# Assessing the sustainability of anticoagulant-based rodent control for wildlife conservation in New Zealand

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*I dedicate this thesis to*

*My Grandmother, Baljinder Kaur Sran*

*who has loved and supported me*

*unconditionally*

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# Abstract

Brodifacoum is used extensively to control invasive rodent pest populations in New Zealand. However, there are major concerns regarding non-target poisoning due to brodifacoum, its high persistence and risk of emergence of resistance in targeted rodents. In the present study, I assessed brodifacoum resistance in ship rats and house mice using blood-clotting response (BCR) tests. Mature ship rats of both sexes were live trapped from Akatarawa forest, an area of no known anticoagulant use history in Wellington. A ranging study was performed whereby healthy ship rats were administered increasing doses of brodifacoum to calculate the effective dose, which in ship rats is considered to be the dose giving a 3.6-fold increase in blood-clotting time (this proportional increase is referred to as the International Normalised Ratio, or INR). An unexpectedly high effective dose of 2.9 and 3.8 mg/kg was calculated for male and female ship rats respectively. The calculated effective dose was used to access brodifacoum susceptibility in ship rats captured from nine areas of known brodifacoum use history in the Wellington region and Palmerston North. A total of 54 ship rats were successfully tested, and there was a significant decrease in INR with increasing number of years of brodifacoum use in an area. Despite this evidence of anticoagulant resistance revealed by BCR tests, no mutations conferring anticoagulant resistance were found in VKORC1 gene sequences in tested ship rats. This suggests that resistance may be caused by other pathways.

Similarly, BCR tests were performed in house mice using the effective dose from published literature, i.e. 0.52 mg/kg for males and 0.46 mg/kg for females. Twenty out of 26 house mice assessed were found to be resistant to administered dose of brodifacoum, meaning the INR was >5. However, no relationship was observed between the INR value and the number of years of brodifacoum use in an area. Seven of the tested mice were found to have a non-synonymous mutation, Tyr139Cys in exon 3 of the VKORC1 gene. The house mouse individuals carrying this mutation are known to be fully resistance to all first-generation anticoagulants and a second-generation anticoagulant, bromadiolone, but only minor resistance is known to occur towards more potent second-generation anticoagulants.

At present, only technical resistance to brodifacoum has been reported in ship rats and house mice, and brodifacoum may still be used effectively to control these rodent populations. However, continual use of brodifacoum may encourage further resistance. Effective long-term control of anticoagulant-resistant populations can only be achieved by use of alternative non-anticoagulant rodenticides.

# Glossary of Key Terms related to Anticoagulant Resistance

1. **Anticoagulant resistance**: "A major loss of efficiency in practical conditions where the anticoagulant has been applied correctly, the loss in efficiency being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant" (Greaves 1994).
2. **Susceptibility:** Lack of resistance to anticoagulants.
3. **Technical resistance:** Refers to cases where resistance to anticoagulants has been identified in laboratory tests but resistance has little or no practical effects in terms of population control in the field (Pelz and Prescott 2015).
4. **Practical resistance:** Refers to the cases where resistance to anticoagulants has been identified in laboratory tests and resistance factors are so high that an acceptable control level cannot be achieved in the field (Pelz and Prescott 2015).
5. **Genetic resistance:** Refers to a heritable factor that allows an animal to survive a dose of an anticoagulant that would kill 99% of susceptible individuals of the population (Bailey *et al.* 2005).
6. **Lethal dose:** A dose of anticoagulant that is expected to kill some proportion of a susceptible population; for example, a $LD_{50}$ will kill 50% of individuals in a susceptible population.
7. **Effