lethality (Buckle & Prescott, 2012). Although there have been occasional reports of House mice resistant to FGARs having an increased level of tolerance to brodifacoum (Greaves, 1994).

Resistance to difenacoum and bromadiolone was reported in the UK and Scandinavia soon after these compounds were first marketed and an increasing tolerance to brodifacoum was also recorded (Lund, 1984). Difenacoum and bromadiolone have been found to not give complete control against House mice resistant to warfarin, this may suggest that resistance to difenacoum and bromadiolone was present before their widespread use (RRAG, 2012). By 1980 a large population of Norway rats resistant to difenacoum was identified in Hampshire, South England. Individuals from this population were trapped and 85% of these animals were identified as 'warfarin resistant' with 14% 'difenacoum resistant' (Lund, 1984). Rodent populations that were resistant to FGARs were found to have a degree of cross-resistance to certain SGARs and were less susceptible to them (Buckle *et al*, 1994).

Rowe *et al* (1981) reported that House mice from a suspected 'difenacoum resistant' population in the UK survived a 21-day feeding test consuming up to 147mg/kg of 0.005% difenacoum. In laboratory tests of UK House mice, Rowe *et al* (1981) reported only 57% mortality during a 21-day feeding test on 0.005% bromadiolone, and it is still found that control of House mice is more difficult to achieve using bromadiolone (RRAG, 2012).

Some populations of rodents may be highly resistant therefore making them difficult to control, whereas other populations may contain individuals of varying degrees of resistance

(FERA, 2002). As with the first-generation compounds, the continued use and reliance on potentially ineffective SGARs creates a selection pressure on rodent populations, with only the most resistant individuals surviving to breed. To date, there is no convincing evidence of practical resistance to brodifacoum or flocoumafen (RRAG, 2012). Practical resistance is where the level of resistance in a population to an anticoagulant is of such a level that use of that anticoagulant is unlikely to achieve control of that rodent population (Pelz & Prescott, 2015).

1.8 Genetic resistance

Genetic resistance to anticoagulants has been recognised and studied since the 1960s in House mice. It is now widespread throughout the UK (Buckle & Prescott, 2012; RRAG, 2012) and a study by the European and Mediterranean Plant Protection Organisation (EPPO) found that House mice resistant to anticoagulants were also widespread throughout Europe (Berny *et al*, 2018). However, House mice also have a natural level of tolerance to anticoagulants (Buckle & Prescott, 2012). Recent studies in Germany found that over 90% of the House mice tested carried genetic resistance mutations (RRAG, 2012). The research in Germany is of significance to the UK as the resistant strains of House mice found in Germany are also present in the UK. A study by Goulois *et al* (2017) in which House mice were trapped at 65 locations throughout France identified *VKORC1* genetic mutations in over 70% of the mice and 80% of these mice were homozygous for the mutations. Nine single mutations were identified, which included those found in the UK, and were associated with resistance to all first-generation anticoagulant active ingredients (Goulois *et al*, 2017). Even though genetic resistance in House mice has been known since the 1960s, it is not well understood. It was thought to be similar to the resistance found in Norway rats in that a single gene was involved, but breeding tests

have shown that there may be several genes and several different resistance mechanisms involved (RRAG, 2012).

Two genetic mutations on chromosome 7 (Pelz & Prescott, 2015) have been identified in House mice in the UK: the 'Cambridge Cream' resistant strain and another strain found in the 1990s around Reading (RRAG, 2012), the 'Reading' strain. The 'Cambridge Cream' strain was studied in a lot of early mouse resistance work and it has been found to be homozygous for the leucine128serine mutation (L128S), where the wild-type amino acid leucine at location 128 is replaced by serine, which confers resistance to warfarin. It is thought this mutation is widespread in the UK and it is known to occur widely in Germany (Buckle & Prescott, 2012). The resistance mutations identified in UK House mice confer resistance to first-generation compounds and the second-generation compounds bromadiolone and difenacoum (Berny *et al*, 2018). The 'Reading' House mouse strain is homozygous for the tyrosine139cysteine (Y139C) mutation, tyrosine is replaced by cysteine at location 139. This mutation has also been identified in Germany (RRAG, 2012) and was also recently found in the Azores (Rost *et al,* 2009). Mice homozygous for the Y139C mutation are fully resistant to first-generation anticoagulants and to bromadiolone and they have a degree of resistance to difenacoum (RRAG, 2012; Buckle & Prescott, 2012). Studies reported by Pelz *et al* in 2005 demonstrated that resistance to warfarin is linked to mutations at tyrosine139 and other mutations were linked to reduced vitamin K epoxide reductase (*VKORC1*) activity (Pelz *et al,* 2005). From studies at the University of Reading using the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C, brodifacoum has been found to be effective against this strain of House mouse (RRAG, 2012). The *VKORC1* genetic mutation Y139C was found in a wild population of House mice trapped around Reading in the 1990s. The gene was transferred through six generations onto a susceptible strain of Swiss mouse (Prescott, 1996; Pelz *et al,* 2005).

The numbered codons in Table 1.3 relate to the location of the genes conferring genetic resistance. A change in nucleotide sequence at these codons changes the amino acid that is produced. This in turn alters the enzyme these amino acids are part of and may confer resistance (Syngenta, 2004). This type of mutation that leads to amino acid mutations is known as missense mutations. Although there may be some other mutations that cause resistance other than those shown in Table 1.3, Norway rats from Hampshire and Berkshire have the same mutation at codon 120 but Hampshire rats are susceptible to bromadiolone whereas Berkshire rats are not (Syngenta, 2004).

Table 1.3: The genetics of resistance in Norway rats and House mice (Syngenta, 2004)

Strain/Area	Codon Position	Wild-type codon	Mutated codon	Wild-type amino-acid	Mutated amino-acid
R. norvegicus					
UK, Hampshire	120	CTG	CAG	Leucine	Glutamine
UK, Berkshire	120	CTG	CAG	Leucine	Glutamine
UK, Scotland	128	CTG	CAG	Leucine	Glutamine
UK, Wales	139	TAT	TCT	Tyrosine	Serine
UK, Yorkshire	139	TAT	TGT	Tyrosine	Cysteine
UK, Yorkshire	128	CTG	CAG	Leucine	Glutamine
Denmark, wild	139	TAT	TGT	Tyrosine	Cysteine
Belgium, wild	139	TAT	TTT	Tyrosine	Phenylalanine
France, wild	139	TAT	TTT	Tyrosine	Phenylalanine
France, wild	35	CCG	CCC	Arginine	Proline
Germany, wild	139	TAT	TGT	Tyrosine	Cysteine
Germany, wild	56	TCC	CCC	Serine	Pro
M. musculus					
UK, CSL	128	TTA	TCA	Leucine	Serine
UK, Reading	139	TAT	TGT	Tyrosine	Cysteine

Vitamin K epoxide reductase complex subunit 1 (*VKORC1*) is a protein molecule located in the endoplasmic reticulum, with 3 to 4 α-helical transmembrane segments, that contributes to the activity of the reductase enzymes (Ishizuka *et al*, 2008). Vitamin K epoxide reductase is targeted by anticoagulants and the gene encoding this protein affects the synthesis of blood clotting factors (Pelz *et al,* 2007). Studies have shown that anticoagulant resistance in Norway rats and House mice is linked to single nucleotide polymorphisms (SNPs) in the coding region

of *VKORC1*(Diaz *et al,* 2010). It has been reported that the 139 mutation causes a change in the structure of *VKORC1* which prevents warfarin from blocking reductase enzymes in the vitamin K cycle (Ishizuka *et al*, 2008).

Single nucleotide polymorphisms in the gene which codes for *VKORC1* are responsible for anticoagulant resistance. The *VKORC1* gene was initially identified on chromosome 1 in rats in 1969, in 1976 it was identified on chromosome 7 in mice , and it was identified on chromosome 16 in humans in 2002 (Lefebvre *et al*, 2016). The vitamin K epoxide reductase subunit 1 gene is a transmembrane protein 163 amino acids long, (Goulois *et* al, 2017) in the endoplasmic reticulum and contributes to the activity of *VKORC1*; one section of *VKORC1* catalyses vitamin K epoxide reduction and another section is crucial in the binding of coumarins (Ishizuka *et* al, 2008). The mutation causes a change in the conformation of *VKORC1* which prevents anticoagulants binding and blocking the vitamin K cycle (Ishizuka *et al*, 2008), anticoagulants have a high affinity for *VKORC1* (Eason & Wickstrom, 2001). In susceptible individuals anticoagulants interfere with the vitamin K cycle in the liver by targeting and non-competitively binding with the vitamin K epoxide reductase enzyme (*VKORC1*) (Lefebvre *et al*, 2016) (Figure 1.6), and thereby inhibiting the reduction of vitamin K epoxide to hydroquinone causing an accumulation of vitamin K epoxide (Meehan, 1984). This leads to under-carboxylation of vitamin K dependent proteins, and thus prevents the activation of certain blood clotting factors – Factors II, VII, IX and X (Buckle, 1994; Gebauer, 2007; Rattner *et al,* 2014; Rost *et al,* 2009). Susceptible animals that consume anticoagulants succumb to lethal internal haemorrhage (Diaz *et al,* 2010) as fibrin is not produced (Bishop, 1981). Most haemorrhages occur in the thoracic cavity, sub cutaneous tissues, stomach and intestine (Eason & Wickstrom, 2001).

Song *et al* reported in 2011 that polymorphisms in *VKORC1* cause anticoagulant resistance, and at least 4 of the 10 polymorphisms (A12T, A26S, A48T and A61L) found in House mice may have been introduced through interspecific mating with the Algerian mouse (*Mus spretus*). The initial interspecific mating of these two species may have occurred where the two species cohabited in North Africa and Spain (Goulois *et* al, 2016). The Algerian mouse has a much higher natural tolerance to anticoagulants than wild type animals. It was found that House mice in Germany and Spain carry the spretus mutation from the Algerian mouse; most House mice in Spain carry this mutation. Mice in Germany that could not be controlled with bromadiolone were analysed and found to carry the spretus mutation; 20% of mice carrying this mutation survived treatment with difenacoum whereas all wild type mice died, wild type mice also had higher mortality to bromadiolone (Song *et al*, 2011). First-generation anticoagulants and the second-generation compounds bromadiolone and difenacoum are ineffective in the control of House mice carrying the spretus mutation (Goulois *et* al, 2016). To date the spretus mutation has not been identified in House mice in the UK. In total there are 15 mutations that have been detected in House mice in Europe (Goulois *et* al, 2017). Double mutations, where House mice carry two resistance mutations of the *VKORC1* gene, have been identified in a study by Goulois *et al* (2017). Double mutations are associated with severe resistance to all anticoagulant rodenticides. These genetic mutations may have already been present in the rodent population before the use of anticoagulants as rodenticides, as the development of resistance has been rapid and use of anticoagulants has selected for these mutations and increased their prevalence (Lefebvre *et al*, 2016).

1.9 The influence of sex and selected breeding on anticoagulant resistance

Numerous studies in both Norway rats and House mice have indicated that females are more tolerant to anticoagulants than males. Greaves and Cullen-Ayres (1988) reported that

mortality was significantly greater in male Norway rats than females throughout a breeding test. They bred a line selected for resistance to difenacoum, although the level of resistance increased in both males and females throughout the generations females were always less susceptible to difenacoum than males (Greaves & Cullen-Ayres, 1988).

Female House mice also have a higher level of tolerance to anticoagulants than males. Studies by Prescott (1996) found that female House mice were more likely to be homozygous resistant than male House mice in that they had a double copy of the resistance gene whereas resistant males were more likely to be heterozygous. This resulted in male offspring having a higher level of mortality in test crosses than females (Prescott, 1996). Results similar to those reported by Prescott (1996) were reported by Scepovic *et al* (2015), females were more resistant than males and had a higher intake of bromadiolone and therefore a higher tolerance than males, and they also lost less weight than males. Female Norway rats have been reported to have higher basal rates of activity of clotting factors VII and X and longer half-lives of clotting factors than males (Lefebvre *et al*, 2016) , and this may explain why females are found to be more tolerant of anticoagulants even when *VKORC1* is completely inhibited blood coagulation continues for longer in females.

The results reported by Scepovic *et* al (2015) were from wild caught House mice and indicates the level of resistance and tolerance to anticoagulants found in the wild population. The results of the breeding test by Greaves and Cullen-Ayres (1988) and the feeding tests by Scepovic *et al* (2015) put into context the issue of continued use of ineffective anticoagulants against resistant populations of rodents; the susceptible and some heterozygous resistant individuals succumb to the anticoagulant treatment whereas the homozygous resistant and

some heterozygous resistant individuals survive to produce the next generation which is likely to be more resistant than previous generations.

1.10 Impacts of anticoagulant rodenticides on the environment and non-target species

At least 95% of the rodenticides used most commonly are believed to be anticoagulants (Hernandez-Moreno *et* al, 2013). Anticoagulants are highly toxic, persistent chemicals making them a risk to the environment and non-target species. Second-generation anticoagulant rodenticides (SGARs) are more persistent than the first-generation anticoagulant rodenticides (FGARs) due to their long half-life and FGARs are metabolised and excreted rapidly in comparison to SGARs (Feinstein *et al*, 2016; Fisher, 2005). The increased half-life of SGARs is due to their increased hydrophobicity which is caused by the addition of a substituted phenyl ring in the structure compared to the structure of warfarin. This causes increased tissue accumulation and retention, especially in the liver (Feinstein *et al*, 2016), kidneys and pancreas (Crowell *et al*, 2013). Studies have shown the mean half-life for warfarin to be 15-58 hours whereas for the SGAR flocoumafen it can be more than 200 days (Sanchez-Barbudo *et al*, 2012). Anticoagulants are absorbed through the gastrointestinal tract and accumulate in the liver, kidneys, lungs, brains, fat and muscles of rodents after consumption and are eliminated via faeces and urine (Crowell *et* al, 2013). Elimination of anticoagulants from the liver is biphasic. There is a rapid initial phase which can last up to a week and then a longer terminal phase that can last months (Shore *et al*, 1999) which means the anticoagulants can persist in the environment for a long period after their initial use potentially poisoning numerous non-target individuals.

Non-target species may be exposed to anticoagulants by either primary or secondary routes of exposure. Primary exposure is when a non-target eats the rodenticide directly and secondary exposure is by eating contaminated prey. Non-targets may experience both primary and secondary exposure (Shore *et al*, 2015). Exposure depends on the feeding behaviour and environment of non-targets. Anticoagulant residues in non-targets may arise from them feeding on organisms exposed to baits or by eating baits themselves (Pitt *et al*, 2015). Scavengers found around human settlements are most at risk of exposure (Ntampakis & Carter, 2005; Shore *et al, 2015*) as they are more likely to come into direct contact with anticoagulants or into contact with prey that has been exposed. The repeated use of anticoagulant rodenticides can lead to bioaccumulation of anticoagulants in rodents which have only ingested a sub lethal dose (Crowell *et* al, 2013). Invertebrates and granivorous vertebrates are most at risk from primary exposure (Shore *et al*, 2015). Invertebrates can consume the anticoagulant baits directly or via rodent faeces, carcases or soil-bound residues (Shore *et al*, 2015). Due to the difference in blood-clotting mechanisms compared to mammals, invertebrates can retain anticoagulant residues in their bodies for longer than 4 weeks possibly leading to the secondary exposure of insectivorous animals and birds (Hernandez-Moreno *et al*, 2013). Small granivorous vertebrates such as wood mice are highly mobile, some moving more than one kilometre in a night which may include several different farms (Tosh *et al*, 2012) and may act as vectors, carrying residues away from the initial site of baiting (Shore *et al*, 2015). Predators and scavengers readily experience secondary exposure. Studies by Sanchez-Barbudo *et al* in 2012 concluded that granivorous birds showed the highest level of primary exposure whereas nocturnal raptors and carnivorous mammals showed the highest level of secondary exposure. Secondary exposure was first considered in the 1960s in the UK but did not come to prominence until the 1980s during a long-term study of barn owl carcasses, then secondary poisoning of mustelids was studied in the 1990s (Shore *et al*, 1999). Barn owls and kestrels very rarely eat commensal rodent species so they are most likely to be

exposed to anticoagulants via non-target small mammals such as wood mice and voles (Tosh *et* al, 2012). Predators and scavengers of target rodent species are the most common non-target species, although species that do not feed on rodents, such as insectivorous birds and other mammals have also been found to have been poisoned (Shore *et al*, 2015).

Elliot *et al* (2014) discussed that the seasonality of rodents may affect the exposure rate of non-targets. Target species tend to move indoors in colder months so the exposure rate to non-targets falls, whereas in warmer months target rodents are more readily found outdoors and the exposure of predators and scavengers increases. Poisoned rodents exhibit altered behaviour and may be found outside their burrows during the day (Mason & Litten, 2003) moving more sluggishly than normal (Cox & Smith, 1992). This often occurs in rodents which have received a sub-lethal dose (Shore *et* al, 2015). They are also more likely to be found dead above ground making them available to scavengers (Elliot *et al*, 2014). Studies by Cox and Smith (1992), found that rats with anticoagulant poisoning spent more time overall in their nests but had a reversal of their normal activity. They spent more time outside their nests during the day thus in the wild this would make them more susceptible to predation. They would also freeze rather than bolt when startled so would be less likely to escape predators. Sub-lethal haemorrhaging may also be linked to sub-lethal doses which may cause liver damage and a loss in body condition (Shore *et al*, 2015).

Secondary poisoning in non-target species is often diagnosed by the presence of haemorrhages which cannot be attributed to any trauma and the detection of anticoagulant residues in tissues (Shore *et al*, 2015). Although haemorrhages may not be visible in some individuals, only detectable microscopically, and some individuals may have no signs of internal haemorrhage at all (Jaffe *et al*, 2016), haemorrhages cannot be linked to residue levels

(Sanchez-Barbudo *et al*, 2012) so it is possible a lot more individuals contain residues than initially thought and it also depends on the anticoagulant being measured (Shore *et al*, 2015). The residues of multiple anticoagulants may be detected in one non-target, this may be from them consuming multiple, highly mobile prey that have spread from different areas, or the bait may have been cross-contaminated (Shore *et al*, 2015). Due to their highly mobile nature wood mice are likely to be exposed to several different anticoagulants in their home range if their range covers several farms which may be using different anticoagulant rodenticides (Tosh *et al,* 2012). The accumulation of liver residues is influenced by diet, the use of anticoagulants, anticoagulant resistance and physiology (Shore *et al*, 2015). Scavengers are most at risk as they are likely to feed on the carcasses of rodents that have died as a result of anticoagulant baiting and they are also more likely to be found in areas where anticoagulant baiting occurs such as around farm buildings and human settlements (Ntampakis & Carter, 2005). Differences in residue accumulation have been found between members of the same species and this is believed to be due to prey distribution (Shore *et al*, 2015).

Resistance to anticoagulants in rodents and the prolonged use of anticoagulant rodenticides can lead to a build-up of residues in the environment, which can have an impact on predators and scavengers (Witmer *et al*, 2013). Resistant rodents have a greater anticoagulant body burden of the less toxic compounds. Some individuals have been found to have a burden 30-40% greater than non-resistant individuals (Shore *et al*, 2015) as they consume more of the anticoagulant which ultimately increases the treatment time to clear an area of rodents (Smith & Shore, 2015). These resistant individuals are more likely to survive an ineffective anticoagulant baiting programme and in turn be available for capture by predators and increase the risk of accidental poisoning of non-targets (Smith & Shore, 2015).

1.11 The role of anthropogenic actions as a driver of behavioural resistance

House mice originated in the Middle East and Asia but now, due to human introductions, they are found worldwide. This has brought them into conflict with humans as they damage buildings, cereals and crops, and they can also threaten the native flora and fauna (Witmer *et al,* 2014). The control of invasive species is of particular importance on islands where they may predate on local species of seabirds and in extreme cases drive them to extinction (Nathan *et* al, 2013; Witmer *et al*, 2014). Control and early detection of invasive rodent species is difficult not only due to neophobia and trap aversion, but also due to the abundance of food resources available which makes baits less attractive (Nathan *et al*, 2013).

In 1986, a population of House mice in a localised area of Birmingham was unable to be controlled using traditional rodenticide baits. Populations were also found in London and Glasgow, but not to the same extent as the Birmingham population (Humphries *et al*, 1992). The Birmingham population of mice had an aversion to cereal based baits and in subsequent experiments this aversion extended to any cereal based foods. No mice could be caught in traps baited with cereals but some were trapped when baited with cheese, fish or chicken (Humphries *et al*, 2000). This aversion to cereal-based foods was classed as 'behavioural resistance'. Due to anthropogenic actions, the traditional environment and foods of House mice had been replaced with a city environment where there was a lot of high fat food waste. These populations of House mice had become used to eating this high fat food which was readily available, so when they were offered cereal based foods they avoided them due to them not being their normal diet, consequently control of these House mice with traditional cereal based baits failed due to this 'behavioural resistance'. Studies by Taylor *et al* (1996) found that these 'behaviourally resistant' House mice had low α-amylase activity in their duodenum so there was a large volume of undigested material in their hindgut, this could

ultimately lead to death due to pressure on the liver and diaphragm. These House mice selected a low carbohydrate diet; they avoided cereals, to avoid the symptoms associated with low α-amylase activity.

Bait avoidance has also been recorded in populations of Norway rats. Norway rats are more neophobic than House mice so they show heightened responses towards changes in their environment such as the introduction of baits (Quy *et al*, 1992a). Populations that were thought to be susceptible to difenacoum baits were found to be problematic to control with difenacoum baits (Quy *et al*, 1992b). These populations were found on farms in Hampshire were there was an abundance of stored grain and fields of crops (Quy *et al*, 1992b). Through anthropogenic actions humans had created a 'behaviourally resistant' population or Norway rats that were difficult to control with anticoagulant rodenticides. These rats avoided the baits due to the abundance of freely available crops and grain, furthermore the constant availability of crops and stored grain allowed the rats to become adapted to that food source so they avoided the baits leading to treatment failure (Quy *et al*, 1992a) and only fed on the crops and stored grain.

These examples of resistance demonstrate how House mice and Norway rats have changed their behaviour in response to human influence. Rodents unintentionally introduced to islands by humans initially prove difficult to trap due to the high abundance of food in comparison to their population size this has allowed some populations to have detrimental effects on the native island species. House mice in Birmingham have altered their diet due to the availability of high fat food waste in their environment and studies found that these mice had difficulties digesting starch so were unlikely to eat grain-based baits (Singleton *et al*,

1999). Norway rats in farm environments are avoiding anticoagulant grain baits due to the high abundance of stored grain and crops that are freely available for them to feed on.

1.12 Recommendations for use of anticoagulants

From previous research it is not recommended to use any first-generation anticoagulants against House mice in the UK due to them being ineffective. Their further use would only increase the resistance problem already found in the UK (RRAG, 2012). Bromadiolone is also not recommended for use against House mice in the UK due to the presence of one resistant strain (Y139C) and its continued use will put a selection pressure on resistant populations of House mice which will lead to the resistance problem being exacerbated (Buckle & Prescott, 2012). Where resistant populations of House mice are suspected, difenacoum products should not be used as mice carrying the Y139C mutation have a natural level of resistance to difenacoum (Buckle & Prescott, 2012). Brodifacoum and flocoumafen are both effective against resistant strains of House mice. However, in the UK current regulations on the use of rodenticides containing these active ingredients is restricted to use against populations of rodents that predominantly live indoors (indoor use) and around buildings. This restriction was put in place primarily to overcome the perceived persistence of these active ingredients in both target and non-target species. This precludes their use against the majority of Norway rat infestations, but (as House mice predominantly live indoors), these active ingredients are often the preferred choice for their control (RRAG, 2012; Buckle & Prescott, 2012). It is also recommended to restrict the availability of vitamin K to rodents, as this is an antidote to anticoagulants and most resistant strains of rodents also have a higher dietary requirement for vitamin K. However, this is difficult to achieve in practical terms as menadione (vitamin K3) is added to most animal feeds.

1.13 Aims

This study aims to:

- Assess the efficacy of first-generation anticoagulants (warfarin, coumatetralyl, chlorophacinone and diphacinone) against a susceptible albino House mouse strain using dose-response data generated using blood clotting response (BCR) methodology. This data can then be used globally as the baseline data for House mouse resistance testing against these first-generation anticoagulants.
- To assess the efficacy of first-generation (warfarin, coumatetralyl, chlorophacinone and diphacinone) and second-generation (brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen) anticoagulants against House mice homozygous for the Y139C mutation using dose-response data generated using BCR methodology, and to quantify the magnitude of resistance by estimating resistance factors using previously published susceptibility baseline data.
- To assess the efficacy of second-generation anticoagulants (brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen) against House mice heterozygous for the Y139C mutation using dose-response data generated using BCR methodology, and to quantify the magnitude of resistance by estimating resistance factors. In combination with data on homozygous resistant animals, this data can then be used to provide a more realistic understanding of the impact of resistance on field populations, where resistant animals will be both homozygous and heterozygous resistant.
- From genetic analysis to determine the geographical distribution of the different *VKORC1* resistance alleles in wild caught House mice trapped in the UK, and to more clearly understand the extent of resistance in the UK wild House mouse population.

The scope of this study depends on the availability of samples provided by pest control operators in the UK.

- To investigate the effect of a selected line of resistant House mice homozygous for the *VKORC1* mutation Y139C, by breeding from survivors of a dose of anticoagulant that does not achieve complete mortality. Such a scenario is likely to occur in the field when rodenticides are used that are not completely effective. In Norway rats, such populations have been shown to have an increased magnitude of resistance.

Chapter 2 –The establishment of four first-generation anticoagulant active ingredient susceptibility baselines against an albino susceptible strain of House mouse

2.1 Introduction

2.1.1 History of resistance testing

The first-generation anticoagulant rodenticides were initially developed in the 1950s. Warfarin was the first active ingredient to be developed and marketed as an anticoagulant rodenticide followed by coumatetralyl and then chlorophacinone and diphacinone (Buckle & Eason, 2015). However, by the late 1950s resistance to warfarin was already being reported in Norway rats (Pelz *et al*, 2005) and in the early 1960s reports of difficulties in controlling House mouse infestations with warfarin were being made (Pelz *et al*, 2005; Rowe & Redfern, 1965) with House mice becoming more of a problem in urban areas than Norway rats (Wallace & MacSwinney, 1976).

Early feeding tests to determine resistance in Norway rats and House mice were crude. 'Resistant', wild individuals which had survived anticoagulant treatments were trapped in the wild and brought back to the laboratory to undergo feeding tests. Rowe and Redfern (1964) were the first laboratory to identify anticoagulant resistance in House mice by studying the toxicity of warfarin against wild-caught House mice. The individuals were deemed 'resistant' if they survived being fed field strength warfarin, 0.0025% (250ppm), for up to 21 days in the laboratory (Buckle & Prescott, 2012). These early feeding tests provided some evidence of resistance that would have an effect on treatment outcome (Berny *et al,* 2018), but they did not take into account that wild-caught individuals did not feed well under laboratory

conditions, so some of those individuals who had survived the feeding tests and consequently were deemed 'resistant' had only survived by not consuming a lethal dose of the active ingredient (MacNicoll, 1995). Rowe and Redfern (1964) also reported that some of the House mice that survived the feeding tests initially showed symptoms of anticoagulant poisoning and stopped feeding for several days and then subsequently recovered. Some of the survivors had consumed large doses of warfarin while some House mice had succumbed to small doses indicating that some individuals were extremely resistant and others were extremely susceptible to warfarin (Rowe & Redfern, 1964). In the wild it is unlikely that House mice will consume large doses of an anticoagulant due to their sporadic feeding behaviour.

2.1.2 Lethal feeding period (LFP) test

Lethal feeding period tests were developed to generate susceptibility baselines. A series of known doses of anticoagulants were given to susceptible rodents in order to generate dose-response data, the groups of rodents were fed anticoagulants under a no-choice situation for fixed periods of time (Pelz & Prescott, 2015). The dose required to achieve a response in 99% of susceptible individuals, i.e. mortality in 99% of the individuals (the LFP_{99}), was estimated from PROBIT analysis of the dose-response data (Berny *et al*, 2018). The estimated dose was then administered to individuals thought to be resistant. If they survived the 99% dose they were deemed to be resistant to that anticoagulant (Berny *et al*, 2018). Lethal feeding period tests are time consuming and like the early feeding tests they still relied on wild-caught animals feeding in a laboratory environment so there was a lot of variation in the dose of anticoagulant consumed by individuals (Pelz & Prescott, 2015). Prescott (1996) used an LFP test to study the level of warfarin resistance in wild-caught House mice from the Reading area of the United Kingdom. The individuals were fed 250ppm warfarin for 21 days and 80% of the House mice survived. Those which did not survive the test were found to have consumed less

warfarin than the survivors indicating a strong level of resistance in 80% of the House mice and a strong level of susceptibility in 20% (Prescott, 1996).

2.1.3 Blood clotting response (BCR) test

In comparison to feeding tests and the LFP test, blood clotting response (BCR) tests used nowadays can produce results 24 hours after the administration of a known dose of anticoagulant active ingredient (Prescott *et al*, 2007). Initial BCR tests determined the dose required to produce prolonged coagulation time 24 to 96 hours after dosing in 99% (the ED_{99}) of susceptible individuals. In the subsequent resistance checking tests, individuals that did not have prolonged coagulation times were classified as resistant (Berny *et al*, 2018). The estimates of ED_{99} had a lot of variation as they were determined using a variety of statistical analyses making it difficult to compare results between tests from different laboratories. To overcome this variation a standardised methodology was developed by Prescott *et al* in 2007 (Berny *et al*, 2018; Pelz & Prescott, 2015). The new standardised methodology measures the blood coagulation response after 24 hours of the administration of a known dose of anticoagulant active ingredient and determines 'responder' and 'non-responder' individuals using the international normalized ratio (INR), 'responders' have an INR equal to or greater than 5 (Pelz & Prescott, 2015) and are classed as susceptible to the active ingredient. The INR is the multiple of normal human blood clotting time if the World Health Organisation standard thromboplastin reagent had been used, it was originally developed to allow comparisons and minimise variations between measures of human prothrombin times taken at different hospitals using different thromboplastin reagents (Deitcher, 2002). In resistance testing, the INR controls for different thromboplastin reagents used by different laboratories. The safe human therapeutic range as quoted by Diagen is an INR of between 2 and 4.5. The resting INR for both Norway rats and House mice was reported to be 1.3, and an INR of 5 was proposed as

the threshold response for all Norway rat and House mouse BCR resistance tests (Prescott *et al*, 2007). For each active ingredient and sex combination of the susceptible House mouse strain dose-response data were statistically analysed to determine the dose required to produce the ED_{50} and a dose of twice the susceptible ED_{50} is used to determine resistance (Pelz & Prescott, 2015). Multiples of the ED_{50} can also be used to estimate the resistance factor of a population (Berny *et al*, 2018) and the impact of resistance on field control (Pelz & Prescott, 2015).

For the purpose of this study, susceptibility baselines for the susceptible albino House mouse strain were produced using the BCR methodology for the active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium; four of the rodenticide active ingredients commonly known as first-generation anticoagulant rodenticides (FGARs). Susceptibility baselines indicate the dose required to cause a response i.e. prolonged clotting time in a certain percentage of the population. Prescott *et* al (2007) had previously produced susceptibility baselines for susceptible albino House mice for the five second-generation anticoagulants (SGARs) brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen. Susceptibility baselines were produced for the four FGARs chlorophacinone, coumatetralyl, diphacinone and warfarin and the five SGARs for Norway rats; but baselines had not been produced for susceptible albino House mice for the four FGARs (Prescott *et al*, 2007). The baseline data produced by Prescott *et* al (2007) allowed the level of resistance to be assessed for any population of Norway rats and House mice globally for the five SGARs, and for Norway rats against the four FGARs. The data produced here will allow the level of resistance in House mice against the four FGARs to be assessed. The BCR methodology is a standardised resistance test which can be used against rats and mice to quantify resistance and estimate resistance factors. This methodology is a mild procedure under the terms of a

Home Office Project Licence in comparison to the lethal feeding period tests and earlier BCR tests which were substantial in their level of severity.

Warfarin sodium was used as high concentrations of warfarin were found to cause toxic effects in House mice within 15 minutes of dosing which lead to the mice having to be euthanised. The toxic effects noted were hunched appearance, gasping and reduced body temperature occurring within 15 minutes of dosing via oral gavage. Normal anticoagulant effects take up to 48 hours to occur and none of the animals displayed any symptoms normally associated with consuming an anticoagulant. It is not known whether the warfarin was becoming denatured during the production of the 5% stock solution and this was causing the toxic effects in the mice or whether the toxicity was due to the high concentration being used. When House mice were dosed with similar high concentrations of warfarin sodium no toxic effects occurred. Warfarin sodium is similar in structure to warfarin however in the hydroxycoumarin base structure the hydrogen of the hydroxide is replaced by sodium (Figure 2.1). Warfarin sodium is fully soluble in water whereas warfarin is highly insoluble in water (Fisher, 2005).

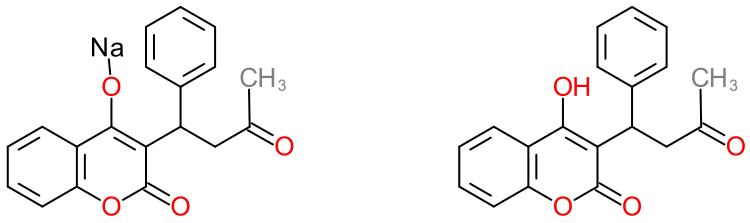


Figure2.1: Warfarin sodium on the left, warfarin on the right

The first-generation anticoagulants can also be classified by their chemical structure (Fisher, 2005); coumatetralyl and warfarin sodium are hydroxycoumarins, chlorophacinone and

diphacinone are indane-diones; hydroxycoumarins and indane-diones are similar in structure and chemical properties but do differ in their toxicity to target species (Buckle & Eason, 2015).

Susceptibility baselines will be produced for the four first-generation anticoagulant active ingredients; chlorophacinone, coumatetralyl, dipahcinone and warfarin sodium for an albino susceptible strain of House mouse.

2.2 Methods

The susceptible albino House mouse strain is an outbred CD-1 strain of House mouse supplied by Charles River UK Ltd. All mice were healthy, active and sexually mature and aged at least 4 weeks old. After arrival from Charles River UK Ltd they were held for a settling in period of at least three days before oral gavage, during this period and the period between oral gavage and blood sampling they were provided with food and water *ad libitum*.

2.2.1 Blood clotting response methodology

The BCR methodology used is as described by Prescott *et al* (2007).

1. The House mice were dosed by oral gavage with an active ingredient of known concentration at a rate of 1ml per 100g body weight; for example if the mice received a 0.1% solution of active ingredient delivered at the rate 1ml per 100g bodyweight they would receive 10mg.kg^{-1} bodyweight of active ingredient. The weight of the animal was recorded to the nearest 0.1g immediately prior to dosing.
    a. The required concentration of active ingredient was produced by diluting a known weight of 1% stock solution of active ingredient with a known weight of

polyethanol glycol 200 (PEG200). Reading Scientific Services Ltd (RSSL) produced 1% stock solutions of chlorophacinone, coumatetralyl and diphacinone.

i. To prepare the 1% stock solution the active ingredient powder was dissolved in triethanolamine and PEG200 at 60°C and then made up to the required volume, the ratio of PEG200:triathanolamine in the final solution was 99:1. All active ingredient powders, triethanolamine and PEG200 were purchased from Sigma Aldrich.

b. The stock solution of warfarin sodium was produced in house by diluting the warfarin sodium powder in distilled water by weight. The warfarin sodium powder was purchased from Sigma Aldrich.

2. 24 hours after dosing 0.9ml of blood was collected by cardiac puncture into a syringe containing 0.1ml of 3.2% sodium citrate under isoflurane terminal anaesthesia.
3. The sample was centrifuged at 5300rpm for 6 minutes and the plasma was collected.
4. Prothrombin time (PT) was determined in triplicate using an Amelung KC4 micro semi-automatic haemostasis machine and Diagen Rabbit Brain Thromboplastin reagent. If prothrombin times were not to be determined immediately the plasma samples were stored at -20^{o}C.

a. 0.05ml rabbit brain thromboplastin was added to four wells in the Amelung machine, the wells each contained a ball bearing, to each well 0.05ml of plasma sample was added. The wells were heated at 37°C for 60 seconds before 0.05ml of 25mM calcium chloride was added to each well using an electronic pipette. The electronic pipette started the timer on the Amelung machine, the timer stopped once a clot had formed. Calcium ions in the calcium chloride solution cause the thromboplastin to initiate coagulation by

activating factor VII which results in the conversion of soluble fibrinogen to insoluble fibrin causing a clot to form (Diagnostic Reagents Ltd, 2010).

5. The mean PT for each animal was converted to an International Normalised Ratio (INR) value using the calibration which was provided with the Diagen reagent.
6. For each active ingredient a ranging study was used and group size was increased gradually, to minimise the number of animals used, when determining appropriate dose-response data.
7. The dose-response data was analysed using GENMOD and PROBIT analysis. PROBIT analysis was used to provide an estimate of the ED_{50}.

Animals were classified as 'responders' when their prothrombin times were prolonged, with an INR greater than 5 (Prescott *et* al, 2007). In the current study where freeze dried Diagen Rabbit Brain Thromboplastin was used, a Prothrombin Time of 47.5 seconds was equivalent to an INR of 5. Animals with an average Prothrombin Time equivalent to an INR less than 5 were classified as 'non-responders' i.e. their blood clotting time was not prolonged.

2.2.2 Generation of dose-response data

House mouse dose-response data was generated for each active ingredient and sex combination. PROBIT data analysis was used to compare the response of the two sexes for each active ingredient. The three possibilities for the PROBIT lines of the two sexes were:

1. That they were completely independent of each other (separate)
2. That they were parallel but not coincident
3. That they were coincident.

PROBIT is a regression analysis which takes independent data points and transforms them into a straight line to enable analysis. PROBIT is used to transform a dose-response curve into a straight line; this allows dose-response lines to be compared for different anticoagulant active ingredients.

Procedure GENMOD was used initially to determine whether the PROBIT lines for the two sexes differed significantly from a parallel response and if they did not differ significantly from a parallel response, to see if they differed significantly from a coincident response. The output from GENMOD generated deviance values for the three assumptions and 'Chi Square' was used to determine whether the differences between the deviance values were significant using the methodology of Prescott *et al* (2007).

- Separate lines – Chi square is significant when comparing deviances of separate and parallel lines ($p<0.05$), and not significant when comparing parallel and coincident
- Parallel lines – Chi square is significant when comparing deviances of parallel and coincident lines ($p<0.05$)
- Coincident lines – Chi square is not significant when comparing deviances of parallel and coincident lines ($p>0.05$), the lines have points in common, and not significant when comparing separate and parallel

Subsequent analysis of the dose-response data for the two sexes was then conducted using Procedure PROBIT taking the results of Procedure GENMOD into account, PROBIT analysis was used to analyse dose-response data to generate dose-response percentiles for each active ingredient/sex combination for both House mouse strains.

2.3 Results

2.3.1 Comparison of the PROBIT response of the two sexes

Using procedure GENMOD to compare the PROBIT lines the response lines were coincident for the two sexes of susceptible albino House mice for chlorophacinone, diphacinone and warfarin sodium, not deviating significantly from a parallel or from a coincident response, and parallel for coumatetralyl, not deviating significantly from a parallel response but deviating significantly from a coincident response (Table 2.1). Coincident lines share points for both sexes whereas parallel lines do not share points.

The PROBIT lines were then analysed as coincident for chlorophacinone, diphacinone and warfarin sodium and as parallel for coumatetralyl for the susceptible albino strain of House mouse to generate dose-response data.

Table 2.1: GENMOD results for susceptible albino House mice dosed with the active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium

FGAR	Sex	Deviance Separate	Deviance Parallel	Deviance Coincident	Chi sq. -from parallel	= p value	Chi sq. - from coincident	= p value
Chlorophacinone	m vs f	30.5013	33.6605	33.6741	3.1592	0.0755	0.0136	0.907162
Coumatetralyl	m vs f	16.9882	19.5248	52.1907	2.5366	0.111234	32.6659	<0.00001
Diphacinone	m vs f	6.1933	8.6217	9.3796	2.4284	0.119	0.7579	0.3840
Warfarin sodium	m vs f	15.8495	16.3355	17.0003	0.486	0.485717	0.6684	0.41361

2.3.2 Generation of the PROBIT dose-response percentiles

The resulting PROBIT dose-response data for each active ingredient sex combination for the susceptible House mouse strain considering the results of Procedure GENMOD are shown in Table 2.2.

Table 2.2: Summary PROBIT dose-response data for male and female susceptible albino House mice for the four active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium

		Effective dose [mg/kg] (lower – upper fiducial limits)			
Effective dose	Sex	Chlorophacinone	Coumatetralyl	Diphacinone	Warfarin sodium
1%	Male	0.34 (0.11-0.49)	0.29 (0.04-0.58)	0.53 (0.07-0.68)	0.42 (0.11-0.68)
40%	Male	0.78 (0.59-0.90)	1.59 (1.03-2.02)	0.86 (0.66-1.07)	1.30 (0.89-1.68)
50%	Male	0.86 (0.70-1.01)	1.95 (1.43-2.51)	0.91 (0.76-1.29)	1.50 (1.10-1.96)
60%	Male	0.94 (0.80-1.16)	2.40 (1.87-3.30)	0.97 (0.83-1.64)	1.72 (1.32-2.35)
99%	Male	2.15 (1.56-5.76)	13.04 (7.00-72.90)	1.57 (1.18-17.02)	5.41 (3.46-17.54)
1%	Female	0.34 (0.11-0.49)	1.55 (0.77-2.20)	0.53 (0.07-0.68)	0.42 (0.11-0.68)
40%	Female	0.78 (0.59-0.90)	4.41 (3.56-5.17)	0.86 (0.66-1.07)	1.30 (0.89-1.68)
50%	Female	0.86 (0.70-1.01)	5.02 (4.18-5.89)	0.91 (0.76-1.29)	1.50 (1.10-1.96)
60%	Female	0.94 (0.80-1.16)	5.70 (4.85-6.80)	0.97 (0.83-1.64)	1.72 (1.32-2.35)
99%	Female	2.15 (1.56-5.76)	16.24 (11.84-30.26)	1.57 (1.18-17.02)	5.41 (3.46-17.54)

The effective dose (ED) values are lower for the indane-diones chlorophacinone and diphacinone than for the hydroxycoumarins coumatetralyl and warfarin sodium for both sexes of the susceptible albino strain of House mice; this indicates that the indane-diones are more potent than the hydroxycoumarins against susceptible albino House mice, a lower dose of an indane-dione active ingredient causes a response i.e. prolonged clotting time, however the difference is limited and was not proven statistically. This research is based on House mice

receiving small volumes of the active ingredients measured to 0.1ml so there were likely to be some individuals that received a lower dose than calculated and conversely others which received a slightly higher dose due to the errors associated with measuring doses to such small volumes.

2.4 Discussion

The effective dose (ED) values for the hydroxycoumarins coumatetralyl and warfarin sodium were greater than the ED values for the indane-diones chlorophacinone and diphacinone, this indicates that the indane-diones and more toxic than the hydroxycoumarins against the albino susceptible House mouse strain.

Indane-dione and hydroxycoumarin anticoagulant active ingredients are similar in structure and have a similar mode of action, they all cause multiple haemorrhages (Meehan, 1984), but vary in their toxicity to rodents (Buckle & Eason, 2015). Warfarin was the first of the first-generation active ingredients to be developed in the 1940s, coumatetralyl was developed soon afterwards in the 1950s following the success of warfarin to control rodent populations (Buckle & Eason, 2015). Diphacinone was developed around the same time as coumatetralyl and is still used widely in the USA to control rats and voles (Buckle & Eason, 2015) and is registered for use against rodents in New Zealand (Fisher, 2005). Diphacinone is more toxic to rats than mice (Meehan, 1984) with Buckle and Eason (2015) quoting the LD_{50} values for Norway rats as 2.3 - 43mg.kg^{-1} and 141 - 340mg.kg^{-1} for House mice, and it has a similar oral toxicity as warfarin for House mice, the LD_{50} of diphacinone is 340mg.kg^{-1} and the LD_{50} of warfarin is 374mg.kg^{-1} (Fisher, 2005). The LD_{50} is the dose at which lethality occurs in 50% of the population. Many early studies quote LD_{50} values for the active ingredients as these

studies were feeding tests in which the lethal dose consumed by the individuals was calculated rather than the ED_{50} (the dose at which 50% of the individuals respond) which is calculated in this study. Chlorophacinone has similar chemical properties to diphacinone and was developed in the early 1960s (Fisher, 2005), it is still used extensively across Europe and USA (Buckle & Eason, 2015).

The indane-diones chlorophacinone and diphacinone had similar effective dose (ED) values for the susceptible albino House mouse strain. Values for diphacinone were not significantly greater than those for chlorophacinone, thus chlorophacinone is more potent than diphacinone against susceptible albino House mice; however the difference is limited and was not proven statistically. The ED_{50} of chlorophacinone for the susceptible albino House mice strain is 0.86mg.kg^{-1} compared to an ED_{50} of 0.91mg.kg^{-1} for diphacinone (Table 2.2).

The effective dose values for coumatetralyl and warfarin sodium were greater than those for the indane-diones for the susceptible House mouse strain. Diphacinone is known to be more toxic than warfarin to rodents as it inhibits the production of vitamin K clotting factors longer than warfarin does (Eason & Wickstrom, 2001). Coumatetralyl and warfarin sodium would be less effective in the control, i.e. requiring a higher dose, of susceptible albino House mice compared to chlorophacinone or diphacinone. The ED_{50} for coumatetralyl for male susceptible albino House mice is 2.0mg.kg^{-1} compared to 0.86mg.kg^{-1} for chlorophacinone and 0.91mg.kg^{-1} for diphacinone. The ED_{50} for coumatetralyl against female susceptible albino House mice is 5.0mg.kg^{-1} compared to 0.86mg.kg^{-1} for chlorophacinone and 0.91mg.kg^{-1} for diphacinone; the ED_{50} for warfarin sodium for both sexes is 1.5mg.kg^{-1}.

Coumatetralyl has previously been reported to be more potent than warfarin to rodents (Eason & Wickstrom, 2001) and it is widely used throughout the world (Buckle & Eason, 2015) but it has been found to be less potent than warfarin sodium in this study against the susceptible albino House mouse strain. Although the ED_{50} is high for coumatetralyl for the susceptible albino House mouse strain this is based on single dose toxicity, the toxicity of all first-generation anticoagulants increases with successive intakes (Fisher, 2005) so several intakes of low doses of coumatetralyl are likely to be more effective than a single intake of a high dose of coumatetralyl. When comparing the relative toxicity of these active ingredients the potency of baits used in the field needs to be considered. Typically baits containing either chlorophacinone or diphacinone have an active ingredient concentration of 50ppm (equivalent to $50mg.kg^{-1}$), baits containing warfarin have an active ingredient concentration of 250ppm ($250mg.kg^{-1}$) and baits containing coumatetralyl have an active bait concentration of between 375ppm ($375mg.kg^{-1}$) and 500ppm ($500mg.kg^{-1}$) (Fisher, 2005). A House mouse normally eats up to 20% of its bodyweight a day (Meehan, 1984), so if a House mouse weighs 25g bodyweight it would typically eat 5g per day. A 25g House mouse which only had access to bait with an active ingredient concentration of 50ppm would typically ingest 0.25mg of the active ingredient per day. If the concentration was 250ppm as found in baits containing warfarin the House mouse may ingest 1.25mg of the active ingredient per day, and if the bait contained the active ingredient at a concentration of 375 - 500ppm then the House mouse may consume 1.88mg per day of the active ingredient in the 375ppm bait or 2.5mg per day of the active ingredient in the 500ppm bait. At these consumption rates of baits, a susceptible albino House mouse would usually consume the ED_{50} dose warfarin sodium within 1-2 days on bait containing 250ppm active ingredient and the ED_{99} dose would be consumed within 5 days. Whereas when feeding on baits of lower potency such as baits containing 50ppm of the active ingredients chlorophacinone or diphacinone it may take up to 4 days for a 25g susceptible albino House mouse to consume an ED_{50} dose, and up to 7 days to consume an ED_{99} dose of

diphacinone or up to 9 days to consume an ED_{99} dose of chlorophacinone. The time taken to consume an effective dose of active ingredient in a bait of higher potency is much shorter, 1-2 days to consume an ED_{50} dose of coumatetralyl when feeding on 500ppm bait and 5-7 days to consume an ED_{99} dose (Table 2.3). House mice are sporadic feeders so in field situations they feed from many food sources so in real terms it will take them longer to consume the effective dose than if they were to feed exclusively on the anticoagulant bait.

The results of this research determined the acute dose required to prolong the clotting times of susceptible albino House mice, whereas in practical conditions mice receive a sub-acute dose over several days. These sub-acute doses are more toxic overall than one large acute dose. Hadler and Buckle (1992) report the sub-acute LD_{50} of warfarin for Norway rats as $1mg.kg^{-1}$ daily over 5 days whereas the acute LD_{50} is quoted as between $10mg.kg^{-1}$ and $323mg.kg^{-1}$, similar findings would be expected in House mice for the first-generation active ingredients as they are all chronic toxicants i.e. they are more toxic over several small doses than one large dose. Rats can survive a single dose of $50mg.kg^{-1}$ of coumatetralyl but succumb to successive doses of $1mg.kg^{-1}$ over several days (Eason & Wickstrom, 2001).

In terms of treatment the indane-diones chlorophacinone and diphacinone initially appear more potent than the hydroxycoumarins coumatetralyl and warfarin sodium but in practical terms the hydroxycoumarins are more potent. Susceptible albino House mice need to consume less of the hydroxycoumarins in field strength baits overall to receive an effective dose leading to prolonged clotting times (Table 2.3).

Table 2.3: Number of days for a 25g susceptible albino House mouse to consume an ED_{50} or ED_{99} dose of active ingredient when delivered at the concentration available in commercial rodenticide baits when eating 5g bait per day

Concentration of active ingredient in field strength bait	Number of days to reach ED_{50}		Number of days to reach ED_{99}	
	male	female	male	Female
Chlorophacinone 50ppm	3.4	3.4	8.6	8.6
Coumatetralyl 375ppm	1.0	2.7	7.0	8.7
Coumatetralyl 500ppm	0.8	2.0	5.2	6.5
Diphacinone 50ppm	3.6	3.6	6.3	6.3
Warfarin (sodium) 250ppm	1.2	1.2	4.3	4.3

The Biocidal Products Directive was established in 2012 to regulate the use of rodenticide active substances in the EU; substances must be registered and their risks to humans, the environment and non-targets listed (Buckle & Eason, 2015), the permitted concentration of active ingredient in any bait is now restricted to 30ppm in the EU. In Europe and the UK the only first-generation anticoagulant active ingredients regulated for the use against House mice are coumatetralyl and warfarin (HSE, 2018). Both of these compounds can be used indoors and outdoors, however coumatetralyl is restricted to use outdoors around buildings. Restrictions have been placed on the purchase of baits containing these compounds, coumatetralyl baits may be purchased by amateurs as well as professionals whereas warfarin baits may only be purchased by professionals (HSE, 2018). A similar registry to the Biocidal Products Directive is in place in the USA, the Federal Insecticides, Fungicides and Rodenticides Act (Buckle & Eason, 2015). In the USA warfarin, chlorophacinone and diphacinone are permitted for use (EPA, 2017). It is possible that the permitted use of any active ingredient may change in the future due to the availability of data such as that produced by this study.

To date no similar study has been undertaken to determine the level of anticoagulant resistance in either of the known anticoagulant resistant strains of House mouse found in the UK, the 'Cambridge' which is homozygous for the *VKORC1* mutation L128S and the 'Reading' strain which is homozygous for the *VKORC1* mutation Y139C, almost all similar research has

concentrated on Norway rats. As House mice are becoming more of a problem and as anticoagulant resistance has been identified it is important to understand the level of resistance and whether there are any active ingredients which will not control a resistant population of House mice.

Chapter 3 –The establishment of susceptibility baselines against a resistant strain of House mouse, homozygous for the *VKORC1* mutation Y139C for four first-generation anticoagulant active ingredients and five second-generation anticoagulant active ingredients, and the assessment of resistance factors

3.1 Introduction

3.1.1 Previous resistance research

The majority of research into resistance to anticoagulant rodenticides has focused on Norway rats as these are seen as the major pest species throughout the world. In 1988, Greaves and Cullen-Ayres published resistance factors for three resistant strains of Norway rats located in the United Kingdom; 'Welsh', 'Scottish' and 'Hampshire' for the active ingredients difenacoum, bromadiolone, brodifacoum, coumatetralyl and warfarin. The 'Welsh' resistant strain of Norway rats carries the *VKORC1* mutation Y139S, the 'Scottish' strain carries the mutation L128Q, and the 'Hampshire' strain carry the mutation L120Q (Pelz & Prescott, 2015). In 2007 Prescott produced dose-response data for the four first-generation active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin , and the five second-generation anticoagulants brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen for susceptible Norway rats , and dose-response data for susceptible House mice against these second-generation anticoagulant active ingredients.

3.1.2 The resistant strain of House mouse homozygous for the VKORC1 mutation Y139C

The House mouse strain homozygous for the *VKORC1* mutation Y139C is a laboratory strain of House mouse in which the homozygous gene has been transferred onto the susceptible strain of Swiss House mouse from wild caught House mice trapped near Reading (Pelz *et al*, 2005). In the 1980s a population of wild House mice trapped from locations around Reading, UK were found to be 'warfarin resistant' (Prescott *et al*, 2017). Wild-caught individuals were fed 250ppm warfarin in a lethal feeding period test of 21 days then maintained for 28 days observation to determine their 'warfarin resistance', 80% of the mice survived (Prescott, 1996). Thirty individuals derived from breeding stocks established at the University of Reading were dosed with bromadiolone via oral gavage, and of the thirty animals dosed only three (10%) died (Prescott, 1996). The level of resistance to both warfarin and bromadiolone suggests that there is a level of cross-resistance to both active ingredients. Breeding studies were established to examine the inheritance of bromadiolone resistance. Wild-caught individuals of unknown genotype were crossed with susceptible Swiss House mice and the progeny tested for resistance by being given a test dose of bromadiolone that would be lethal to susceptible individuals; it was assumed that some of the wild-caught individuals would be homozygous resistant (RR), some would be heterozygous resistant (Rr) and some would be susceptible (rr) (Prescott, 1996). Assuming the unifactorial dominant inheritance of resistance, in a test cross 100% of the progeny of homozygous resistant parents, and 50% of the progeny of heterozygous resistant parents would be expected to survive the test dose of bromadiolone (Prescott, 1996). A breeding nucleus of House mice homozygous for the *VKORC1* mutation Y139C was created from individuals identified as homozygous resistant in the breeding studies (Prescott, 1996). The resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C is maintained at University of Reading. To date no dose-response data or resistance factors have been produced for the resistant strain of House mouse homozygous for the *VKORC1* mutation.

3.1.3 Susceptibility baselines

Susceptibility baselines for the House mouse strain homozygous for the *VKORC1* mutation Y139C were produced using the BCR methodology for the anticoagulant active ingredients chlorophacinone, coumatetralyl, diphacinone, warfarin sodium, brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen. Susceptibility baselines have not previously been produced for House mice homozygous for the *VKORC1* mutation Y139C for anticoagulants but they have been produced for the second-generation anticoagulant rodenticides (SGARs) brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen for a susceptible albino strain of House mice by Prescott *et al* (2007). Due to the level of resistance to the first-generation anticoagulants, SGARs are routinely used to try to control populations of House mice. Currently the level of resistance in House mice homozygous for the *VKORC1* mutation Y139C is not understood and pest control operators are reporting failures of some SGARs in the control of House mice populations. Use of first-generation anticoagulant rodenticides (FGARs) for the control of House mice in the UK is not permitted due to them being ineffective, however in some countries including Germany and USA their use by amateurs is still permitted (Buckle & Smith, 2015); this leads to inadequate House mouse control and an increase in the resistance problem in these countries.

The baselines were compared with the susceptibility baselines for a susceptible albino House mouse strain presented in Chapter 2 and those produced by Prescott *et al* (2007), and the resistance factors (RF) determined for each sex/active ingredient combination. The magnitude of resistance in House mice homozygous for the *VKORC1* mutation Y139C to the anticoagulant active ingredients has not been previously quantified.

3.1.4 Calculation of resistance factors

The resistance factor (RF) is determined by calculating the dose required to produce the ED_{50} (the dose at which 50% of the population respond) in the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C as a multiple of the dose required to produce the ED_{50} in the susceptible albino strain of House mouse. The ED_{50} is used as the Fiducial Limits are narrower than at the ED_{99} (the dose at which 99% of the population respond) so it is much more accurate. At the ED_{99} there will still be some House mice that do not respond i.e. do not have prolonged clotting times, they are highly resistant to the active ingredients. Resistance may be termed as 'practical' or 'technical'. High resistance factors (>5) are associated with 'practical resistance', adequate levels of control of a rodent population in the field is highly unlikely; 'technical resistance' is associated with low resistance factors, the level of resistance in the rodent population is unlikely to affect the treatment outcome (Pelz & Prescott, 2015) but it can still be demonstrated in the laboratory (Buckle *et al*, 2007).

Susceptibility baselines for the four first-generation anticoagulant active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium, and the five second-generation anticoagulant active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen will be produced for the House mouse strain homozygous for the *VKORC1* mutation Y139C. The ED_{50} value for each active ingredient will be compared with the corresponding ED_{50} value for the albino susceptible House mouse strain to determine the resistance factor, resistance factors were determined for each sex/active ingredient combination.

3.2 Methods

The blood clotting response methodology and methodology to generate dose-response data used in sections 2.2.1 and 2.2.2 were used to generate the susceptibility baselines and the dose-response data for the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C for each anticoagulant active ingredient.

3.2.1 Calculation of resistance factors

The resistance factor (RF) was determined by calculating the dose required to produce the ED_{50} (the dose at which 50% of the population respond) in the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C as a multiple of the dose required to produce the ED_{50} in the susceptible albino strain of House mouse.

Data generated by Prescott *et al* (2007) for the susceptible albino strain of House mice against the second-generation anticoagulant active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen , and the data generated in Chapter 2 of this study for the four first-generation anticoagulant active ingredients warfarin sodium, chlorophacinone, coumatetralyl and diphacinone were used to determine the resistance factor (RF) for either sex for each anticoagulant active ingredient.

3.3 Results

3.3.1 Comparison of the PROBIT response of the two sexes

Using procedure GENMOD to compare the PROBIT lines for the resistant strain of House mice homozygous for the *VKORC1* mutation Y139C the response lines for the two sexes were separate for coumatetralyl, they deviated significantly from a parallel response; coincident for chlorophacinone and diphacinone, they did not deviate significantly from a parallel or coincident response, and parallel for warfarin sodium, they did not deviate significantly from a parallel response but did deviate significantly from a coincident response (Table 3.1).

Table 3.1: GENMOD results for House mice homozygous for the *VKORC1* mutation Y139C dosed with the active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium

FGAR	Sex	Deviance Separate	Deviance Parallel	Deviance Coincident	Chi sq. -from parallel	= p value	Chi sq. - from coincident	= p value
Chlorophacinone	m vs f	0.0000	0.0000	1.0168	0.0000	1	1.0168	0.313279
Coumatetralyl	m vs f	5.0464	11.3869	39.1044	6.3405	0.0118	27.7175	<0.00001
Diphacinone	m vs f	0.0000	0.0000	1.639	0.0000	1	1.1639	0.280658
Warfarin sodium	m vs f	16.8213	16.8550	23.1261	0.0337	0.8543	6.2711	0.012272

The PROBIT response lines for the two sexes of the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C were separate for bromadiolone and flocoumafen, they deviated significantly from a parallel response; were parallel for difenacoum, not deviating significantly from a parallel response but deviating significantly from a coincident response; and were coincident for brodifacoum and difethialone, not deviating significantly from a parallel or from a coincident response (Table 3.2).

Table 3.2: GENMOD results for the House mouse strain homozygous for the *VKORC1* mutation Y139C dosed with the active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen

SGAR	Sex	Deviance Separate	Deviance Parallel	Deviance Coincident	Chi sq. - from parallel	= p value	Chi sq. - from coincident	= p value
brodifacoum	m vs f	4.6072	5.0272	5.0636	0.42	0.5169	0.0364	0.8487
bromadiolone	m vs f	3.2091	15.2203	15.2633	12.011	<0.0005	0.043	0.8357
difenacoum	m vs f	11.9217	12.3073	31.5551	0.3856	0.5346	19.2478	<0.0005
difethialone	m vs f	10.7915	11.2236	11.6482	0.4321	0.5110	0.4246	0.5147
flocoumafen	m vs f	22.3893	36.7823	39.8354	14.393	<0.0005	3.0531	0.0806

The PROBIT response lines for the first-generation anticoagulant active ingredients for the two sexes of the susceptible albino strain of House mice are presented previously in Chapter 2; the lines were coincident for chlorophacinone, diphacinone and warfarin sodium, and parallel for coumatetralyl. The PROBIT response lines for the second-generation anticoagulant active ingredients for the two sexes of the susceptible albino strain of House mice were derived from Prescott *et al* (2007). The PROBIT lines were separate for bromadiolone; parallel for difenacoum, flocoumafen and brodifacoum; and coincident for difethialone.

3.3.2 Generation of PROBIT dose-response percentiles

For each active ingredient and sex combination the dose-response data was analysed using PROBIT analysis taking into account the results of the GENMOD analysis, the data was analysed for the two sexes as separate, parallel or coincident.

Table 3.3: Summary PROBIT dose-response data for male and female House mice homozygous for the *VKORC1* mutation Y139C for the four active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium. Results for susceptible albino House mice are shown in italics

		Effective dose [mg/kg] (lower – upper fiducial limits)			
Effective dose	Sex	Chlorophacinone	Coumatetralyl	Diphacinone	Warfarin sodium
1%	Male	302.38 *0.34 (0.11-0.49)*	1.61 (0.00-6.39) *0.29 (0.04-0.58)*	283.38 *0.53 (0.07-0.68)*	96.13 *0.42 (0.11-0.68)*
40%	Male	506.76 *0.78 (0.59-0.90)*	41.37 (20.40-62.77) *1.59 (1.03-2.02)*	461.84 *0.86 (0.66-1.07)*	351.73 *1.30 (0.89-1.68)*
50%	Male	539.78 *0.86 (0.70-1.01*)	61.50 (40.10-119.49) *1.95 (1.43-2.51)*	490.24 *0.91 (0.76-1.29)*	412.16 *1.50 (1.10-1.96)*
60%	Male	574.94 *0.94 (0.80-1.16)*	91.43 (60.63-295.76) *2.40 (1.87-3.30)*	520.40 *0.97 (0.83-1.64)*	482.97 *1.72 (1.32-2.35)*
99%	Male	963.56 *2.15 (1.56-5.76)*	2345 (517.38-1697868) *13.04 (7.00-72.90)*	848.11 *1.57 (1.18-17.02)*	1767 *5.41 (3.46-17.54)*
1%	Female	302.38 *0.34 (0.11-0.49)*	83.19 *1.55 (0.77-2.20)*	283.38 *0.53 (0.07-0.68)*	186.68 *0.42 (0.11-0.68)*
40%	Female	506.76 *0.78 (0.59-0.90)*	104.80 *4.41 (3.56-5.17)*	461.84 *0.86 (0.66-1.07)*	683.08 *1.30 (0.89-1.68)*
50%	Female	539.78 *0.86 (0.70-1.01*)	107.80 *5.02 (4.18-5.89)*	490.24 *0.91 (0.76-1.29)*	800.43 *1.50 (1.10-1.96)*
60%	Female	574.94 *0.94 (0.80-1.16)*	110.89 *(5.70 (4.85-6.80)*	520.40 *0.97 (0.83-1.64)*	937.94 *1.72 (1.32-2.35)*
99%	Female	963.56 *2.15 (1.56-5.76)*	139.70 *16.24 (11.84-30.26)*	848.11 *1.57 (1.18-17.02)*	3432 *5.41 (3.46-17.54)*

Summary PROBIT dose-response data for the male and female resistant House mice homozygous for the *VKORC1* mutation Y139C against the four FGAR active ingredients are presented in Table 3.3; the dose-response data for the susceptible albino strain of House mice produced in Chapter 2 are also shown in italics. Summary PROBIT dose-response data for the male and female resistant House mice homozygous for the *VKORC1* mutation Y139C against the five second-generation anticoagulant active ingredients are presented in Table 3.4; the dose-response data for the susceptible strain of House mice produced by Prescott *et al* (2007) are shown in italics.

No Fiducial Limits were calculated for chlorophacinone, diphacinone or warfarin sodium for the resistant strain of House mice homozygous for the *VKORC1* mutation Y139C due to the lack of animals dosed. When the resistant strain of House mice homozygous for the *VKORC1* mutation Y139C were dosed with a 5% solution of either chlorophacinone or diphacinone, which when delivered at a rate of 1ml per 100g bodyweight provided the animals with a dose of 500mg.kg^{-1} bodyweight, less than 50% of the animals had prolonged clotting times. This was the highest concentration of these active ingredients which was available so the dose could not be increased above 500mg.kg^{-1} bodyweight. Ethical and welfare considerations have to be taken into account when working with animals and the generation of scientific data has to be weighed against the use of animals in accordance with Home Office guidelines. In the case of producing more data for chlorophacinone, diphacinone and warfarin sodium, it was considered unethical to dose more resistant House mice homozygous for the *VKORC1* mutation Y139C with lower concentrations of the active ingredient when it is already known that very few of these animals would have prolonged clotting times.

Table 3.4: Summary PROBIT dose-response data for male and female House mice homozygous for the *VKORC1* mutation Y139C against the active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen. The results produced by Prescott *et al* (2007) for susceptible albino House mice are shown in italics

		Effective dose [mg/kg] (lower – upper fiducial limits)				
Effective dose	Sex	Brodifacoum	Bromadiolone	Difenacoum	Difethialone	Flocoumafen
1%	Male	0.31 (0.18-0.40)	1.70 (0.06-5.23)	0.52 (0.31-0.65)	0.30 (0.13-0.43)	0.09 (0.01-0.17)
40%	Male	0.63 (0.56-0.68) *0.38 (0.35-0.39)*	23.59 (10.70-34.18) *1.89 (1.75-2.00)*	0.97 (0.84-1.07) *0.81 (0.72-0.88)*	1.09 (0.92-1.44) *0.78 (0.71-0.82)*	0.37 (0.21-0.46) *0.49 (0.45-0.52)*
50%	Male	0.68 (0.62-0.75) *0.39 (0.37-0.40)*	32.54 (18.75-46.21) *1.96 (1.84-2.09)*	1.05 (0.94-1.15) *0.85 (0.76-0.92)*	1.27 (1.06-1.85) *0.83 (0.77-0.87)*	0.44 (0.32-0.56) *0.51 (0.47-0.55)*
60%	Male	0.74 (0.69-0.84) *0.40 (0.38-0.41)*	44.88 (30.21-67.93) *2.03 (1.91-2.19)*	1.13 (1.03-1.26) *0.89 (0.80-0.97)*	1.49 (1.19-2.41) *0.88 (0.82-0.93)*	0.53 (0.42-0.75) *0.53 (0.49-0.57)*
99%	Male	1.49 (1.18-2.62) *0.51 (0.47-0.57)*	623.13 (244.39-9726) *2.72 (2.42-3.58)*	2.13 (1.73-3.40) *1.27 (1.12-1.61)*	5.34 (3.00-22.96) *1.46 (1.28-1.83)*	2.32 (1.25-29.62) *0.74 (0.65-0.96)*
1%	Female	0.31 (0.18-0.40)	16.43 (8.15-21.49)	0.74 (0.47-0.90)	0.30 (0.13-0.43)	0.32 (0.23-0.37)
40%	Female	0.63 (0.56-0.68) *0.34 (0.32-0.35)*	32.49 (26.93-37.76) *1.66 (1.62-1.70)*	1.38 (1.25-1.53) *0.54 (0.49-0.58)*	1.09 (0.92-1.44) *0.78 (0.71-0.82)*	0.48 (0.45-0.52) *0.42 (0.37-0.45)*
50%	Female	0.68 (0.62-0.75) *0.35 (0.33-0.36)*	35.31 (30.11-41.87) *1.68 (1.64-1.73)*	1.49 (1.36-1.68) *0.56 (0.52-0.60)*	1.27 (1.06-1.85) *0.83 (0.77-0.87)*	0.51 (0.48-0.56) *0.44 (0.39-0.47)*
60%	Female	0.74 (0.69-0.84) *0.36 (0.34-0.37)*	38.38 (33.16-47.15) *1.70 (1.66-1.76)*	1.61 (1.47-1.87) *0.59 (0.54-0.64)*	1.49 (1.19-2.41) *0.88 (0.82-0.93)*	0.54 (0.50-0.60) *0.45 (0.41-0.49)*
99%	Female	1.49 (1.18-2.62) *0.46 (0.42-0.51)*	75.88 (57.36-158.24) *1.87 (1.79-2.06)*	3.03 (2.40-5.18) *0.84 (0.74-1.07)*	5.34 (3.00-22.96) *1.46 (1.28-1.83)*	0.81 (0.69-1.21) *0.63 (0.56-0.81)*

3.3.3 Determination of the resistance factor

The ED_{50} of the four FGAR active ingredients were determined for both sexes of the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C and both sexes of the

susceptible albino House mouse strain. The resistance factors (RF) for each active ingredient were calculated and all were high and would be associated with practical resistance (Table 3.5) so these anticoagulant active ingredients would not be expected to provide adequate control of House mice homozygous for the *VKORC1* mutation Y139C.

Table 3.5: Calculated resistance factors (RF) at ED_{50} for male and female House mice homozygous for the *VKORC1* mutation Y139C against the four active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium

Active Ingredient	ED_{50} for Y139C strain ($mg.kg^{-1}$)		ED_{50} for susceptible strain ($mg.kg^{-1}$)		RF	
	Male	Female	male	female	male	Female
Chlorophacinone	540	540	0.86	0.86	628	628
Coumatetralyl	61.5	108	2.0	5.0	30.8	21.6
Diphacinone	490	490	0.91	0.91	538	538
Warfarin sodium	412	800	1.5	1.5	275	533

The ED_{50} of the five SGAR active ingredients were determined for both sexes of the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C and both sexes of the susceptible House mouse albino strain. The resistance factors (RF) were calculated for the five active ingredients (Table 3.6). All the SGAR active ingredients would be expected to control House mice homozygous for the *VKORC1* mutation Y139C with the exception of bromadiolone which has an RF of 16.3 for males and 20.8 for females; bromadiolone would be ineffective in the control of House mice carrying the Y139C mutation.

Table 3.6: Calculated resistance factors (RF) at ED_{50} for male and female House mice homozygous for the *VKORC1* mutation Y139C against brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen

Active Ingredient	ED_{50} for Y139C strain ($mg.kg^{-1}$)		ED_{50} for susceptible strain ($mg.kg^{-1}$)		RF	
	male	Female	male	Female	Male	Female
Brodifacoum	0.68	0.68	0.39	0.35	1.7	1.9
Bromadiolone	32.5	35.3	2.0	1.7	16.3	20.8
Difenacoum	1.1	1.5	0.85	0.56	1.3	2.7
Difethialone	1.3	1.3	0.83	0.83	1.6	1.6
Flocoumafen	0.44	0.51	0.51	0.44	0.86	1.2

3.4 Discussion

The estimated resistance factor values were significantly high for all the FGAR active ingredients ranging from 22 for coumatetralyl for female House mice to 628 for chlorophacinone against both sexes. Resistance factors of these levels would be associated with complete failure to control a House mouse population homozygous for the *VKORC1* mutation Y139C. The resistance factors for the SGAR active ingredients ranged from 0.86 for flocoumafen against male House mice to 21 for bromadiolone against female House mice. All the SGARs with the exception of bromadiolone would be expected to control a population of House mice homozygous for the *VKORC1* mutation Y139C.

Coumatetralyl is the most potent of the four first-generation anticoagulant active ingredients to House mice homozygous for the *VKORC1* mutation Y139C, the ED_{50} being lower for coumatetralyl than for the other active ingredients. Coumatetralyl has been reported to be more potent than warfarin in previous literature (Eason & Wickstrom, 2001). Warfarin sodium was more potent than the indane–diones chlorophacinone and diphacinone against male resistant House mice homozygous for the *VKORC1* mutation Y139C, however against female resistant House mice homozygous for the *VKORC1* mutation Y139C warfarin sodium was the least potent active ingredient. The ED_{50} for warfarin sodium was 800mg.kg^{-1} for female resistant House mice homozygous for the *VKORC1* mutation Y139C, compared to 540mg.kg^{-1} for chlorophacinone, 108mg.kg^{-1} for coumatetralyl and 490mg.kg^{-1} for diphacinone. Eason and Wickstrom (2001) report that diphacinone is more toxic than warfarin to rodents; this was demonstrated previously in Chapter 2 against the susceptible House mouse strain and is shown here against female mice homozygous for the *VKORC1* mutation Y139C.

No previous studies have clarified the resistance factor for any anticoagulant active ingredient against House mice homozygous for the *VKORC1* mutation Y139C; most studies have concentrated on Norway rat strains which have *VKORC1* mutations that make them anticoagulant resistant. This study shows the magnitude of resistance demonstrated by House mice homozygous for the *VKORC1* mutation Y139C in comparison to susceptible albino House mice.

The resistance factor for coumatetralyl for both sexes is high, 30.8 and 21.6 for males and females respectively (Table 3.5). A resistance factor of this magnitude is known as 'practical resistance' and is expected to be associated with the failure in treatment of coumatetralyl against House mice homozygous for the *VKORC1* mutation Y139C, control of these House mice would be unexpected when using the active ingredient coumatetralyl. The resistance factor for chlorophacinone, diphacinone and warfarin sodium are extremely high for both sexes, 628 for chlorophacinone, 538 for diphacinone, and 275 for males and 533 for females for warfarin sodium (Table 3.5); total failure of control using chlorophacinone, diphacinone or warfarin sodium against resistant House mice homozygous for the *VKORC1* mutation Y139C would be expected. In some countries chlorophacinone and diphacinone are still licensed for use by amateurs against House mouse infestations. Use of anticoagulant rodenticides which contain these ineffective active ingredients against House mice known or suspected to be homozygous for the *VKORC1* mutation Y139C will exacerbate the resistance problem with only House mice that are susceptible succumbing to the treatment while resistant individuals survive to produce the next generation; this is a form of selective breeding and has the potential to create a wild population which is fully resistant to the active ingredients chlorophacinone and diphacinone.

It has been recognised since the 1980s that House mice have a degree of resistance to difenacoum and bromadiolone (Rowe *et al*, 1981) but the magnitude of the resistance was not clarified. The wild House mice from which the laboratory mice used in this study were derived were known to be highly resistant to warfarin and bromadiolone (Pelz & Prescott, 2015), 80% survived a lethal feeding period test on 250ppm warfarin and 90% survived bromadiolone intubation at doses lethal to susceptible House mice (Prescott, 1996). Difenacoum and bromadiolone are the most popular rodenticides used in the UK as they can be used both indoors and outdoors and due to the restriction on the use of the more potent SGARs (Buckle & Prescott, 2012; Shore *et al*, 2003),; it is possible that the over-reliance on difenacoum and bromadiolone by amateurs has exacerbated the growth in resistance to them. To date difenacoum has been used successfully in the control of House mice although it is known that House mice homozygous for the *VKORC1* mutation Y139C do have a degree of resistance to this active ingredient (Prescott *et al*, 2017).

The resistance factors for brodifacoum, difenacoum, difethialone and flocoumafen were all below 5 for both sexes; these four anticoagulants would be expected to successfully control House mice homozygous for the *VKORC1* mutation Y139C. Flocoumafen had the lowest ED_{50} and resistance factor for both sexes (Table 3.6); brodifacoum was believed to be the most potent SGAR active ingredient achieving 100% mortality in warfarin resistant House mice and Norway rats after 1 days feeding, no other SGAR active ingredient achieved this level of mortality (Buckle & Eason, 2015) but results here have indicated that flocoumafen is the most potent SGAR active ingredient against House mice homozygous for the *VKORC1* mutation Y139C, in this study however the significance was not proven. Similarly, difethialone was also found to have a lower resistance factor than brodifacoum; difethialone is known to be highly potent against warfarin-resistant rodents and results here have demonstrated how potent. Resistance to brodifacoum, difenacoum, difethialone and flocoumafen may be termed

'technical resistance' as it is unlikely to have an effect of the treatment outcome (Pelz & Prescott, 2015) when any of these active ingredients are used but it can be detected in the lab using sensitive tests. Interestingly, the resistance factor for male mice was lower for difenacoum than for brodifacoum and difethialone which are both more potent active ingredients. Some resistance to difenacoum has been reported in House mice in the UK but overall this compound seems to be effective (Buckle & Eason, 2015); although difenacoum is not advised to be used against House mice populations suspected of carrying the mutation Y139C (Buckle & Prescott, 2012).

The resistance factor for bromadiolone for both sexes was above 10, confirming resistance to bromadiolone that has been reported for the strain in previous studies in the UK and Germany; the identical *VKORC1* mutation in Norway rats is also associated with resistance to bromadiolone (Buckle & Eason, 2015). Resistance to bromadiolone may be termed 'practical resistance' as a population of House mice homozygous for the *VKORC1* mutation Y139C is unlikely to be controlled by bromadiolone treatment (Pelz & Prescott, 2015). The use of difenacoum and bromadiolone to control Norway rats homozygous for the *VKORC1* mutation Y139C has been almost ineffective when there has been a high level of resistance within the population, however difenacoum is slightly more effective than bromadiolone (Pelz & Prescott, 2015), which is similar to the results presented in this study for House mice homozygous for the *VKORC1* mutation Y139C. Continued use of bromadiolone to control House mice homozygous for the *VKORC1* mutation Y139C will exacerbate the resistance problem, as only susceptible House mice will be controlled whereas the majority of the population, which carry the *VKORC1* genetic mutation Y139C, will survive to breed the next resistant generation. Brodifacoum, difethialone and flocoumafen are the most potent of the second-generation anticoagulant active ingredients; only a small dose of the active ingredient

is required for a lethal dose which is important in House mice due to their sporadic feeding behaviour.

A similar study has not been undertaken for House mice homozygous for the *VKORC1* mutation L128S which is also present in the UK House mouse population. It was known in the 1990s that some of these House mice could not be controlled with difenacoum, so it may be that where anticoagulant resistance is suspected both bromadiolone and difenacoum baits should not be used (Prescott *et al* 2017) as their use would increase the selection for homozygous resistant individuals.

In the wild, House mouse populations will consist of some susceptible individuals, some homozygous resistant individuals, and some individuals which are heterozygous for the *VKORC1* resistance mutation. It may be plausible to consider that heterozygous individuals will be more resistant to anticoagulants than susceptible individuals but the level of resistance is unknown, and conversely, they may be less resistant than individuals homozygous for the *VKORC1* mutation.

Chapter 4 –The establishment of second-generation susceptibility baselines against a strain of House mouse heterozygous for the *VKORC1* mutation Y139C, and the assessment of resistance factors

4.1 Introduction

All current published studies of blood clotting response focus on homozygous resistant and susceptible strains of rodents. In wild House mouse populations it is highly likely that the population will contain homozygous resistant, heterozygous resistant and susceptible individuals. Before the use of anticoagulant rodenticides, rodent populations were susceptible to these active ingredients and it is highly likely that the vast majority of the individuals were either susceptible or heterozygous. However, the continued use and reliance on ineffective anticoagulants against these rodent populations resulted in susceptible individuals being selectively killed and the majority of the surviving population being heterozygous, and over time homozygous individuals would become more frequent. The BCR studies against homozygous resistant animals are looking at the worst case scenario, as in most populations the majority of individuals will be heterozygous. To date the effect of heterozygosity on the degree of resistance in any House mouse species is unknown although it is suspected that heterozygous resistant animals may be less resistant than homozygous resistant animals. Grandemange *et al* (2009) have investigated the effect of heterozygosity in Norway rats carrying the *VKORC1* mutation Y139F and found that they were more resistant than a susceptible strain of Norway rat to the FGAR active ingredient chlorophacinone and the SGAR active ingredients bromadiolone and difenacoum.

4.1.1 Heterozygous advantage – vitamin K requirement

Although suspected to be less resistant than homozygous resistant rodents, heterozygous resistant rodents are potentially fitter than homozygous resistant and anticoagulant susceptible individuals in the field. Homozygous resistant rodents require about 20 times the dietary vitamin K that anticoagulant susceptible individuals require, whereas heterozygous resistant individuals only require two to three times the amount (Bishop, 1981). The activity of the altered *VKORC1* in homozygous resistant individuals is lower than in heterozygous resistant individuals, the enzyme has a lower affinity to anticoagulants so is less inhibited but conversely it has a lower affinity for vitamin K analogues such as vitamin K epoxide therefore it is less efficient in converting vitamin K epoxide to vitamin K (Berody & Smith, 1993; Greaves *et al*, 1977) and ultimately is unable to produce clotting factors when consuming normal levels of dietary vitamin K (Smith & Greaves, 1986), so homozygous resistant individuals are in a constant state of vitamin K deficiency and have a higher requirement for dietary vitamin K (Ishizuka *et al*, 2008) to maintain normal levels of clotting factors. Hermodson *et al* (1969) maintained anticoagulant susceptible, homozygous resistant and heterozygous resistant rats on a vitamin-K deficient diet for 3 days which caused the prothrombin concentrations of all the individuals to become less than 30 units/ml. To return prothrombin levels to normal (200-230 units/ml) anticoagulant susceptible individuals required 1.0µg/day vitamin K for 4 days, heterozygous resistant individuals required 3.0µg/day for 7 days, and homozygous resistant individuals required 20.0µg/day for 3 days (Hermodson *et al*, 1969).

4.1.2 Determining heterozygous resistant individuals

Early resistance tests could distinguish between anticoagulant resistant and anticoagulant susceptible individuals but could not distinguish between homozygous and heterozygous resistant individuals. Laboratory tests developed in the 1970s by Martin *et al* (1979), initially on laboratory strains and then later on wild-caught rodents, have been able to distinguish between homozygous resistant, heterozygous resistant and anticoagulant susceptible individuals. In the early tests individuals were injected with a warfarin-vitamin K-oxide combination and their blood clotting time measured; 'susceptible' individuals had prolonged clotting times and 'resistant' individuals had normal clotting times. The 'resistant' individuals were fed on a vitamin-K deficient diet for the following 4 days, at the end of the 4 day period their blood clotting time was measured and homozygous resistant individuals had prolonged clotting times. Martin *et al* (1979) successfully classified laboratory strains of rats as 'susceptible', 'homozygous resistant' and 'heterozygous resistant' using this two-stage method. This two-stage test was then used to classify wild-caught individuals into three groups; susceptible individuals which had prolonged clotting times caused by warfarin, heterozygous resistant individuals which did not suffer from vitamin K deficiency after feeding on vitamin-K deficient diet for 4 days, and homozygous resistant individuals which became vitamin K deficient after feeding for 4 days on vitamin-K deficient diet; the 'homozygous resistant' genotype was confirmed by backcrossing the individuals with susceptible individuals and all the resulting offspring were anticoagulant resistant (Martin *et al,* 1979).

4.1.3 Anticoagulant resistance and body weight

The high requirement for vitamin K is a severe disadvantage to homozygous resistant individuals whilst giving birth, as females are unlikely to survive haemorrhage if there is a lack of available dietary vitamin K (Bishop *et al*, 1977; Smith & Greaves, 1986); vitamin K is also a requirement for bone formation so reproductive function may not be supported in highly resistant females (Ishizuka *et al*, 2008). The inability of homozygous resistant individuals to produce normal levels of clotting factors at normal levels of dietary vitamin K intake is a severe disadvantage in the wild where there are many opportunities for injury to occur which may cause internal haemorrhaging (Smith & Greaves, 1986). Heterozygous resistant rats have been found to be larger than homozygous resistant individuals and anticoagulant susceptible individuals (Smith *et al*, 1994); this is most likely due to the requirement for vitamin K in bone formation and other key proteins in the body (Berody & Smith, 1993). Growth may be compromised in homozygous resistant individuals, vitamin K being prioritised in maintaining levels of blood clotting factors at the expense of growth and fitness. Body size is related to social dominance in rodents (Smith *et al*, 1994), especially in rat populations, heavier individuals are more dominant so it may be that homozygous resistant individuals are less dominant than heterozygous resistant individuals, but it may also be that homozygous resistant individuals are less competitive and weaker in a social situation due to increased risk of internal haemorrhage and bruising (Berody & Smith, 1993).

In the presence of anticoagulants there is a selection against anticoagulant susceptible individuals, homozygous resistant and heterozygous resistant individuals have an advantage; but in the absence/limited availability of dietary vitamin K homozygous resistant individuals are at a disadvantage due to their increased requirement for dietary vitamin K, and are

selected against (Pelz & Prescott, 2015). Many farm animal feeds have menadione (vitamin K3) included, this provides an important source of dietary vitamin K to rodenticide resistant rodents and effectively neutralises the cost of anticoagulant resistance.

The heterozygous strain in this study was created by breeding resistant male House mice homozygous for the *VKORC1* mutation Y139C with susceptible albino female House mice and all the F1 progeny were heterozygous for the *VKORC1* mutation Y139C. Anticoagulant resistance in male and female House mice heterozygous for the *VKORC1* mutation Y139C against the five second-generation active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen was determined by the generation of BCR dose-response data and estimating the resistance factors. The magnitude of resistance of the heterozygous strain was compared to the data generated for the homozygous resistant strain of House mice in Chapter 4 and with previously published data for susceptible House mice from Prescott *et al* (2007).

Susceptibility baseline data will be produced for a House mouse strain heterozygous for the *VKORC1* mutation Y139C against the second-generation active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen. The resistance factors will be estimated for the active ingredients to determine the level of resistance in comparison to the albino susceptible House mouse strain.

4.2 Methods

4.2.1 Establishment of the House mouse strain heterozygous for the VKORC1 mutation Y139C

Female CD-1 albino House mice were obtained from Charles River UK Ltd; this is a fully anticoagulant susceptible strain of House mouse. After a settling in period breeding pairs were established, each female CD-1 albino House mouse was paired with a male House mouse homozygous for the *VKORC1* mutation Y139C. The homozygous resistant strain is the same strain as used in Chapters 3 and 6; it is a laboratory strain of House mouse that possesses the resistant gene derived from wild caught House mice and is homozygous for the *VKORC1* mutation Y139C. The F1 progeny were heterozygous for the *VKORC1* mutation Y139C; the F1 progeny were weaned at 21 days and were at least 4 weeks old at the time of oral dosing with a pre-determined dose of active ingredient.

4.2.2 Testing procedure

The planned initial doses for each active ingredient were 66% of the established ED_{50} dose for House mice homozygous for the *VKORC1* mutation Y139C calculated in Chapter 3 (Table 4.1). Initially 12 male and 12 female mice were allocated to each active ingredient dose. Subsequent doses and group sizes were determined in light of the previous results to generate data for PROBIT analysis. The initial doses for the sexes changed as results were often completed for one sex before dosing started for the next dose, this ensured excessive groups of animals were not dosed with concentrations which would not yield results of value.

Table 4.1: Initial doses for the active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen

	Homozygous Y139C ED_{50} (mg/kg)		66% ED_{50} (mg/kg)	
	Male	Female	Male	Female
Brodifacoum	0.68	0.68	0.45	0.45
Bromadiolone	32.5	35.3	21.5	23.3
Difenacoum	1.1	1.5	0.73	0.99
Difethialone	1.3	1.3	0.86	0.86
Flocoumafen	0.44	0.51	0.29	0.34

4.2.3 Generation of dose-response data and calculation of resistance factors

The methodology in section 2.2.2 to generate dose-response data for each sex/active ingredient combination was used. GENMOD analysis was used to determine whether the PROBIT lines were separate, parallel or coincident for the two sexes for each second-generation anticoagulant active ingredient. PROBIT analysis was used to generate the dose-response data for each sex/active ingredient combination depending upon the GENMOD results.

The methodology in section 3.2.1 was used to calculate the estimated resistance factors (RF) for the heterozygous resistant strain for each second-generation active ingredient, the data for the susceptible albino strain was calculated by Prescott *et al* (2007). The RF for the heterozygous resistant strain was compared to the RF calculated for the homozygous resistant strain in Chapter 3.

4.3 Results

4.3.1 Comparison of the PROBIT response of males and females

The PROBIT lines of the two sexes were separate for brodifacoum, they deviated significantly from a parallel response; coincident for bromadiolone, difethialone and flocoumafen, not deviating significantly from a parallel or from a coincident response, and parallel for difenacoum, not deviating significantly from a parallel response but deviating significantly from a coincident response for male and female House mice heterozygous for the *VKORC1* mutation Y139C (Table 4.2).

Table 4.2: GENMOD results for House mice heterozygous for the *VKORC1* mutation Y139C dosed with the active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen

SGAR	Strain	Deviance Separate	Deviance Parallel	Deviance Coincident	Chi sq. - from parallel	= p value	Chi sq. - from coincident	= p value
brodifacoum	h vs s	3.5053	17.9915	21.3369	14.4862	0.0001	3.3454	0.0674
bromadiolone	h vs s	1.506	4.5214	4.5728	3.0154	0.08248	0.0514	0.82065
difenacoum	h vs s	3.9359	7.2068	40.292	3.2709	0.0705	33.0852	<0.00001
difethialone	h vs s	7.8804	9.222	10.1919	1.3416	0.2468	0.9699	0.32471
flocoumafen	h vs s	1.2402	3.4265	3.6701	2.1863	0.1392	0.2436	0.6216

4.3.2 Generation of PROBIT dose-response percentiles

For each active ingredient and sex combination the dose-response data was analysed using PROBIT analysis taking into account the results of the GENMOD analysis, the data was analysed for the two sexes as separate, parallel or coincident.

4.3.3 Determination of the resistance factor

The ED_{50} of all five active ingredients were determined for both sexes of the House mice strain heterozygous for the *VKORC1* mutation Y139C. The dose-response data for the heterozygous resistant strain (Table 4.3) was compared to the equivalent data generated by Prescott *et al* (2007) for the susceptible House mouse strain to calculate the estimated resistance factors (RF) for the heterozygous resistant strain (Table 4.4).

Table 4.3: Summary PROBIT dose-response data for male and female House mice heterozygous for the *VKORC1* mutation Y139C against the active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen

		Effective dose [mg/kg] (lower – upper fiducial limits)				
Effective dose	Sex	Brodifacoum	Bromadiolone	Difenacoum	Difethialone	Flocoumafen
1%	Male	0.24 (0.10-0.34)	4.5 (2.7-6.0)	0.75 (0.68-0.79)	0.32 (0.16-0.44)	0.36 (0.32-0.39)
40%	Male	0.56 (0.42-0.67)	9.9 (8.1-11.5)	0.87 (0.84-0.91)	0.79 (0.63-0.92)	0.46 (0.44-0.48)
50%	Male	0.62 (0.49-0.74)	10.9 (9.2-12.6)	0.89 (0.86-0.93)	0.89 (0.74-1.02)	0.47 (0.46-0.50)
60%	Male	0.69 (0.57-0.83)	12.0 (10.3-13.9)	0.91 (0.88-0.95)	1.0 (0.85-1.1)	0.49 (0.47-0.51)
99%	Male	1.64 (1.24-3.11)	26.4 (21.3-38.6)	1.1 (1.0-1.2)	2.5 (2.0-4.1)	0.62 (0.58-0.70)
1%	Female	0.38 (0.31-0.42)	4.5 (2.7-6.0)	0.61 (0.56-0.65)	0.32 (0.16-0.44)	0.36 (0.32-0.39)
40%	Female	0.48 (0.45-0.52)	9.9 (8.1-11.5)	0.72 (0.69-0.74)	0.79 (0.63-0.92)	0.46 (0.44-0.48)
50%	Female	0.50 (0.46-0.54)	10.9 (9.2-12.6)	0.73 (0.71-0.75)	0.89 (0.74-1.0)	0.47 (0.46-0.50)
60%	Female	0.51 (0.48-0.56)	12.0 (10.3-13.9)	0.75 (0.73-0.77)	1.0 (0.85-1.1)	0.49 (0.47-0.51)
99%	Female	0.65 (0.59-0.84)	26.4 (21.3-38.6)	0.88 (0.83-0.96)	2.5 (2.0-4.1)	0.62 (0.58-0.70)

Table 4.4: Calculated resistance factors (RF) for male and female House mice heterozygous for the *VKORC1* mutation Y139C

Active Ingredient	**ED_{50} for heterozygous strain**		**ED_{50} for susceptible strain**		**Resistance factor heterozygous strain**	
	male	**female**	**male**	**female**	**male**	**female**
Brodifacoum	0.62	0.50	0.39	0.35	1.6	1.4
Bromadiolone	10.9	10.9	2.0	1.7	5.5	6.4
Difenacoum	0.89	0.73	0.85	0.56	1.0	1.3
Difethialone	0.89	0.89	0.83	0.83	1.1	1.1
Flocoumafen	0.47	0.47	0.51	0.44	0.9	1.1

4.4 Discussion

The heterozygous House mouse strain had ED_{50} values below values for the homozygous strain and similar values to the susceptible strain for all the active ingredients apart from bromadiolone. Similar results were found in a study by Grandemange *et* al (2009) for a strain of Norway rat heterozygous for the *VKORC1* mutation Y139F, prothrombin times (PT) for the heterozygous strain were lower than or near basal levels the PT for the susceptible strain for bromadiolone, chlorophacinone and difenacoum. The estimated resistance factor values were lower for the heterozygous strain than for the homozygous strain for all the active ingredients.

House mice heterozygous for the *VKORC1* mutation Y139C only carry one copy of the mutated gene whereas the homozygous strain carry two copies, therefore it would be expected that the homozygous strain would be more resistant to the anticoagulant active ingredients.

The heterozygous resistant strain of House mouse was found to have lower resistance factors than the homozygous resistant strain for all five second-generation active ingredients. The resistance factor for the House mouse strain heterozygous for the *VKORC1* mutation Y139C was 5.5 for males and 6.4 females for the active ingredient bromadiolone; this is considerably less than the calculated resistance factor for the homozygous resistant strain, 16.3 and 20.8 for males and females respectively (Table 4.5). The high resistance factors of the homozygous resistant strain would suggest that this strain would not be controlled using an anticoagulant rodenticide that contains the active ingredient bromadiolone.

Table 4.5 Calculated resistance factors (RF) for House mice heterozygous and House mice homozygous for the *VKORC1* mutation Y139C

Active Ingredient	Resistance factor heterozygous strain		Resistance factor for homozygous strain	
	male	female	male	female
Brodifacoum	1.6	1.4	1.7	1.9
Bromadiolone	5.5	6.4	16.3	20.8
Difenacoum	1.0	1.3	1.3	2.7
Difethialone	1.1	1.1	1.6	1.6
Flocoumafen	0.92	1.1	0.86	1.2

It is not possible to predict the treatment outcome of the use of bromadiolone against a House mouse population heterozygous for the *VKORC1* mutation Y139C but from the calculated resistance factors bromadiolone would be more efficient in the control of heterozygous resistant individuals than the control of homozygous resistant individuals. However, bromadiolone would act as a selection factor if used to control a population of House mice of unknown resistance status; initially the susceptible individuals would be eradicated, then the heterozygous resistant individuals would be controlled, this would lead to an increase in the incidence of the resistance gene and ultimately the population would be expected to become homozygous resistant. The continued use of rodenticides containing bromadiolone would be expected to produce a 'selected line' with the individuals being highly resistant; this is similar to the 'selected line' of Norway rats created by Greaves and Cullen-Ayres (1988), and to the initial generations produced in Chapter 6 of this thesis.

The calculated resistance factors for the other four active ingredients; brodifacoum, difenacoum, difethialone and flocoumafen, against both sexes of the heterozygous resistant strain were all below 1.6. Similarly, low resistance factors, all below 2.7, for both sexes of the homozygous resistant strain were calculated (Table 4.5).

All of the second-generation anticoagulant active ingredients tested, with the possible exception of bromadiolone would be expected to successfully control House mice heterozygous for the *VKORC1* mutation Y139C. Where bromadiolone is used to control a population of House mice that possess the *VKORC1* genetic mutation Y139C susceptible individuals would be controlled effectively and in the resulting population heterozygous resistant individuals would be more effectively controlled than homozygous resistant individuals. After the completion of treatment there would be no susceptible individuals and an increasing number of homozygous resistant individuals in the population; subsequent breeding would result in some susceptible individuals (25% of offspring from heterozygous x heterozygous breeding) but the majority of the population would be resistant and the incidence of the *VKORC1* genetic mutation Y139C in the population would increase.

The data calculated in Chapters 2 to 4 is of use to pest control operators who know the genetic make-up of the House mouse populations they are trying to control. In combination with genetic analysis of tissue samples from any House mice trapped, this data may be used to develop a pest control strategy specific to the House mouse population; the population is likely to contain susceptible, heterozygous resistant and homozygous resistant individuals.

Chapter 5 – The identification and distribution of the *VKORC1* resistant mutations in tissue samples derived from UK wild caught House mice

5.1 Introduction

5.1.1 Determining anticoagulant resistance in rodents

The development of genetic analysis has allowed the rapid identification of *VKORC1* resistant mutations in rodent populations and the presence of specific mutations has been found to correlate well with previous known distributions of anticoagulant resistance, particularly with Norway rats (Pelz *et al*, 2005; Rymer thesis 2017). However, the identification of the different *VKORC1* resistance mutations provides no information on their likely impact in practical terms on treatment outcome for the different anticoagulant active ingredients.

When anticoagulant resistance was first identified the only tests to confirm the presence of resistant individuals were laboratory no-choice feeding tests, as previously described in Chapter 2. These tests had limitations to their level of accuracy as many wild rodents will not feed consistently in the laboratory environment, making it difficult to distinguish between 'resistant' animals and poor feeders. These tests were time consuming, labour intensive and inhumane, as many animals died as a result of the anticoagulant (Prescott *et al*, 2007). Furthermore, 'anticoagulant resistance' is a highly variable trait and difficult to identify reliably using feeding tests (Greaves & Ayres, 1977).

With blood clotting response (BCR) tests, the active ingredient was administered by oral gavage, thus overcoming the major disadvantage of lethal feeding period (LFP) feeding tests. BCR tests are also considered more humane, as the test animals are humanely killed 24 hours after dosing, prior to the development of any symptoms of anticoagulant toxicity. However, BCR tests still require the capture of wild rodents and are thus both time consuming and expensive (Buckle & Prescott, 2012). A major advantage of recent BCR tests is that they provide a means of estimating the resistance factor for each species/sex/active ingredient combination (see Chapters 3 and 4), and can be used to quantify the magnitude of the resistance, thus providing insight into the likely impact of the resistance on treatment outcome (Buckle *et al*., 2007). Resistance level may also be estimated by measuring VKOR activity, VKOR activity may be modified in resistant strains of rodents and work is still ongoing in various laboratories to identify the impact of *VKORC1* mutations on VKOR activity (Berny *et al*, 2018).

5.1.2 DNA sequencing to determine anticoagulant resistance

The use of DNA sequencing to identify rodents carrying genetic mutations for anticoagulant resistance is a recent technique and has been used to show the distribution of anticoagulant resistance in several countries in Europe (Buckle & Prescott, 2012; Prescott *et al*, 2017). Genetic testing is quick and more cost effective than BCR testing. DNA can be mapped from small pieces of tissue from a tail tip or from fresh faecal pellets; although the procedure using faecal pellets has a lower success rate and is more prone to contamination, and it is impossible to distinguish between individuals so it is difficult to assess the level of resistance in a population (Berny *et* al., 2018; Pelz & Prescott, 2015). Genetic testing can identify and monitor the presence of anticoagulant resistant individuals in a population but it does not

quantify the severity of the resistance in terms of treatment outcome. To evaluate the severity of resistance, the resistance factor of the identified genetic mutation must be calculated for the anticoagulant active ingredients being used in order to control the rodent populations (Pelz & Prescott, 2015) for each sex and species.

Anticoagulant rodenticides were introduced in the 1950s at around the same time as anticoagulants were beginning to be used to treat and prevent thrombosis in humans (Pelz *et al*, 2005). Anticoagulants have similar modes of action in both humans and rodents; they target the vitamin K epoxide reductase complex, disrupting the recycling of vitamin K which in turn inhibits the carboxylation of clotting factors and hence compromises blood coagulation (Pelz *et al*, 2005; Rost *et al*, 2004). The activity of vitamin K epoxide reductase was first described in the 1970s but the gene (*VKORC1)* which encodes for this enzyme was not identified until 2004 (Rost *et al*, 2004). The vitamin K epoxide reductase enzyme is an important component of the vitamin K cycle, involved in the recycling of the oxidised form of vitamin K (vitamin K-2,3-epoxide) back to the reduced form of vitamin K (hydroquinone). Genetic mutations in the *VKORC1* gene lead to a reduction in the activity of the vitamin K epoxide reductase enzyme and the enzyme becomes more resistant to anticoagulant inhibition (Pelz *et* al., 2005). Rost *et al* (2004) described the link between mutations in the *VKORC1* gene in humans and the human blood coagulation disorders, and the link between mutations in the *VKORC1* gene in Norway rats and warfarin resistance. The *VKORC1* gene has been mapped to a region on chromosome 16 in humans, it has three exons which code for a protein 163 amino acids long and mutations in the third exon have been linked with anticoagulant resistance in humans (Rost *et al*, 2004). Exons are the part of the *VKORC1* gene which code for mRNA (messenger RNA), the mRNA undergoes translation to synthesise the protein vitamin K epoxide reductase, any mutation in an exon causes the base pair to change and hence a different amino acid is coded for. This region of chromosome 16 in humans is

similar to the region on chromosome 1 in Norway rats and the region on chromosome 7 in House mice, which are linked to anticoagulant resistance (Li *et al*, 2004). These regions of chromosomes are orthologous in the three different species, and combined with the link between missense mutations in the *VKORC1* gene of humans being linked with warfarin resistance led to the conclusion that the *VKORC1* gene may be associated with warfarin resistance in Norway rats and House mice (Pelz *et al*, 2005).

Breeding experiments have led to the conclusion that the gene responsible for anticoagulant resistance in both Norway rats and House mice is a single dominant autosomal gene and is located on chromosome 1 and chromosome 7 in Norway rats and House mice respectively (Pelz *et al*, 2005). Pelz *et al* (2005) identified at least seven different missense mutations associated with anticoagulant resistance in Norway rats and at least two in House mice throughout Europe, with some Norway rats being heterozygous for two different mutations. Some mutations have been found in several different Norway rat populations in Europe, the spread of resistance is most likely due to the migration of individuals throughout Europe (Pelz *et al*,. 2005). Following the advent of DNA sequencing of tissue samples from rodents to identify genetic mutations, 'Hampshire' Norway rats were found to carry the *VKORC1* mutation L120Q, which is also found to be present in 'Berkshire' Norway rats (Pelz & Prescott, 2015). Norway rats which have the *VKORC1* mutation L120Q are now found throughout southern England and in some parts of France and Belgium.

5.1.3 Genetic mutations in the VKORC1 gene

The main mutations identified in Norway rats to date are Y139S (Welsh), L128Q (Scottish), L120Q (Hampshire and Berkshire), Y139C (Denmark, north-west Germany), and Y139F (France,

Belgium, Netherlands) (Pelz & Prescott, 2015). Two single nucleotide polymorphisms (SNPs) linked to anticoagulant resistance have so far been identified in House mice in the UK, Y139C (Reading resistance) and L128S (Cambridge resistance). There is a possibility of further unidentified resistance SNPs in UK House mice (Pelz & Prescott, 2015), but to date only Y139C and L128S mutations have been identified.

House mice with the mutation L128S were first identified in individuals trapped near Cambridge, UK which were resistant to warfarin (Pelz *et* al., 2011). A laboratory strain of House mice that were homozygous for the *VKORC1* mutation L128S, was established by crossing wild-caught House mice with warfarin-susceptible LAC-grey laboratory mice and breeding from offspring that were found to be warfarin resistant (Pelz *et al*, 2011). House mice homozygous for the mutation L128S that have been found to be resistant to warfarin, have been reported to be resistant to all other first-generation anticoagulant active ingredients, and have survived no-choice feeding tests with bromadiolone and difenacoum (Pelz & Prescott, 2015). However, there has been little research conducted on the magnitude of the resistance for the different anticoagulant active ingredients in House mice homozygous for the *VKORC1* mutation L128S.

The House mouse strain carrying the mutation Y139C was derived from wild-caught individuals from the area around Reading, UK which were crossed six generations onto warfarin-sensitive laboratory Swiss mice and then backcrossed to produce a homozygous strain (Pelz *et al*, 2011). House mice homozygous for the *VKORC1* mutation Y139C are highly resistant to warfarin and the second-generation compound bromadiolone (Prescott, 1996). However, their susceptibility to the other anticoagulants was currently unknown before this study.

The *Spretus* mutation has been identified in House mice in Europe; but to date it has not been identified in House mice in the UK. The *Spretus* mutation is a group of linked amino acid substitutions; R12W, A26S, A48T and R61L (Pelz *et al*, 2011). The *Spretus* mutation is believed to have transferred from *Mus spretus* to *Mus musculus/domesticus* via hybridization in southern Europe and individuals carrying the mutation have now been identified in Germany, Switzerland, Spain and France (Pelz & Prescott, 2015). As seen in the L128S homozygous and Y139C homozygous House mouse strains, first-generation anticoagulants have little effect in controlling House mice which carry the *Spretus* mutations, the main concern is that all three different resistant strains display varying levels of resistance to the second-generation active ingredients bromadiolone and difenacoum (Pelz & Prescott, 2015). Female House mice carrying the *Spretus* mutation can survive doses up to 10 times the LD_{50} of bromadiolone and some individuals can also survive high doses of difenacoum. However, the three second-generation anticoagulants difethialone, brodifacoum and flocoumafen have been reported to successfully control House mice carrying the *Spretus* mutation (Goulois *et al*, 2016).

In total 15 mutations have been identified in House mice in Europe to date; the single *VKORC1* mutations L128S, Y139C and *Spretus* are associated with severe resistance to the first-generation anticoagulants and limited resistance to the second-generation anticoagulants (Goulois *et al*, 2017). In studies throughout Europe high proportions of wild-caught House mice have been found to possess *VKORC1* mutations (Goulois, *et al*, 2017), which may be due to the high use of ineffective anticoagulant rodenticides; thus creating a selective pressure on the population whereby the homozygous resistant individuals have an advantage over the susceptible and heterozygous resistant individuals. Ineffective application of rodenticides may also be partly to blame for the increase in resistance; mice are sporadic feeders so if they have access to many food sources including rodenticides, over generations, they may be able to build up a tolerance to many of the less potent anticoagulant active ingredients.

By combining the resistance factors for all first and second-generation anticoagulant active ingredients which have been determined in Chapters 3 and 4 for House mice homozygous and heterozygous for the *VKORC1* mutation Y139C, genetic analysis of individuals from wild populations will give insight into the active ingredients that are likely be efficacious against mice possessing the Y139C mutation. For the other *VKORC1* mutations, further work is required. With this data set, pest control operators will be able to target resistant House mice that possess the *VKORC1* mutation Y139C more effectively, using reduced quantities of effective anticoagulant rodenticide. The use of effective anticoagulants would reduce the selection for resistance, and reduce the risk to non-target species (as successful treatment outcomes would be expected to occur more quickly). Extensive research has been undertaken to identify the distribution of anticoagulant resistant Norway rats but similar research with House mice has been limited. This chapter describes the initial stages of research into the distribution of anticoagulant resistant House mice in the UK, but further work in collaboration with pest controllers throughout the UK is required.

Tissue samples will be collected from wild caught House mice throughout parts of the UK in order to identify and determine the distribution of House mice that convey *VKORC1* mutations.

5.2 Methods

5.2.1 Genetic sequencing

Pest control operators were contacted to ask for their assistance in the collection of tail samples from House mice. It was requested that the areas selected for collection were a random representation of the House mouse population in the pest controller's area and a

maximum of two samples per site were collected to reduce the risk of sampling mice from the same family group. Pest control operators were provided with vials containing 80% ethanol to place the tail samples in after collection, samples had to come from House mice that had been trapped to ensure samples were fresh and had not come from mice that had succumb to poisoning; (to reduce any selection for the wild-type susceptible genotype). A sample of 2-3cm in length of tail was taken from the mice and placed in the vial; the samples were either delivered to the University of Reading within 24 hours of collection, or frozen and shipped at a later date. The postcode/GPS location of each sample was provided by the pest control operators.

The DNA from these samples was purified and amplified then regions; exon 1, exon 2 and exon 3, of the *VKORC1* gene were sequenced to identify any mutations which may confer anticoagulant resistance. The base sequence of the *VKORC1* gene of wild-type anticoagulant susceptible House mice is known and any sequence deviations were used to identify anticoagulant resistance (Table 5.1). The mutations associated with resistance to first and second-generation anticoagulants are located on exon 3; the *Spretus* mutation is located on exon 1.

Table 5.1: Original (wild-type) and new (resistant) codons for House mice for the two *VKORC1* mutations identified in the UK, L128S and Y139C

Mutation	Original (wild-type) codon	Codon Sequence (wild-type)	New (resistant) codon
L128S	TTA	TTT-GTG-**TTA**-TAT	TCA
Y139C	TAT	ACC-ACC-**TAT**-GCC	TGT

The following methodology was used to extract, purify, amplify and sequence the DNA:

1. Tail tip samples were collected and preserved in 80% alcohol in individual tubes. If the samples were not going to be processed upon arrival in the laboratory they were stored at -20^{o}C.
2. The Qiagen DNeasy tissue extraction kit was used to extract genomic DNA following the manufacturers guidelines (Qiagen Ltd, UK).
    2.1. Approximately 4mm of tissue was shaved from the end of the tail tip using a sterile sharp razor blade.
    2.2. The tissue shaving was placed in a 1.5ml microtube and 180µl of pre-warmed lysis buffer ATL and 20µl of proteinase K was added. The tubes were vortexed and incubated at 55˚C overnight.
3. The genomic DNA was then purified and eluted.
    3.1. 400µl of buffer AL-ethanol mixture was added to the sample and the tubes were vortexed.
    3.2. 600µl of the mixture was pipetted into a DNeasy mini spin column and placed in a new 2ml spin column and centrifuged at 8000 rpm for 1 minute; the flow through and collection tube were discarded.
    3.3. The mini column was placed in a new collection tube; 500µl of wash buffer AW1 was added and the sample was centrifuged at 8000 rpm for 1 minute to elute waste products from the spin column.
    3.4. The mini column was placed in a new collection tube; 500µl of wash buffer AW2 was added, the sample was centrifuged at 13000 rpm for 3 minutes.
    3.5. The mini column was placed in a new microcentrifuge tube; 70µl of buffer AE was added directly to the DNeasy membrane, incubated at room temperature for 2 minutes then centrifuged for 1 minute at 8000 rpm to elute.

3.6. Step 3.5. was repeated once to ensure the extracted DNA was sufficiently purified.

4. The extracted DNA could now undergo Polymerase Chain Reaction (PCR). Two primers were used to sequence the DNA forwards (primer F) and backwards (primer R), primers are short pieces of single-stranded DNA that are complementary to the target sequence. Primers used for detecting SNPs in exon 1 (*Spretus* resistance) were F: CTG GAC TCG TGC GGC TTG and R: GAG AGG AGA AGA CGC GGG; primers used for exon 3 (FGAR and SGAR resistance) were F: TAC TGG TGC TGA GTT CCC TG and R: TTA GTC TGG CAT GAG GTG GG. The DNA polymerase synthesised new DNA from the ends of each primer by adding nucleotide bases to the ends. The extracted DNA was used as a template and new double strand DNA was produced.

   4.1. The sample was put in the PCR machine and the program 'Rat Exons' was selected. The 'Rat Exons' programme is: two minutes at 94^{o}C; then 35 cycles of denaturation at 94^{o}C, each cycle is thirty seconds; annealing at 57^{o}C for thirty seconds; elongation at 72^{o}C for thirty seconds; then a final extension at 72^{o}C for three minutes. The temperature was then reduced to 4^{o}C and held there until the samples were removed from the PCR machine.

5. The PCR products were evaluated using electrophoresis and examined under UV light.

6. The PCR products were purified using the Qiagen PCR purification kit.

   6.1. Buffer PB was added at a ratio of 5:1 to the successful PCR products.

   6.2. The solution was transferred to a spin column (in a 2ml tube) and centrifuged at 13000 rpm for 40 seconds.

   6.3. The flow through was discarded.

   6.4. 750µl of Buffer PE was added to the tube and centrifuged for 40 seconds at 13000 rpm, the flow through was discarded and the sample was centrifuged for one minute.

6.5. The column was transferred to a 1.5ml Eppendorf and 25µl Buffer EB was added. After a few minutes the sample was centrifuged at 13000 rpm for one minute.

6.6. The elution was the final DNA product to be sent for sequencing.

7. The PCR products were sequenced by Source Biosciences, Cambridge.
8. The results of the sequencing were analysed by eye using 'FinchTV' software. The codon sequences are known for wild-type (susceptible) individuals and individuals carrying mutations for the *VKORC1* gene so it is possible to use 'FinchTV' to search for the codon sequence before the wild-type sequence and identify any changes in the codon; for example when searching for the mutation L128S the codon 'TTT-GTG' would be searched for, an individual carrying the L128S mutation would have the codon sequence TTT-GTG-TCA-TAT. When searching for the Y139C mutation the codon 'ACC-ACC' would be searched for, an individual carrying the Y139C mutation would have the codon sequence ACC-ACC-TGT-GCC, the original and mutated codons are shown in Table 5.1.
9. House mice can carry more than one mutation so it was important to check for all mutations.

5.2.2 Mapping samples

After sequencing an online postcode converter (https://gridreferencefinder.com/the location) was used to convert the x and y coordinates of the samples to latitude and longitude for mapping. The sample locations were added to an ordnance survey base map. The *VKORC1* mutations were assigned different symbols dependent upon whether they were homozygous or heterozygous for the mutation; susceptible samples were also assigned a symbol.

5.3 Results

5.3.1 Genetic sequencing

Tail samples collected by pest control operators and the British Wildlife Centre while working in collaboration with Dr D. Rymer underwent DNA sequencing and their genetic mutation identified (Table 5.2). The samples were collected from areas around London and Reading, UK; unfortunately genetic mutations for the two samples collected from Reading, UK (RG postcodes) were not identified. Further samples were collected and analysed by the Vertebrate Pests Unit of the University of Reading, in collaboration with the Greater London Pest Control Group (Table 5.3).

Of the samples analysed in collaboration with Dr D Rymer 58.3% were homozygous for the *VKORC1* mutation L128S, 16.7% were homozygous for the *VKORC1* mutation Y139C and 1 sample was heterozygous for the *VKORC1* mutation Y139C.

Table 5.2: *VKORC1* mutations identified in House mouse tail samples collected in 12 locations from London and Reading, UK.

Sample	Location Postcode	Mutation(s)
1	London	L128S (hom)
2	London	L128S (hom)
3	London	L128S (hom)
4	London	L128S (hom)
5	EN9 3BZ	L128S (hom)
6	EN9 3BZ	L128S (hom)
7	EN9 3BZ	Y139C (het)
8	EN9 3BZ	Y139C (hom)
9	SW6 2SY	L128S (hom)
10	RG4 6AA	-
11	HA8 7BQ	Y139C (hom)
12	RG1 7HL	-

In total 32 samples were collected and analysed by the University of Reading, of these samples 84.4% were identified as carrying genetic mutations. The L128S *VKORC1* mutation was most prevalent; 43.8% (14) of samples of which 34.4% (11) of all the samples were homozygous and 9.4% (3) were heterozygous. The Y139C *VKORC1* mutation was identified in 34.4% (11) of the samples; 18.8% (6) of the total were homozygous and 15.6% (5) were heterozygous. Double mutations were identified in 2 (6.2%) of the 32 samples collected; one sample was homozygous for the *VKORC1* mutation L128S and heterozygous for Y139C, the other sample was heterozygous for both *VKORC1* mutations. Only 5 of the 32 (15.6%) samples collected and analysed were identified as susceptible.

Table 5.3: *VKORC1* mutations identified in House mouse tail samples analysed by the Vertebrate Pest Unit, University of Reading

Mutation	Homozygous or Heterozygous	% Total of Samples Collected
L128S	Homozygous	34.4
L128S	Heterozygous	9.4
Y139C	Homozygous	18.8
Y139C	Heterozygous	15.6
L128S + Y139C	Homozygous + Heterozygous	3.1
L128S + Y139C	Heterozygous	3.1
Susceptible	Homozygous	15.6

5.3.2 Mapping of samples

For the House mouse tail samples analysed by the University of Reading, their locations were mapped to show the distribution of the *VKORC1* genetic mutations across London and Greater London, plus one sample from East Sussex (Figure 5.1).

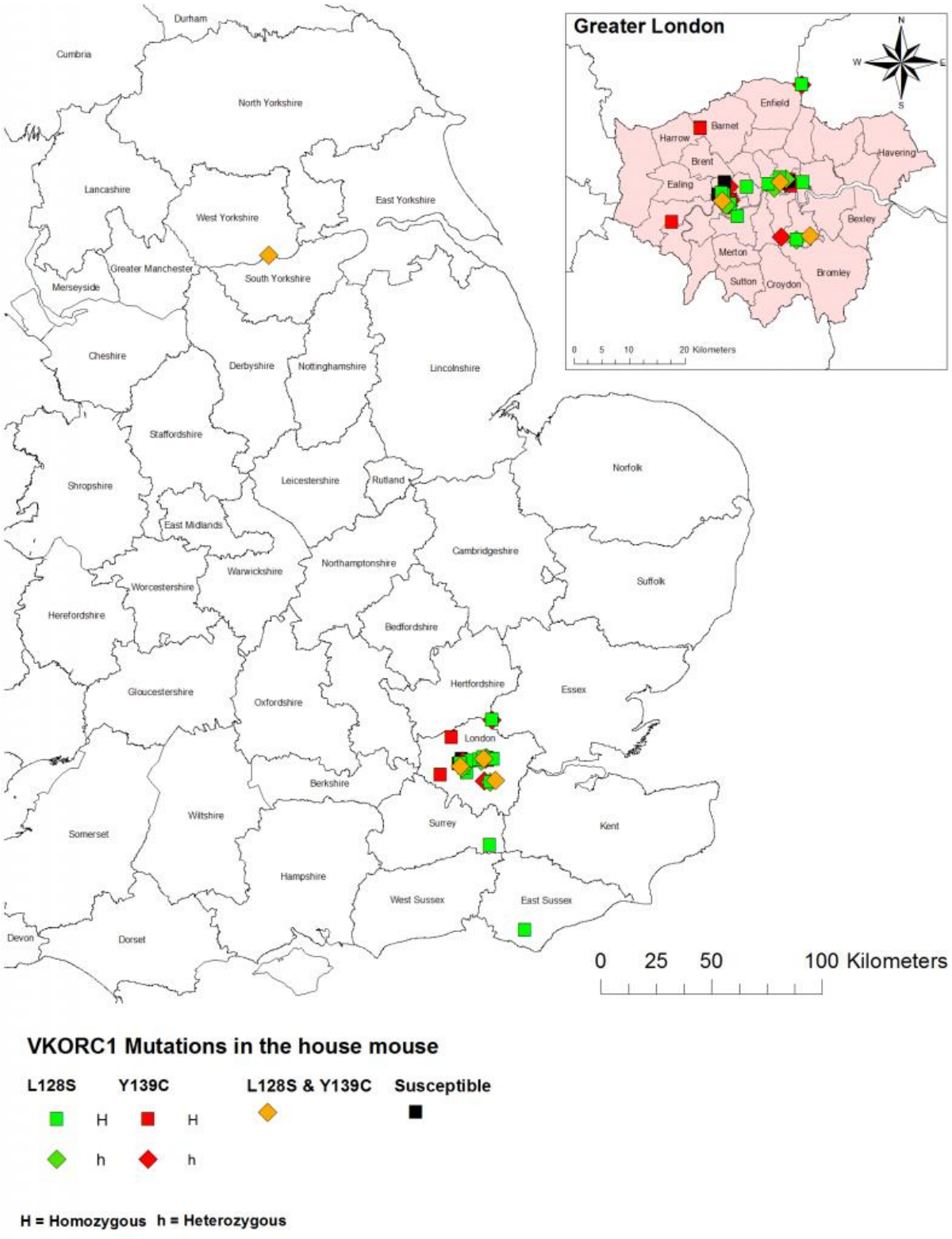


Figure 5.1: Distribution of House mice carrying *VKORC1* mutations throughout southern England (Prescott, Coan, Jones, Baxter and Rymer, unpublished data)

5.4 Discussion

The *VKORC1* mutations Y139C and L128S were identified in tissue samples, and over 58% of these samples were homozygous for either mutation which indicates a high degree of selection for anticoagulant resistance in the wild House mouse populations that were surveyed.

Recent advances in molecular methodology has revolutionised the detection of resistance in rodent populations and in the UK it has been extensively used against resistant Norway rats. Preliminary molecular resistance testing of wild House mice trapped in the UK has to date identified the *VKORC1* mutations Y139C and L128S. The molecular methodology is very good at identifying mutations of the main resistance gene *VKORC1* and additionally provides information about the genotype of the test animals (whether they are heterozygous or homozygous resistant), thus providing insight into the degree of selection that has been occurring in the wild populations. In combination with the known resistance factors of the molecular sequence variants for the different anticoagulant active ingredients (see Chapter 3 and Chapter 4), it is possible to determine the magnitude of the resistance and the impact of the molecular mutation on field control (Pelz & Prescott, 2015). As such, this can allow rodent control strategies to be developed to target rodents which carry the specific molecular sequence variants.

There have been many reports of anticoagulant treatment failures throughout the UK since the 1960s, when it was first discovered that warfarin was unsuccessful in the control of some House mouse populations. The rapid spread of anticoagulant resistance throughout the wild House mouse population suggests that there may have been resistant genotypes already present in the wild population. Even with resistant genotypes at a low frequency, the wide

use of anticoagulant rodenticides may have selected for these genotypes, resulting in them becoming increasingly widespread. Thus, it is also likely that 'bromadiolone resistance' has been present in wild populations before the introduction of bromadiolone as a rodenticide.

In total 7 samples were analysed in collaboration with Dr D Rymer and 37 samples were analysed by Ms E Coan and Ms C Jones of the Vertebrate Pest Unit, University of Reading (Figure 5.1). The majority, 46.9% of the samples were identified as carrying the *VKORC1* mutation L128S, 31.8% of the samples carried the mutation Y139C, 4.6% carried a double mutation (L128S + Y139C), and 11.4% were susceptible (Table 5.4). Over 58% of samples were identified as homozygous resistant which indicates a high degree of selection for anticoagulant resistance; homozygous resistant individuals have an advantage over heterozygous resistant and susceptible individuals. A similar situation has been observed in Germany; both *VKORC1* mutations identified in UK House mice plus the *Spretus* mutation have been found in wild populations of House mice in Germany. Pelz *et al* (2011) analysed tissue samples from wild House mice throughout Germany and the *VKORC1* mutations were found to be common and widespread, and a high proportion were homozygous so there had been a degree of selection in the wild population. The continued use of ineffective anticoagulants containing the active ingredient bromadiolone selects for homozygous resistant House mice as it is only effective against susceptible and heterozygous resistant individuals.

Table 5.4: *VKORC1* mutations identified in all House mouse tail samples analysed in collaboration with Dr D Rymer and the Vertebrate Pest Unit, University of Reading

Mutation	Homozygous or Heterozygous	% of Total Samples Analysed
L128S	Homozygous	40.1
	Heterozygous	6.8
Y139C	Homozygous	18.2
	Heterozygous	13.6
L128S + Y139C	Homozygous + Heterozygous	2.3
	Heterozygous	2.3
Susceptible	Homozygous	11.4

It is highly unlikely that House mouse infestations throughout the Greater London area would be controlled with any first-generation anticoagulant active ingredients or the second-generation active ingredient bromadiolone. The resistance factor has been previously calculated as between 5.5 and 6.5 for heterozygous resistant House mice and greater than 16.5 for homozygous resistant House mice (Table 5.5) so treatment failure is highly likely with the use of rodenticides containing the active ingredient bromadiolone where it was known that the local House mouse population carries the *VKORC1* mutation Y139C. House mice homozygous for the *VKORC1* mutation L128S have previously been reported as having the ability to survive choice and no-choice feeding tests with bromadiolone and difenacoum (Pelz Prescott, 2015), it is advisable not to use rodenticides containing either of these active ingredients where the House mouse population is known to be homozygous for the *VKORC1* mutation L128S.

Table 5.5 Calculated resistance factors (RF) for House mice heterozygous and House mice homozygous for the *VKORC1* mutation Y139C

Active Ingredient	Resistance factor heterozygous strain		Resistance factor for homozygous strain	
	male	female	male	female
Brodifacoum	1.59	1.43	1.74	1.94
Bromadiolone	5.57	6.50	16.60	21.02
Difenacoum	1.05	1.30	1.24	2.66
Difethialone	1.07	1.07	1.53	1.53
Flocoumafen	0.92	1.07	0.86	1.16

The initial genetically resistant populations of House mice were identified around Cambridge and then Reading, and now across London and the south coast of England (Figure 5.1). Therefore, it may be expected that further anticoagulant resistant House mouse populations will be found throughout the UK. In the early 1960s, populations of House mice around Harrogate in North Yorkshire were found to be resistant to warfarin, prior to this no control problems were reported (Dodsworth, 1961). These populations were discovered prior to the knowledge of genetic mutations in *VKORC1* and use of genetic analysis. It was speculated that the failure of warfarin to control these House mice was either due to the sporadic feeding behaviour of House mice or the poor application of the anticoagulant. Their resistance to warfarin was confirmed by trapping several individuals and feeding them warfarin in the laboratory for eight weeks after which time they were still healthy (Dodsworth, 1961).

Currently, there is no equivalent data set for either the L128S or *Spretus* mutation; but due to the similarly high incidence of mice being homozygous for the L128S mutation in Greater London it is possible that this mutation is also being selected for in a similar way as the Y139C mutation. It is plausible to consider that House mice that carry copies of both the genetic mutations would be very difficult to control using first-generation anticoagulant active ingredients and the second-generation active ingredient bromadiolone. Although no samples were identified as being homozygous for both mutations, it is possible that there are some House mice that are and that they would be likely to be highly resistant to all first-generation

active ingredients, bromadiolone and possibly the other second-generation active ingredients. This is due to the fact that it is possible the mutations have a cumulative effect, so the magnitude of resistance would possibly be higher in House mice homozygous for the double mutations.

Detailed studies have only been undertaken on the resistant populations identified in Cambridge and Reading, so it is possible that there are many more resistant populations throughout the UK. Due to the lack of tail samples being collected from various regions in the UK for genetic analysis, it is impossible to clearly define the genetic status of House mouse populations across the UK. It may be that the resistance status of House mice found in Greater London is found throughout the UK, similar to the situation found in Germany. Without further research this cannot be clarified, it is also plausible to consider that the *Spretus* mutation may be found in the UK House mouse population in the future. The incidence of homozygous resistant House mice is likely to increase with the continued use of ineffective rodenticides containing the first-generation anticoagulant active ingredients or bromadiolone. This is because they act as a selection agent, so it is possible that we may get to a situation where the wild House mouse population is homozygous resistant and is impossible to control without the use of increasingly potent rodenticides.

Chapter 6 - The establishment of a selected line of House mice homozygous for the *VKORC1* mutation Y139C, and its effect on the BCR dose-response to a range of anticoagulant active ingredients

6.1 Introduction

6.1.1 Anticoagulant resistant rodents

Since the 1960s it has been known that anticoagulant resistance is a highly heritable trait and breeding experiments have demonstrated its heritability (Greaves & Ayres, 1977), and the possibility of creating laboratory strains of anticoagulant resistant Norway rats and House mice that are homozygous for a dominant autosomal resistance gene. Greaves and Cullen-Ayres (1988) produced homozygous anticoagulant resistant strains of 'Welsh', 'Scottish', and 'Hampshire' Norway rats; these strains were derived from crossing wild-caught Norway rats which had survived resistance tests with albino Wistar rats. The 'Welsh' strain is homozygous for the *VKORC1* mutation Y139S, the 'Scottish' strain is homozygous for the mutation L128Q, and the 'Hampshire' strain is homozygous for the mutation L120Q. After several generations of outcrossing progeny that possessed the 'resistance gene' with white Wistar rats, the animals were back-crossed to produce the homozygous resistant strain (Greaves & Cullen-Ayres, 1988). The same method was used to produce the laboratory strain of House mouse homozygous for the *VKORC1* mutation Y139C, which were derived from wild-caught House mice trapped near Reading in the UK (Prescott, 1996).

6.1.2 'Hampshire' Norway rats

The 'Hampshire' anticoagulant resistant strain of Norway rats was first identified in the late 1970s in north east Hampshire, UK (Greaves & Cullen-Ayres, 1988). This strain of Norway rats was found to be resistant to the first-generation active ingredient warfarin and the second-generation active ingredient difenacoum; difenacoum had previously been found to be unsuccessful in the control of a strain of Norway rats in an adjacent area, Berkshire (Greaves & Cullen-Ayres, 1988). It is quite possible that the high level of resistance in these wild populations, particularly when compared with the 'Hampshire' strain tested in 1988 by Greaves and Cullen-Ayres, is a result of the continued selection for the homozygous resistant strain by the use of difenacoum and bromadiolone. Indeed, this was demonstrated in the laboratory by Greaves and Cullen-Ayres (1988), who created a selected line by breeding from animals that survived a difenacoum resistance test. The rats were maintained for five days on a diet containing 50ppm difenacoum and those which survived for twenty-one days following the withdrawal of the diet were classed as 'difenacoum resistant' and selected for breeding.

6.1.3 Selected breeding of 'Hampshire' Norway rats

In any population of rodents, the dose required to achieve mortality will differ between individuals, with some able to survive higher doses of anticoagulant than the majority of the population. In populations where there is resistance to the anticoagulant being used, there is a greater likelihood that these more tolerant individuals will survive the treatment and go on to breed in the population. The rodenticide acts as a selection agent; individuals resistant to the rodenticide are selected for, individuals susceptible to the rodenticide are selected against.

The laboratory strain of 'Hampshire' Norway rats was established by crossing the 'difenacoum resistant' wild individuals with white Wistar laboratory rats and then backcrossing the progeny with the resistant parents, after several generations a laboratory strain homozygous for the *VKORC1* mutation L120Q was established (Greaves & Cullen-Ayres, 1988).

The LD_{50}, the lethal dose required to kill 50% of the population, for the selected line was higher for females for difenacoum compared to the 'Hampshire' strain of Norway rats, 29.3mg.kg^{-1} compared to 14.0mg.kg^{-1} (Greaves & Cullen-Ayres, 1988). Similar results were demonstrated for bromadiolone for both sexes, the LD_{50} for the selected line was 8.3mg.kg^{-1} and 12.7mg.kg^{-1} for males and females respectively compared to 2.6mg.kg^{-1} and 4.3mg.kg^{-1} for male and female 'Hampshire' Norway rats; with the more potent active ingredient brodifacoum there was no obvious difference in the LD_{50} (Greaves & Cullen-Ayres, 1988). Greaves and Cullen-Ayres (1988) also demonstrated that the degree of resistance increased (Table 6.1).

Greaves and Cullen-Ayres (1988) were selecting for difenacoum resistance but there also seemed to be a degree of cross-resistance. The resistance factor for bromadiolone was higher for the selected line than the 'Hampshire' strain for both sexes, with the resistance factor for brodifacoum higher for selected line females than 'Hampshire' females (Table 6.1). Anticoagulant resistance within a population of rodents is not specific to an active ingredient; there is a range of resistance to all the active ingredients. (Pelz & Prescott, 2015) and 'cross-resistance' was being demonstrated by the selected line of Norway rats bred by Greaves and Cullen-Ayres. 'Cross-resistance' is not surprising as many of the active ingredients have similar chemical structures. Such 'cross-resistance' was first described in 'Scottish' Norway rats in the

1960s, where rats which had been fed diphacinone and also survived being fed warfarin (Meehan, 1984).

Table 6.1 Resistance factors at the LD_{50} level for 'Hampshire' Norway rats and a selected line of Norway rats, adapted from Greaves & Cullen-Ayres (1988)

		Resistance factor		
Strain	Sex	Difenacoum	Bromadiolone	Brodifacoum
Hampshire	Male	3.9	1.5	2.0
	Female	4.1	2.9	2.0
Selected line	Male	3.7	4.9	1.9
	Female	8.6	8.5	2.7

6.1.4 Selected breeding in wild rodent populations

In the wild, House mouse populations undergo continuous selection with the continued use of ineffective anticoagulants; where animals which are resistant to the anticoagulant active ingredient survive the rodenticide treatment and continue to breed whereas susceptible animals succumb to the treatment. However, it has been reported that removing the selection pressure caused by anticoagulant use leads to a reduction in the incidence of resistance found in a population (Greaves *et al*, 1977) and the population returns to a normal level of susceptibility to the anticoagulant. However, to date there are no known populations of House mice or Norway rats which are resistant to either brodifacoum or flocoumafen.

This study aims to create a selected line of resistant House mice homozygous for the *VKORC1* mutation Y139C and determine the magnitude of resistance in the offspring of individuals selected for 'bromadiolone resistance' and their possible 'cross-resistance' to other anticoagulant active ingredients, along similar lines to the work of Greaves and Cullen-Ayres (1988) with the selected line of Norway rats. The work by Greaves and Cullen-Ayres (1988) relied on mortality tests which are a severe procedure but the response is clear cut; the

objective of this study is to use the BCR methodology, which is a mild procedure, to measure anticoagulant resistance. It is expected that the resistance factor of the selected line of resistant House mice homozygous for the *VKORC1* mutation Y139C will increase with increasing selection pressure, and once the selection pressure is removed the resistance factor will decrease over several generations.

6.2 Methods

The BCR methodology was used in this study to select for individuals that are more resistant rather than using lethal feeding test as used by Greaves and Cullen-Ayres (1988). The BCR methodology was chosen for welfare reasons; it is a mild procedure unlike the lethal feeding test which is a severe procedure as it determines resistance based on mortality.

6.2.1 Bromadiolone checking test

1. House mice homozygous for the *VKORC1* mutation Y139C were dosed with the ED_{50} concentration of bromadiolone established from the susceptibility baselines in Chapter 3, via oral gavage at a rate of 1ml per 100g body weight. At this dosage rate male House mice received 32.5mg.kg^{-1} bromadiolone and females received 35.3mg.kg^{1} bromadiolone. The solutions were prepared by diluting a 1% (a 1% solution delivers a 100mg per kilo body weight dose when delivered at a rate of 1ml per 100g bodyweight) solution of bromadiolone with PEG200 to produce the required concentrations.
2. 24 hours after dosing the INR of the mice was determined using the CoaguChek XS System.

a. The tip of the tail was removed using a scalpel blade and a drop of blood was applied to the test area of the test strip in the CoaguChek XS System.
b. The CoaguChek XS System started measuring as soon as the drop of blood was applied. The result was displayed after about one minute.

3. House mice with an INR value of 4.5 or lower were selected for breeding.
4. Five male and five female House mice homozygous for the *VKORC1* mutation Y139C underwent the *Bromadiolone Checking Test.*
5. The male and female House mice with the lowest INR values were paired. Three breeding pairs were initially established, these were known as generation 0.
6. The first-generation offspring subsequently underwent the *Bromadiolone Checking Test* and individuals with the lowest INR values were selected for breeding. The offspring of subsequent generations underwent the *Bromadiolone Checking Test* and selected for breeding as detailed above.
7. After several generations, the resistance factor of the mice was assessed using the BCR methodology described in Chapter 2 and the methodology to calculate resistance factors in Chapter 3.
8. House mice from the selected line were also dosed with other second-generation active ingredients and the resistance factor (RF) determined to evaluate the degree of 'cross-resistance'.
9. The selection pressure was then removed from the population and the RFs determined after several generations.

The CoaguChek XS System was introduced by Roche in the 1990s to allow patients to monitor their INR values at home or to be used in outpatient clinics, results can be obtained rapidly compared to the time taken for traditional laboratory testing (Dinkova *et al,* 2016). The activity of thrombin is detected by the system and the prothrombin time (PT) determined by

an electrochemical method (Iijima *et al*, 2014). The test strips contain human recombinant thromboplastin which activates coagulation producing an electrical signal, the human recombinant thromboplastin has an international sensitivity index (ISI) of 1 (Dinkova *et al*, 2016). The ISI is a measure of the responsiveness of the thromboplastin reagent to a decrease in coagulation proteins; the lower the ISI the more sensitive the reagent (Smith & Morrissey, 2004). The PT is converted to an INR value using the ISI for the test strip, the INR range is 0.8-8.0 and the 'normal INR' range is 0.9-1.1 (Iijima *et al*, 2014).

6.3 Results

Initially five male and five female house mice homozygous for the *VKORC1* mutation Y139C (Generation 0) were subjected to the *Bromadiolone Checking Test* and were found to have INR values of between 2.9–7.2 and 1.8-5.6 for males and females respectively. Three males and three females were paired for breeding although one female was subsequently euthanised (Table 6.2).

Generation 0 produced thirteen male and seven female offspring (Generation 1). They were subjected to the *Bromadiolone Checking Test* and were found to have INR values of between 3.2–7.2 and 1.7-3.9 for males and females respectively (Table 6.2). Five males and five females were paired for breeding although one male was found deceased 13 days after the pairs were established. The cause of death was unknown.

Equivalent data for Generations 2, 3 and 4 are presented in Table 6.2. As the INR values of the male progeny did not decrease as expected in Generation 3 it was decided to select males with an INR value of 5.5 and below for breeding.

Table 6.2: INR range of individuals tested and individuals selected for breeding for each generation

	MALES					FEMALES			
Generation	n	INR range	Breeding (n)	INR breeding		n	INR range	Breeding (n)	INR breeding
0	5	2.9-7.2	3	2.9-4.0		4	1.8-5.6	3	1.8-4.0
1	13	3.2-7.2	5	3.2-4.4		7	1.7-3.9	5	1.7-2.8
2	7	1.6->8.0	3	1.6-3.2		11	1.1->8.0	3	1.2-2.1
3	5	3.5->8.0	4	3.5-5.5		6	1.3-4.2	5	1.3-2.4
4	11	>8.0	0			9	3.5->8.0	0	

6.4 Discussion

Initially the selected line of House mice were more 'resistant' than the unselected line however after several generations of selection the line became less 'resistant'.

The results of the BCR methodology utilised looked promising initially. The INR values of all females decreased from generation 0 to 2 and INR values of males decreased between generations 1 and 2. This indicated that the mice were potentially becoming more resistant. However, after several generations of selected breeding the results were contrary to expectation and so the study was terminated (Table 6.3, Figure 6.1). From generation 3, the INR values increased for both males and females and after generation 4 the mean INR values were greater than 8 for males and 6 for females, it was decided to stop the study at generation 4. These results would suggest that maximum resistance in both sexes had possibly been selected for by generation 2. Male House mice selected for breeding had a mean INR of 2.4 and female House mice selected for breeding from generation 2 had a mean INR of 1.7, in comparison to 3.6 and 3.1 respectively for the original male and female parent stock selected for breeding (Table 6.3).

Table 6.3: The mean INR ranges for all progeny and the individuals selected as parents of the next generation of House mice homozygous for the *VKORC1* mutation Y139C selected for 'bromadiolone resistance'

Generation	Males			Females	
	Mean INR of all males	Mean INR of selected males		Mean INR of all females	Mean INR of selected females
0	4.6	3.6		3.7	3.1
1	5.3	3.9		2.5	2.1
2	4.2	2.4		1.6	1.7
3	5.4	4.8		2.3	1.9
4	>8.0	-		6	-

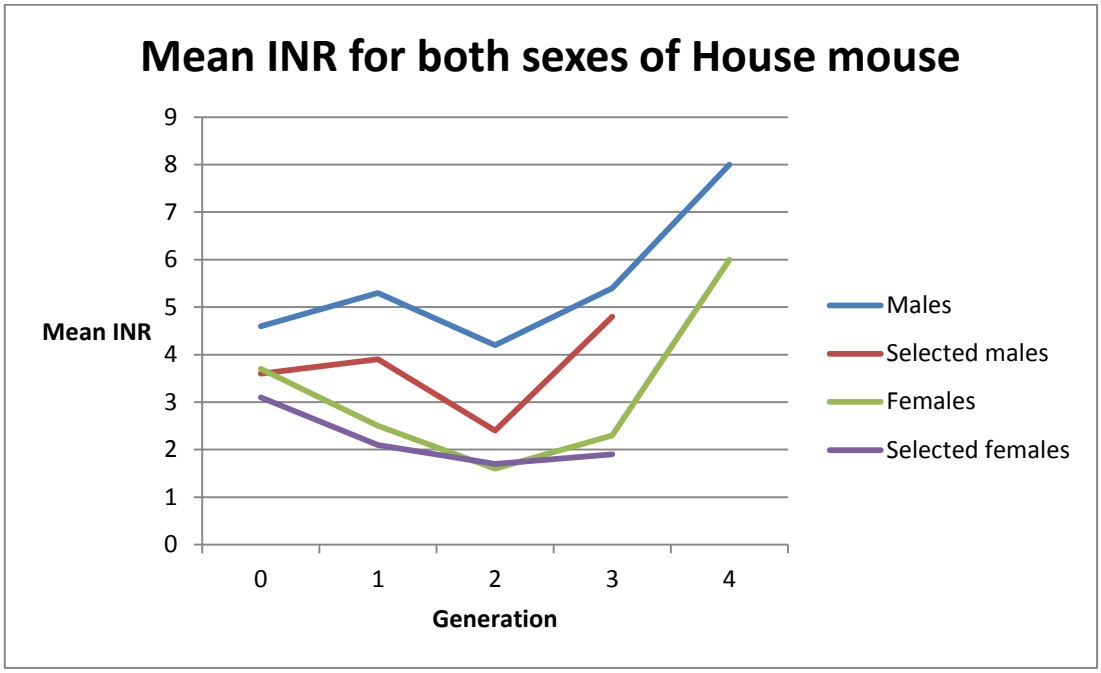


Figure 6.1: The changes in INR across the generations of all House mice homozygous for the *VKORC1* mutation Y139C undergoing the '*Bromadiolone Checking Test*' and those selected for breeding

The methodology utilised to determine resistance in this study does not truly replicate what happens in field conditions. In this study, House mice received a large, single dose of the anticoagulant active ingredient bromadiolone via oral gavage and their INR was determined 24 hours later. In the field, House mice ingest multiple small doses of the active ingredient which over time have a cumulative action and those which survive can be considered to possess "practical resistance" (Pelz & Prescott, 2015).

For this study individuals with a low INR value were classed as 'resistant' and were selected to become parents of the next generation. However, some of these individuals may have had minor haemorrhaging. Haemorrhaging is highly variable and those individuals with an INR value greater than 5 most likely had a rapid, major bleed (for example in the abdominal cavity) whereas individuals with minor bleeds would have lower INR values and would have been classed as 'resistant' whereas they were actually susceptible. The BCR methodology used in previous chapters uses an INR value of 5 to differentiate responders (susceptible individuals) from non-responders ('resistant' individuals) , and the higher the dose of active ingredient given, the more responders there are. However, in this study all generations were given the same dose of active ingredient and the differences in the INR values more likely reflected the severity and timing of resulting haemorrhages rather than the magnitude of resistance.

A lethal feeding study would be more accurate in determining whether individuals were resistant or susceptible. House mice would be fed a fixed dose of anticoagulant and then observed for up to 21 days and either survive or die, those individuals who survived would be selected to become parents of the next generation. The feeding study would then be repeated for several generations of offspring. After several generations the offspring of the pairs would then receive a dose of active ingredient via oral gavage and their prothrombin time determined 24 hours later using BCR methodology. The results from such a laboratory feeding study would be expected to indicate that resistance does increase in subsequent selected generations and more accurately reflect what happens in wild rodent populations due to the use of ineffective anticoagulant active ingredients. It is possible to conclude from this study that we cannot use the INR value of blood samples taken 24 hours after dosing as a measure of lethality. Measuring the INR value using a Coaguchek is crude in comparison to the more sensitive BCR methodology in previous chapters and more likely reflects the severity of the haemorrhage that occurs in the 24 hours after dosing.

There is a clear sex difference in the results; females are more tolerant to bromadiolone as shown by their lower mean INR values in comparison to male House mice (Table 6.3). Rowe and Redfern (1967) demonstrated that male House mice were more susceptible than females to warfarin at various concentrations and at different ages. More male mice succumbed to warfarin bait and the average time to death was longer for females, although at 6 weeks of age the difference in mortality was not significantly different between the sexes, but susceptibility to warfarin increased with age in male House mice. Fisher (2005) also reported that female House mice were more resistant to bromadiolone than males; the oral LD_{50} for females was 1.13mg.kg^{-1}, and the oral LD_{50} for males was 0.86mg.kg^{-1}.

It was originally planned to determine the resistance factor of the selected offspring after several generations of selected breeding using the '*Bromadiolone Checking Test'*. This was not completed due to the INR values increasing and the offspring potentially being more susceptible to bromadiolone than the original parent stock House mice. This is similar to the results reported by Greaves and Cullen-Ayres (1988) where the response in the selected Norway rat line became unstable after nine generations of selection , and they suggested that the maximum level of resistance had been reached within the first few generations of selection. Research by Rymer (2017) determined that most Norway rats in Berkshire and Hampshire, SE England were almost impossible to control with either bromadiolone or difenacoum. The populations of Norway rats in these areas had undergone generations of selection due to the use of these ineffective anticoagulant active ingredients. This resulted in highly resistant populations which were more resistant than the 'Hampshire' strain studied by Greaves and Cullen-Ayres (1988). This suggests that selection across south east England for anticoagulant resistance in wild populations of Norway rats is quite stable in comparison to the selected line of Norway rats of Greaves and Cullen-Ayres (1988) and the selected line of House mice in this study.

The genetics of anticoagulant resistance are not as well understood in House mice as they are in Norway rats. Initially it was thought that the genetics were similar in both species, but it is now thought that several different genes and mechanisms may be involved in House mice (RRAG, 2012). If different mechanisms are involved in House mice it may be that anticoagulant resistance is not inherited in the way that was shown in the selected Norway rat strain bred by Greaves and Cullen-Ayres (1988). This may go some way to explain why in the selected line of House mice homozygous for the *VKORC1* mutation Y139C, the initial generations seemed to be more resistant than the original parents but then after a few generations the offspring became more susceptible. More research into the heritability of anticoagulant resistance in House mice needs to take place to fully understand the mechanisms. Anticoagulant resistance is also affected by the activity of *VKORC1* and the activity of cytochrome p450. Cytochrome p450 metabolises external substances, especially in the liver, and has been shown to be involved in the metabolism of warfarin (Berny *et al*, 2018). Metabolic resistance is not fully understood and as yet the ability of cytochrome p450 to metabolise the SGARs has not been studied (Berny *et al*, 2018) however it is possible that it has an influence on anticoagulant resistance.

Once the mechanisms and genetics of anticoagulant resistance are fully understood in House mice, it may be possible to devise control strategies that specifically target anticoagulant resistant populations of House mice. Any control strategy needs to be selective against resistant individuals to have any chance of eliminating anticoagulant resistant rodents. Preventing the use of ineffective anticoagulant rodenticides and restricting the access of rodents to vitamin K rich diets would therefore be a logical first step in any control strategy.

Chapter 7 – General Discussion

7.1 Overview

Rodents have been recognised as pests for hundreds of years, being found in almost every environment throughout the world. They live in close proximity of human habitation causing damage and contamination to crops, stored food and structures, and they are also vectors of zoonotic diseases (Lefebvre *et al*, 2017; Tomlinson *et al*, 2017). The House mouse (*Mus musculus*) is the most wide spread rodent species and is found worldwide in all climates and environments (Meehan, 1984).

Following the introduction of anticoagulant rodenticides, populations of House mice that could not be successfully controlled with the anticoagulants available were soon being identified throughout the UK. Some populations have now been identified that cannot even be controlled by some of the more potent second-generation anticoagulant active ingredients. There has been a lack of research to quantify anticoagulant resistance in House mice, with the majority of research concentrating on Norway rats. However, anticoagulant resistance in House mice is becoming much more of a prominent problem. The work presented in this thesis focuses on anticoagulant resistance in House mice, and in particular animals that are homozygous or heterozygous for the *VKORC1* genetic mutation Y139C. This genetic mutation has been found in wild House mice throughout the UK, Europe and the USA and is already known to be associated with resistance to first-generation anticoagulant active ingredients and the second-generation anticoagulant active ingredient bromadiolone (Prescott, 1996).

Many non-anticoagulant rodenticides have been removed from the market in the EU following the introduction of the Biocidal Products Directive, so there are few alternative rodenticides

available for the control of anticoagulant resistant rodents (Pelz & Prescott, 2015). Also, the more effective second-generation active ingredients are no longer available for use by amateurs in many European countries and so they are forced to use the ineffective first-generation anticoagulant active ingredients (Buckle & Smith, 2015). It has already been recognised in the UK and across Europe that the first-generation anticoagulant active ingredients are ineffective in controlling anticoagulant resistant House mice and their use for such is now restricted, although these restrictions are not uniformly applied elsewhere (Buckle & Smith, 2015). Both the prevention of use of non-anticoagulant rodenticides and the restriction on the use of the effective second-generation anticoagulants pose the risk of increasing the anticoagulant resistance problem throughout Europe. As such, within a few years it may become almost impossible to control anticoagulant resistant House mice and Norway rats. The situation is similar in the USA, resistant populations of House mice which carry the *VKORC1* genetic mutation Y139C have been identified but amateurs are restricted to only using first-generation anticoagulants for the control of House mice (Buckle & Smith, 2015). These regulations in both the EU and USA have been applied to prevent the exposure of both non-target species and humans to the more potent second-generation anticoagulant active ingredients. However, most House mouse populations that amateurs want to control live predominantly indoors where there is little opportunity of non-targets being exposed, and if baits are provided in tamper-proof boxes humans and pets are unlikely to be at risk.

7.2 The refinement and replacement of animal tests

In the 1960s, feeding tests were used to determine the toxicity of anticoagulants against wild-caught rodents; individuals would either die or survive the anticoagulant treatment and those which survived were deemed resistant (Rowe & Redfern, 1964). These feeding tests were classed as severe in severity as individuals would die, and they were also crude as they did not

take into account that wild-caught rodents do not feed well in the laboratory. Due to this, some of the 'resistant' individuals would have only survived due to not consuming a lethal dose of the anticoagulant. Feeding tests were replaced by blood coagulation response (BCR) tests. The early BCR tests measured coagulation time up to 96 hours after a rodent received a dose of anticoagulant, so some susceptible individuals would be expected to die within this time, these early BCR tests still had a severe severity limit. The BCR methodology was refined by Prescott *et al* (2007) with the introduction of a standardised BCR test. The refined BCR methodology developed by Prescott *et al* (2007) is a mild procedure, test animals do not develop symptoms of anticoagulant toxicity as their coagulation is measured 24 hours after the administration of the active ingredient.

Due to advances in technology, anticoagulant resistance can now be identified genetically by the presence of the *VKORC1* genetic mutation and this has replaced the requirement for laboratory animal studies. A limiting factor, however, is that such molecular tests cannot quantify resistance. These genetic resistance tests are only of practical use once the magnitude of the resistance has been determined for each strain/sex/active ingredient combination. This study has identified the presence of anticoagulant resistant House mice in various locations throughout Greater London and the south of England. Additionally, levels of resistance to the first-generation anticoagulant active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium in House mice homozygous for the *VKORC1* mutation Y139C have been quantified. Resistance levels have also been quantified for the second-generation anticoagulant active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen in House mice homozygous or heterozygous for the *VKORC1* mutation Y139C.

7.3 Susceptibility baselines

Susceptibility baselines and resistance factors for the first-generation anticoagulant active ingredients chlorophacinone, coumatetralyl, diphacinone and warfarin sodium had not been previously produced for House mice homozygous for the *VKORC1* genetic mutation Y139C, or the susceptible House mouse strain. Results in Chapter 2 indicated that susceptible House mice would have to feed for over 4 days to consume a lethal dose of any of these first-generation active ingredients. However, the time required to consume a lethal dose depends upon the concentration of the bait and its toxicity to susceptible albino House mice.

The results of this study shown in Chapters 2 and 3 have quantified the level of resistance in the resistant House mouse strain homozygous for the *VKORC1* mutation Y139C in response to the four first-generation anticoagulant active ingredients studied. Resistance factors of the four first-generation anticoagulant active ingredients studied were high for both sexes of the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C. This demonstrates that these active ingredients would not be effective in the control of this strain of House mouse. The resistance factor values were between 20 and 35 for coumatetralyl for both sexes, and over 600 for chlorophacinone for the resistant strain of House mouse homozygous for the *VKORC1* mutation Y139C. In practical terms, there is a 600-fold loss of toxicity of chlorophacinone and a 20-35-fold loss in toxicity of coumatetralyl in comparison to the toxicity against the susceptible House mouse strain. Sufficient control of a population of House mice homozygous for the *VKORC1* genetic mutation Y139C is highly unlikely to be achieved with any first-generation anticoagulant active ingredient studied. Laboratory feeding tests had previously concluded that first-generation anticoagulant active ingredients were ineffective in the control of House mice homozygous for the *VKORC1* genetic mutation Y139C (Prescott, 1996) but the levels of resistance had not been quantified until this study.

The second-generation anticoagulant active ingredients brodifacoum, difenacoum, difethialone and flocoumafen would be expected to be highly successful in the control of a population of House mice homozygous for the *VKORC1* genetic mutation Y139C, whereas bromadiolone would be ineffective in the control of these House mice. The resistance factors for the active ingredients brodifacoum, difenacoum, difethialone and flocoumafen were all below 3, however, the resistance factor was over 16 for bromadiolone. High resistance factors are associated with ineffective rodent control in the field (Pelz & Prescott, 2015). In any case, the resistance factor associated with treatment failure will depend on two things; the LD_{50} of the active ingredients and the formulation strength. The more toxic the active ingredients, the higher the resistance factor required for treatment failure and the higher the formulation strength, the higher the resistance factor required for treatment failure. The resistance factors were calculated in this study using ED_{50} values and it cannot be guaranteed that the levels of resistance will be the same as resistance factors calculated using LD_{50} values from lethal feeding tests. In the presence of a population of House mice homozygous for the *VKORC1* genetic mutation Y139C, it would not be advisable to use the four first-generation anticoagulant active ingredients investigated in this study or the second-generation anticoagulant active ingredient bromadiolone. These active ingredients would only be successful in the control of susceptible individuals, whereas the resistant individuals would survive and the resistance problem would increase.

To date, most research against anticoagulant resistant rodents has concentrated on individuals homozygous for the *VKORC1* genetic mutations; however Grandmange *et al* (2009) did investigate the effect of heterozygosity on anticoagulant resistance in a strain of Norway rats heterozygous for the *VKORC1* mutation Y139F. In Chapter 4, the level of resistance to the second-generation anticoagulant active ingredients brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen in a strain of House mice heterozygous for the *VKORC1* genetic

mutation Y139C was investigated. The resistance factors were all below 2 for both sexes of this strain for the active ingredients brodifacoum, difenacoum, difethialone and flocoumafen, these active ingredients would be expected to be successful in the control of a population of House mice heterozygous for the *VKORC1* mutation Y139C. The resistance factor for bromadiolone was above 5 for both sexes, bromadiolone would not be as effective in the control of these House mice in comparison to the other second-generation anticoagulant active ingredients. As expected, the resistance factors were higher for the homozygous strain than for the heterozygous strain and some level of control may be afforded against a heterozygous population when using the active ingredient bromadiolone. Even so, there would be some individuals in the population that would survive and bromadiolone would be actively selecting for those more resistant individuals. These results provide some evidence that resistance may have an effect on treatment failure, but they must be interpreted carefully.

7.4 Selective breeding and anticoagulant resistance

The continued use of ineffective anticoagulant active ingredients in the control of anticoagulant resistant strains of rodents only serves to exacerbate the resistance problem. They select for anticoagulant resistance and against anticoagulant susceptibility and with every successive treatment, more susceptible individuals succumb to the treatment whereas heterozygous and homozygous resistant individuals survive. With subsequent treatments the rodent population becomes more resistant, there are fewer susceptible individuals in the population and eventually only homozygous resistant individuals will survive meaning the population will be much more difficult to control. It has been reported by Rymer (2017), that Norway rats in both Berkshire and Hampshire, UK have become extremely difficult to control with baits containing the active ingredients bromadiolone or difenacoum. The over reliance on

baits containing either bromadiolone or difenacoum in these areas has selected for Norway rats that are resistant to these active ingredients. When resistance to an anticoagulant active ingredient is of a significantly high magnitude (i.e. a resistance factor >15) it is recommended to stop using that active ingredient in any further pest control management strategy in that area. For House mice homozygous for the *VKORC1* mutation Y139C the estimated resistance factor for brodifacoum is <2 for both sexes and would not be considered to be sufficient to cause treatment failure, but the resistance factor for bromadiolone is >16 for both sexes and would be considered to be sufficient to cause treatment failure. When resistance is identified in a population of rodents using the molecular method, the advice of both RRAG and RRAC is to stop using anticoagulant rodenticides which have high resistance factors against that strain of rodent and to only use anticoagulant rodenticides that have low resistance factor values. The advice of both RRAG and RRAC for House mice homozygous for the *VKORC1* mutation Y139C is based on the work presented here.

Unlike the study by Greaves and Cullen-Ayres (1988) where survival of a feeding test was used to determine resistance, this study relied on measuring the INR value of blood samples 24 hours after House mice homozygous for the *VKORC1* genetic mutation Y139C had received a large, oral dose of bromadiolone. Greaves and Cullen-Ayres (1988) produced a selected line by using a lethal feeding period test to determine the magnitude of resistance in individual rats. Animals that survived had a high magnitude of resistance and animals that died had a low magnitude of resistance. By breeding from rats that had been identified as having a high magnitude of resistance, over several generations, a line of rats with a much higher resistance factor than the original animals was produced.

In this study INR values were measured 24 hours after dosing with a specified amount of anticoagulant and individual INR values were used as a measure of the magnitude of resistance. Low INR values indicated a high magnitude of resistance and high INR values indicated a low magnitude of resistance. To produce a selected line of mice over several generations, individuals that were identified as having a high magnitude of resistance were selected for breeding. Initially there was some evidence that the selected line of mice was becoming more resistant, but by the third generation the INR values of the mice were prolonged. This suggests that the magnitude of resistance had declined. From these results it would appear that the INR values determined 24 hours after dosing with an anticoagulant are not a good measure of the magnitude of resistance. It is quite possible that the differences in INR values between individual mice is more a reflection of the time delay between dosing and lethal haemorrhage, rather than a measure of the magnitude of resistance. After administration of a lethal dose of anticoagulant, mice typically die of haemorrhage between 3 and 11 days after dosing. Therefore, it is likely that animals which haemorrhage more rapidly after dosing will have a more prolonged INR value 24 hours after dosing than animals that haemorrhage less rapidly after dosing. The INR value may have been more a measure of haemorrhage speed rather than of 'resistance'.

Further study into the possibility of ineffective anticoagulant active ingredients acting as selection agents on 'anticoagulant resistant' populations of House mice may be undertaken using a similar method to that used by Greaves and Cullen-Ayres (1988). This would allow individuals that are more 'resistant' to be definitively identified and selected for breeding.

7.5 Genetic identification of anticoagulant resistant House mouse populations

The identification of the *VKORC1* gene and the evolution of the methodology to identify rodents carrying this gene has allowed the study of the distribution and spread of anticoagulant resistant rodent populations without the use of anticoagulant rodenticide baits (Blazic *et al*, 2018). Use of the genetic methodology to identify any *VKORC1* genetic mutations in a House mouse population, in combination with the resistance factors calculated in Chapters 3 and 4 for House mice homozygous or heterozygous for the *VKORC1* genetic mutation Y139C, may allow pest control operators to plan their rodent control management more accurately. This would aid in reducing the use of ineffective anticoagulants which pose a risk of poisoning to non-target individuals.

In Greater London, House mice carrying the *VKORC1* genetic mutations Y139C and L128S have been identified (Chapter 5). Some individuals were homozygous for either mutation, some were heterozygous for either mutation, but worryingly some individuals were identified that carried both mutations. Due to the widespread distribution of these individuals carrying *VKORC1* genetic mutations it would be expected that no first-generation anticoagulant active ingredient used in this study or the second-generation active ingredient bromadiolone would be successful in the control of House mice populations in London. This widespread distribution of anticoagulant resistant House mice is most likely caused by the over reliance on ineffective anticoagulant active ingredients, particularly by amateurs. This may be due to the fact that the more potent and effective second-generation active ingredients, brodifacoum and flocoumafen, have been restricted for use to professional pest control operators. Further detailed study of House mouse populations throughout the UK must be undertaken to understand the full extent of anticoagulant resistance in the UK House mouse population. To

date studies have only been undertaken on very restricted populations around Reading and Cambridge, and more recently across Greater London. It is therefore likely that anticoagulant resistant populations are more widespread across the UK. It is also possible that other *VKORC1* genetic mutations may be identified. Any study relies on the cooperation of pest controllers, with full cooperation and the use of data produced in this study it may be possible to create a pest control strategy for anticoagulant resistant House mouse populations throughout the UK, across Europe, and elsewhere.

7.6 The impact of anticoagulant resistance in the future control of House mice

The more potent second-generation active ingredients brodifacoum and flocoumafen are potentially lethal to House mice in a single feed and this includes House mice carrying the *VKORC1* genetic mutations Y139C and L128S (Blazic *et al*, 2018). The risk to non-target species is an important concern when considering the control of resistant House mice. However, as the populations of House mice are most likely to be found indoors the potential risk is lower than when controlling Norway rats, but there is still a risk to pets and children. Single-dose active ingredients reduce the chance of the accidental primary and secondary poisoning of non-targets as only small amounts of bait are required in comparison to the less potent active ingredients bromadiolone and difenacoum. In the control of resistant populations of rats and mice, it is common practice to use much more rodenticide than when dealing with susceptible animals, and this will increase the potential risk to non-target species (see Blazic *et al*, 2018). Resistant rodents are also reservoirs of anticoagulants, with active ingredient stored in tissues, particularly the liver, and when consumed by predators and scavengers, they pose a significant non-target risk (Daniells *et al*, 2011).

The over-reliance on ineffective first-generation anticoagulant active ingredients and the second-generation active ingredient bromadiolone have acted as a selection pressure on wild House mouse populations. This has caused an increase in the number of 'anticoagulant resistant' individuals which are more difficult to control and will result in populations of House mice that are much more difficult to control. In any wild population of House mice there will be a mixture of susceptible, heterozygous resistant and homozygous resistant individuals. Initially, susceptible individuals will succumb to the anticoagulant treatment along with some heterozygous resistant individuals. Ultimately, the final population after treatment will be composed mainly of homozygous resistant individuals together with some more resilient heterozygous resistant individuals. For effective control of wild resistant House mouse populations in the UK, the recommendation is now to use the more potent second-generation active ingredients brodifacoum, flocoumafen and difethialone (CropLife International, 2016). As homozygous resistant House mice have a much higher dietary requirement for vitamin K than heterozygous animals, a sensible resistance management strategy might be to reduce the availability of vitamin K, particularly in animal feeds. However, for practical reasons, this has proved to be very difficult.

For House mice that possess the *VKORC1* genetic mutation L128S, similar studies to those described in Chapters 3 and 4 with House mice homozygous and heterozygous for the mutation should be undertaken. This will lead to a more thorough understanding of the potential impact of this type of anticoagulant resistance in the UK House mouse population. This future study alongside results here, would allow pest control operators to accurately target populations of House mice in the UK using anticoagulant active ingredients known to be effective, whilst also reducing the incidence of secondary poisoning of non-targets.

The identification of anticoagulant resistance in House mice and Norway rats has been revolutionised by the development of the new molecular methodology that identifies mutations of the *VKORC1* gene. However, the main drawback of this new molecular methodology is that it provides no information on the magnitude of the resistance conferred by the different *VKORC1* mutations that have been identified to date. The work presented in this thesis has made a significant contribution to this limitation of the new molecular method. The blood clotting response test, developed by Prescott *et al* (2007) has provided a method to determine the magnitude of the resistance for each *VKORC1* resistant strain against each active ingredient; with susceptibility baseline data presented for both Norway rats and House mice against the five second-generation anticoagulants, and for Norway rats against the four first-generation anticoagulants, chlorophacinone, coumatetralyl, diphacinone and warfarin.

In the current work, susceptibility baseline data is presented for susceptible House mice against the four first-generation anticoagulants, chlorophacinone, coumatetralyl, diphacinone and warfarin sodium, thus providing the baseline data required to determine the magnitude of the resistance for any of these first-generation anticoagulants.

Specifically, for House mice that are homozygous for the *VKORC1* mutation Y139C, the current work presents resistance factors for the five second-generation anticoagulant active ingredients, brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen, and the four first-generation anticoagulants, chlorophacinone, coumatetralyl, diphacinone and warfarin sodium. This data is presented separately for each sex, thus providing a measure of the magnitude of the resistance for all nine active ingredients. This data has been made available online by the Rodenticide Resistance Action Committee of CropLife International,

where it can be used globally to quantify the magnitude of the resistance in any House mouse populations that possess the *VKORC1* mutation Y139C.

The current work also presents resistance factors for the five second- generation anticoagulant active ingredients, brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen generated against House mice that are heterozygous for the *VKORC1* mutation Y139C. The lower resistance factors, compared with House mice that are homozygous for the *VKORC1* mutation Y139C, demonstrates the high potential for selection of homozygosity, which in wild populations would lead to the development of a selected line of highly resistant animals. The geographical distribution of *VKORC1* mutations that have been identified across Greater London, with a high incidence of homozygous animals, provides support for this view.

References

Auffray J-C, Vanlerberghe F & Brotton-Davidian J. 1990. The house mouse progression in Eurasia: a palaeontological and archaeozoological approach. *Biological Journal of the Linnean Society* 41:13-25.

Bailey CI & Eason CT. 2000. Anticoagulant resistance in rodents. *Conservation Advisory Science Notes No. 297.* Department of Conservation, Wellington.

Bayer. 2007. Rodents – their biology and control [Online]. Bayer Environmental Science. Available www.bayerhome.co.za/docs/Rodent%20editorial%20complete.pdf [Accessed 4/3/13].

Bedroy M & Smith P. 1993. Arms race and rat race: adaptations against poisoning in the brown rat. *Revue d'Écologie* 48:215-228.

Bell RG & Caldwell PT. 1973. Mechanism of warfarin resistance. Warfarin and the metabolism of vitamin K_1. *Biochemistry* 12:1759-1762.

Bentley EW. 1972. A review of anticoagulant rodenticides in current use. *Bulletin World Health Organisation.* 47:275-280.

Berny P, Esther A, Jacob J & Prescott C. 2018. Development of resistance to anticoagulant rodenticides in rodents. In: van den Brink N, Elliot JE, Shore RF & Rattner BA (eds) *Anticoagulant rodenticides and wildlife. Springer International Publishing.* pp259-286.

Berry RJ & Scriven PN. 2005. The house mouse: a model and motor for evolutionary understanding. *Biological Journal of the Linnean Society* 84:335-347.

Bishop JA. 1981. A NeoDarwinian approach to resistance: examples from mammals. In: Bishop JA, Cook LM (eds) *Genetic consequences of man made change. Academic Press,* London, UK. pp37-51.

Bishop JA, Hartley DJ & Partridge GG. 1977. The population dynamics of genetically determined resistance to warfarin in *Rattus norvegicus* from mid Wales. *Heredity* 39:389-398.

Blazic T, Jokic G, Esther A, Vuksa M & Dedovic S. 2018. Brodifacoum as a first choice rodenticide for controlling bromadiolone-resistant *Mus musculus*. *Journal of Stored Products Research* 79:29-33.

Boursot P, Auffray J-C, Britton-Davidian J & Bonhomme F. 1993. The evolution of house mice. *Annual Review of Ecology and Systematics* 24:119-152.

Buckle AP. 1994. Control methods: chemical. In: Buckle AP, Smith RH (eds) *Rodent Pests and Their Control. CAB International,* Wallingford, UK. pp 127-160.

Buckle AP & Eason CT. 2015. Control methods: chemical. In: Buckle AP, Smith RH, (eds) *Rodent Pests and Their Control 2nd edition*. CAB International, Wallingford, UK. pp 123-154.

Buckle AP, Endepols S & Prescott CV. 2007. Relationship between resistance factors and treatment efficacy when bromadiolone was used against anticoagulant-resistant Norway rats (*Rattus norvegicus* Berk.) in Wales. *International Journal of Pest Management* 53:291-297.

Buckle A & Prescott C. 2012. The Current Status of Anticoagulant Resistance in Rats and Mice in the UK. RRAG 2012 Report from the Rodenticide Resistance Action Group of the United Kingdom to the Health and Safety Executive.

Buckle AP, Prescott CV & Ward KJ. 1994. Resistance to the first and second generation anticoagulant rodenticides – a new perspective. Proceedings of the Sixteenth Vertebrate Pest Conference [Online]. Available: http://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1006dcontext=vpc16 [Accessed 25/4/13].

Buckle AP & Smith RH. 2015 Rodent control: back to the future (the sequel). In: Buckle AP, Smith RH, (eds) *Rodent Pests and Their Control 2nd edition.* CAB International, Wallingford, UK. pp 397-402.

Chu K, Wu S-M, Stanley T, Stafford DW & High KA. 1996. A mutation in the propeptide of factor IX leads to warfarin sensitivity by a novel mechanism. *Journal of Clinical Investigation* 98:1619-1625.

Cox P. & Smith RH. 1992. Rodenticide ecotoxicology: pre-lethal effects of anticoagulants on rat behaviour. Proceedings of the Fifteenth Vertebrate Pest Conference, ed by Borrecco JE & March RE. University of California, Davis. pp 165-170.

CropLife International. 2016. RRAC guidelines on anticoagulant rodenticide resistance management [Online]. Available http://about.rrac.info/fileadmin/downloads/RRAC_Guidelines_Resistance.pdf. [Accessed 29/11/18].

Crowell MD, Broome KG, Eason CT, Fairweather AAC, Ogilvie S & Murphy EC. 2013. How long do vertebrate pesticides persist in living mammals? Priorities for research. DOC Research and Development Series 337, Department of Conservation, Wellington, NZ.

Daniells L, Buckle A & Prescott CV. 2011. Resistance as a factor in environmental exposure of anticoagulant rodenticides – a modelling approach. Proceedings of the Eighth European Vertebrate Pest Management Conference, ed by Jacob J & Ester A. Julius Kuhn Institute and Humboldt University, Berlin, Germany. pp 58-59.

Deitcher SR. 2002. Interpretation of the International normalised ratio in patients with liver disease. *The Lancet* 359:47-48.

Diagnostic Reagents Ltd. 2010. Freeze Dried Rabbit Brain Thromboplastin. *Product insert leaflet (version 4)*.

Diaz JC, Song Y, Moore A, Borchert JN & Kohn MH. 2010. Analysis of *vkorc1* polymorphisms in Norway rats using the roof rat as outgroup. *BMC Genetics* 11:43-53.

Dinkova A., Vladimirova-Kitova L. & Deneva T. 2016. Comparative study of the results of capillary blood international normalized ratio on coagucheck VX POC and conventional coagulation blood test. *International Journal of Pharmaceutical and Clinical Research* 8:322-325.

Dodsworth E. 1961. Mice are spreading despite such poisons as warfarin. *Municipal Engineering* 3746:1668.

Eason CT & Wickstrom M. 2001. *Vertebrate pesticide toxicology manual (poisons): Information on poisons used as vertebrate pesticides, edition 2.* Department of Conservation: Wellington, New Zealand. pp 41-74.

Elliot JE, Hindmarch S, Albert CA, Emery J, Mineau P & Maisonneuve F. 2014. Exposure pathways of anticoagulant rodenticides to nontarget wildlife. *Environmental Monitoring & Assessment* 186:895-906.

EPA. 2017. Rodent control pesticide safety review [Online]. Available https://www.epa.gov/rodenticides/rodent-control-pesticide-safety-review. [Accessed 28/10/18].

ExpertsMind. 2013. Define vitamin k-dependent carboxylation, biology [Online]. Available http://www.expertsmind.com/questions/define-vitamin-k-dependent-carboxylation-30184084.aspx. [Accessed 25/11/18].

Feinstein DL, Akpa BS, Ayee MA, Boullerne AI, Braun D, Brodsky SV, Gidalevitz D, Hauck Z, Kalinin S, Kowal K, Kuzmenko I, Lis K, Marangoni N, Martynowycz MW, Rubinstein I, van Breemen R, Ware K & Weinberg G. 2016. The emerging threat of superwarfarins: history, detection, mechanisms, and countermeasures. *Annals of the New York Academy of Sciences* 1374:111-122.

FERA. 2002. The control of rats with rodenticides: A complete guide to best practice [Online]. Available http://www.naturalengland.org.uk/Images/ratcontrolguidelines_tcm6-11216.pdf [Accessed 15/8/12].

Fisher P. 2005. Review of House mouse (*Mus musculus*) susceptibility to anticoagulant poisons. Department of Conservation Technical Series 198. Department of Conservation, Wellington, New Zealand.

Gabriel SI, Stevens MI, da Luz Mathias M & Searle JB. 2011. Of mice and 'convicts': origin of the Australian house mouse, *Mus musculus*. Available: www.plosone.org/article/fetchObject.action?uri=info:doi/10.1371/journal.pone.0028622&representation=PDF [Accessed 20/10/15].

Gebauer M. 2007. Synthesis and structure-activity relationships of novel warfarin derivatives. *Bioorganic & Medicinal Chemistry* 15:2414-2420.

Goulois J, Hascoet C, Dorani K, Besse S, Legros L, Benoit E & Lattard V. 2016. Study of the efficiency of anticoagulant rodenticide to control *Mus musculus domesticus* introgressed with *Mus spretus Vkorc1.* Available: http://onlinelibrary.wiley.com/doi/10.1002/ps.4319/full [Accessed 28/07/16].

Goulois J, Lambert V, Legros L, Benoit E & Lattard V. 2017. Adaptative evolution of the *Vkorc1* gene in *Mus musculus domesticus* is influenced by the selective pressure of anticoagulant rodenticides. *Ecology and Evolution* 7:2767-2776.

Grandemange A, Kohn MH, Lasseur R, Longin-Sauvageon C, Berny P & Benoit E. 2009. Consequences of the *Y139F Vkorc1* mutation on resistance to AVKs: in-vivo investigation in a 7th generation of congenic *Y139F* strain of rats. *Pharmacogenet Genomics* 19:742-750.

Greaves JH. 1994. Resistance to anticoagulant rodenticides. In: Buckle AP, Smith RH (eds) *Rodent Pests and Their Control.* CAB International, Wallingford, UK. pp 197-217.

Greaves JH. 1995. Managing resistance to anticoagulant rodenticides: an appraisal. *Pesticide Science* 43:79-82.

Greaves JH & Ayres PB. 1977. Unifactorial inheritance of warfarin resistance in *Rattus norvegicus* from Denmark. *Genetic Research* 29:215-222.

Greaves JH & Cullen-Ayres PB. 1988. Genetics of difenacoum resistance in the rat. Current Advances in Vitamin K Research. In Suttie JW, editor: Elsevier Science Publishing Co. pp 389-397.

Greaves JH, Redfern R, Ayres PB & Gill JE. 1977. Warfarin resistance: a balanced polymorphism in the Norway rat. *Genetic Research* 30:257-263.

Hadler M & Buckle AP. 1992. Forty five years of anticoagulant rodenticides – past, present and future trends. Proceedings Fifteenth Vertebrate Pest Conference, ed. by Borrecco JE & Marsh RE. Newport Beach, California. pp 149-155.

Hermodson MA, Suttie JW & Link KP. 1969. Warfarin metabolism and vitamin K requirement in the warfarin-resistant rat. *American Journal of Physiology* 217:1316.

Hernandez-Moreno D, De La Casa-Resino I, Lopez-Beceiro A, Fidalgo LE, Soler F & Perez-Lopez M. 2013. Secondary poisoning of non-target animals in an Ornithological Zoo in Galicia (NW Spain) with anticoagulant rodenticides: a case report. *Veterinarni Medicina* 58:553-559.

HSE. 2004. HSE information sheet MISC515: Urban rodent control; and the safe use of rodenticides by professional users [Online]. Health and Safety Executive. Available http://www.hse.gov.uk/pubns/misc515.pdf [Accessed 15/1/13].

HSE. 2018. UK authorised rodenticide product database – search database [online]. Available https://webcommunities.hse.gov.uk/connect.ti/pesticides/viewdatastore?dsid=10116. [Accessed 28/10/18].

Humphries RE, Meehan AP & Sibly RM. 1992. The characteristics and history of behavioural resistance in inner-city house mice (*Mus domesticus*) in the U.K. Proceedings Fifteenth Vertebrate Pest Conference, ed. by Borrecco JE & Marsh RE. Newport Beach, California. pp 161-164.

Humphries RE, Sibly RM & Meehan AP. 2000. Cereal aversion in behaviourally resistant house mice in Birmingham, UK. *Applied Animal Behaviour Science* 66:323-333.

Iijima S., Baba T., Ueno D. & Ohishi A. 2014. International normalized ratio testing with point-of-care coagulometer in healthy term neonates. *BMC Pediatrics* 14:179-184.

Ishizuke M, Tanikawa T, Tanaka KD, Heewon M, Okajima F, Sakamoto KQ & Fujita S. 2008. Pesticide resistance in wild mammals – Mechanisms of anticoagulant resistance in wild rodents. *Journal of Toxicological Sciences* 33:283-291.

Jaffe J, Molenaar F, Rowcliffe M, Sainsbury A, Carter I, Barnett E & Shore R. 2016. Poisoning of reintroduced red kites (*Milvus milvus*) in England. 12th Conference of the European Wildlife Disease Association (EWDA), Berlin.

Lefebvre S, Benoit E & Lattard V. 2016. Comparative biology of the resistance to vitamin K antagonists: an overview of the resistance mechanisms. In: Basaran O & Biteker M (eds) *Pharmacology, toxicology and pharmaceutical science*. INTech, Rijeka, Croatia. pp19-45.

Lefebvre S, Fourel I, Queffélec S, Vodovar D, Mégarbane B, Benoit E, Siguret V & Lattard V. 2017. Poisoning by anticoagulant rodenticides in humans and animals: causes and consequences. In: Malangu N (ed) *Poisoning – from specific toxic agents to novel rapid and simplified techniques for analysis.* INTech, Rijeka, Croatia. pp11-32.

Li T, Chang C-Y, Jin D-Y, Lin P-J, Khvorova A & Stafford DW. 2004. Identification of the gene for vitamin K epoxide reductase. *Nature* 427:541-544.

Li W, Schulman S, Dutton RJ, Boyd D, Beckwith J & Rapoport TA. 2010. Structure of a bacterial homologue of vitamin K epoxide reductase. *Nature* 463:507-512.

Lund M. 1972. Rodent resistance to the anticoagulant rodenticides, with particular reference to Denmark. *Bulletin World Health Organisation* 47:611-618.

Lund M. 1984. Resistance to the second-generation anticoagulant rodenticide. Proceedings of the Eleventh Vertebrate Pest Conference, ed. By Clark DO. University of California. pp 89-94.

Lund M. 1994. Commensal Rodents. In Buckle AP, Smith RH (eds) *Rodent Pests and Their Control*. CAB International, Wallingford, UK. pp 23-43.

Lund M. 2015. Commensal Rodents. In: Buckle AP, Smith RH, (eds) *Rodent Pests and Their Control 2nd edition.* CAB International, Wallingford, UK. pp 19-32.

MacDonald DW & Fenn MGP. 1994. Chapter 1. The Natural History of rodents: Preadaptations to Pestilence. In Buckle AP, Smith RH, editors, Wallingford (Oxon UK): CAB International. pp 1-21.

Macdonald DW, Fenn MGP & Gelling M. 2015. The Natural History of Rodents: Preadaptations to Pestilence. In: Buckle AP, Smith RH, (eds) *Rodent Pests and Their Control 2nd edition.* CAB International, Wallingford, UK. pp 1-18.

MacNicoll AD. 1995. A review of biochemical mechanisms of warfarin resistance in the house mouse. *Pesticide Science* 43:57-59.

Martin AD, Linda C, Steed R, Redfern JE, Gill JE & Huson LW. 1979. Warfarin-resistance genotype determination in the Norway rat, *Rattus norvegicus. Laboratory Animals* 13:209-214.

Mason G & Littin KE. 2003. The humaneness of rodent pest control. *Animal Welfare* 12:1-37.

Meehan AP. 1984. Rats and Mice: their biology and control. East Grinstead: Rentokil Limited. pp 11-30, 112-140, 177-188.

Meerburg BG, Brom FWA & Kijlstra A. 2008. Perspective The ethics of rodent control. *Pest Management Science.* 64:1205-1211.

Nathan HW, Clout MN, Murphy EC & MacKay JWB. 2013. Strategies for detection of house mice on a recently invaded island. *New Zealand Journal of Ecology* 37:26-32.

Natural England. 2012. Natural England Technical Information Note TIN034 House mice: options for management and control [Online] Available: www.naturalengland.org.uk [Accessed 15/8/12].

Ntampakis D & Carter I. 2005. Red kites and rodenticides – a feeding experiment. *British Birds* 98:411-416.

Pelz H-J, Rost S, Hunerberg M, Fregin A, Heiberg A-C, Baert R, MacNicoll AD, Prescott CV, Walker A-S, Oldenburg J & Muller CR. 2005. The genetic basis of resistance to anticoagulants in rodents. *Genetics* 170:1839-1847.

Pelz H-J, Rost S & Muller CR. 2007. DNA-based field monitoring of warfarin resistance in rats (*Rattus norvegicus*). *International Journal of Pest Management* 53:281-284.

Pelz H-J, Rost S, Muller E, Esther A, Ulrich RG & Muller CR. 2011. Distribution and frequency of VKORC1 sequence variants conferring resistance to anticoagulants in *Mus musculus. Pest Management Science* 68:254-259.

Pelz H-J & Prescott CV. 2015. Resistance to anticoagulant rodenticides. In: Buckle AP, Smith RH, (eds) *Rodent Pests and Their Control 2nd edition*. CAB International, Wallingford, UK. pp 187-208.

Pitt WC, Berentsen AR, Shiels AB, Volker SF, Eisemann JD, Wegmann AS & Howald GR. 2015. Non-target species mortality and the measurement of brodifacoum rodenticide residues after a rat (*Rattus rattus*) eradication on Palmyra Atoll, tropical Pacific. *Biological Conservation* 185:36-46.

Pocock MJO, Hauffe, HC & Searle JB. 2005. Dispersal in house mice. *Biological Journal of the Linnean Society* 84:565-583.

Prescott CV. 1996. Preliminary Study of the Genetics of Resistance in the House Mouse. Proceedings of the Seventeenth Vertebrate Pest Conference, ed. by Timm RM & Cribb AC. University of California. pp 83-87.

Prescott CV. 2011. Development of Rodenticides and the Impact of Resistance on Anticoagulant Rodenticides. Proceedings of the Seventh International Conference on Urban Pests, ed. By Robinson WH & de Carvalho Campos AE. Instituto Biologico, Sao Paulo. pp 33-36.

Prescott C, Baxter M, Coan E, Jones C, Rymer D & Buckle A. 2017. Anticoagulant resistance in rats and mice in the UK – current status in 2017 [Online]. Available https://www.thinkwildlife.org/downloads/ [Accessed 1/2/18].

Prescott CV, Buckle AP, Hussain I & Endepols S. 2007. A standardized BCR resistance test for all anticoagulant rodenticides. *International Journal of Pest Management* 53:265-272.

Quy RJ, Cowan DP, Haynes P, Inglis IR & Swinney T. 1992a. The influence of stored food on the effectiveness of farm rat control. Brighton Crop Protection Conference – Pests and Diseases. pp 291-300.

Quy RJ, Shepherd DS & Inglis IR. 1992b. Bait avoidance and effectiveness of anticoagulant rodenticides against warfarin- and difenacoum-resistant populations of Norway rats (*Rattus norvegicus*). *Crop Protection* 11:14-20.

Rattner BA, Lazarus RS, Elliott JE, Shore RF & van den Brink N. 2014. Adverse outcome pathway and risks of anticoagulant rodenticides to predatory wildlife. *Environmental Science & Technology* 48:8433-8445.

Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz H-J, Lappegard K, Seifried E, Scharrer I, Tuddenham EGD, Muller CR, Strom TM & Oldenburg J. 2004. Mutations in *VKORC1* cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 427:573-541.

Rost S, Pelz H-J, Menzel S, MacNicoll AD, Leon V, Song K-I, Jakel T, Oldenburg J & Muller CR. 2009. Novel mutations in the VKORC1 gene of wild rats and mice – a response to 50 years of selection pressure by warfarin? *BMC Genetics* 10:4.

Rowe FP, Plant CJ & Bradfield A. 1981. Trials of the anticoagulant rodenticides bromadiolone and difenacoum against the house mouse (*Mus musculus* L.). *Journal of Hygiene 87:171-177*.

Rowe FP & Redfern R. 1964. The toxicity of 0.025% warfarin to wild house-mice (*Mus musculus* L.). *Journal of Hygiene 62*:389-393.

Rowe FP & Redfern R. 1965. Toxicity tests on suspected warfarin resistant house mice (*Mus musculus* L.). *Journal of Hygiene* 63:417-425.

Rowe FP & Redfern R. 1967. The effect of sex and age on the response to warfarin in a non-inbred strain of mice. *Journal of Hygiene* 65:55-60.

RRAG. 2010. Anticoagulant resistance in the Norway rat and Guidelines for the management of resistant rat infestations in the UK [Online]. Rodenticide Resistance Action Group. Available: http://www.bpca.org.uk/rrag/downloads/RRAG_Resistance_Guideline.pdf [Accessed 15/8/12].

RRAG. 2012. RRAG House mouse resistance guideline [Online]. Rodenticide Resistance Action Group. Available: www.bpca.org.uk/assets/RRAG_Housemouseresistanceguideline1.pdf [Accessed 20/3/13].

Rymer D. 2017. Anticoagulant resistance in Norway rats conferred by VKORC1 mutations in South-East England. *University of Reading.*

Sanchez-Barbudo I, Camarero PB & Mateo R. 2012. Primary and secondary poisoning by anticoagulant rodenticides of non-target animals in Spain. *Science of the Total Environment* 420:280-288.

Scepovic T, Jokic G, Esther A, Kataranovski D, Vuksa P, Dedovic S & Vuksa M. 2015. VKOR variant and sex are the main influencing factors on bromadiolone tolerance of the house mouse (*Mus musculus* L.). *Pest Management Science* 72:574-579.

Shore RF, Birks JDS, Afsar A, WIenburg CL & Kitchener AC. 2003. Spatial and temporal analysis of second-generation anticoagulant rodenticide residues in polecats (*Mustela putorius*) from throughout their range in Britain, 1992-1999. *Environmental Pollution* 122:183-193.

Shore RF, Birks JDS & Freestone P. 1999. Exposure of non-target vertebrates to second-generation rodenticides in Britain, with particular reference to the polecat *Mustela putorius*. *New Zealand Journal of Ecology* 23(2):199-206.

Shore RF, Pereira MG, Potter ED & Walker LA. 2015. Chapter 17. Monitoring Rodenticide Residues in Wildlife. In Buckle AP, Smith RH, editors, Wallingford (Oxon UK): CAB International. pp. 346-365.

Singleton GR, Leirs H, Hinds LA & Zhang Z. 1999. Ecologically-based management of rodent pests – re-evaluating our approach to an old problem. *ACIAR Monograph 59*, Australian Centre for International Agricultural Research, Canberra. p p17-29.

Singleton GR & Redhead TD. 1990. Structure and biology of house mouse populations that plague irregularly: an evolutionary perspective. *Biological Journal of the Linnean Society* 41:285-300.

Smith P, Berody M & Smith RH. 1994. Body weight and social dominance in anticoagulant-resistant rats. *Crop Protection* 13:311-315.

Smith RH & Greaves JH. 1986. Resistance to anticoagulant rodenticides: the problem and its management. Proceedings of the 4th International Conference of Stored-Product Protection, ed. by Donahaye E & Navarro S. Tel Aviv, Israel. pp 302-315.

Smith SA & Morrissey JH. 2004. Properties of recombinant human thromboplastin that determine the International Sensitivity Index (ISI). *Journal of Thrombosis and Haemostasis* 2:1610-1616.

Smith RH & Shore RF. 2015. Chapter 16. Environmental Impacts of Rodenticides. In Buckle AP, Smith RH, editors, Wallingford (Oxon UK): CAB International. pp. 330-345.

Song Y, Endepols S, Klemann N, Richter D, Matuschka F-R, Shih C-H, Nachman MW & Kohn MH. 2011. Adaptive introgression of anticoagulant rodent poison resistance by hybridization between old world mice. *Current Biology* 21:1296-1301.

Stafford DW. 2005. The vitamin K cycle. *Journal of Thrombosis and Haemostasis* 3:1873-1878

Sutcliffe FA, MacNicoll AD & Gibson GG. 1987. Aspects of anticoagulant action: A review of the pharmacology, metabolism and toxicology of warfarin and congeners. *Review on Drug Metabolism and Drug Interaction* 5:226-261.

Syngenta. 2004. Rodent Pest Management – the Syngenta Experience [Online]. Syngenta. Available: www3.syngenta.com/country/au/SiteCollectionDocuments/Pest/Talon-Technical-%20Brochure.pdf [Accessed 4/3/13].

Taylor RC, Stephens AG & Sibly RM. 1996. The physiological basis of dietary preference in West Midlands behaviourally resistant house mice. *Proceedings of the Nutrition Society* 55:223A.

Thijssen HH. 1995. Warfarin-based rodenticides: mode of action and mechanism of resistance. *Pesticide Science* 43:73-78.

Tie J-K, Jin D-Y, Straight DL & Stafford DW. 2011. Functional study of the vitamin K cycle in mammalian cells. *Blood* 117:2967-2974.

Tie J-K & Stafford DW. 2015. Structural and functional insights into enzymes of the vitamin K cycle. *Journal of Thrombosis and Haemostasis* 14:236-247.

Tomlinson AJ, Rayner A, Bright A, Cowan DP, Littin KE & Layton R. 2017. Exploring drivers of the usage of anticoagulant rodenticides on UK farms. *International Journal of Pest Management* [Online] Available: http://dx.doi.org/10.1080/09670874.2017.1396378 [Accessed 9/1/18].

Tosh DG, McDonald RA, Bearhop S, Llewellyn NR, Montgomery WI & Shore RF. 2012. Rodenticide exposure in wood mice and house mice populations on farms and potential secondary risk to predators. *Ecotoxicology* 21:1325-1332.

Valchev I, Binev R, Yordanova V & Nikolov Y. 2008. Anticoagulant rodenticide intoxication in animals – a review. *Turkish Journal of Veterinary & Animal Sciences* 32:237-243.

Wallace ME & MacSwinney FJ. 1976. A major gene controlling warfarin-resistance in the house mouse. *Journal of Hygiene Cambridge 76*:173-181.

Whitlon DS, Sadowski JA & Suttie JW. 1978. Mechanism of coumarin action: Significance of vitamin K epoxide reductase inhibition. *Biochemistry* 17:1371-1377.

Witmer GW & Burke PW. 2009. Influence of vitamin K-rich plant foods on anticoagulant baiting in wild House Mice, wild Norway Rats, and wild Black Rats. *Pacific Conservation Biology* 15:87-91.

Witmer G, Horak K, Moulton R & Baldwin RA. 2013. New Rodenticides: An Update on Recent Research Trials. Proceedings of the Wildlife Damage Management Conference, ed by Armstrong JB & Gallagher GR. Clemson, South Carolina. pp 79-85.

Witmer GW, Snow NP & Moulton RS. 2014. Responses by wild house mice (*Mus musculus*) to various stimuli in a novel environment. *Applied Animal Behaviour Science* 159:99-106.

Wood BJ. 1994. Chapter 3. Rodents in Agriculture and Forestry. In Buckle AP, Smith RH, editors, Wallingford (Oxon UK): CAB International. pp 45-83.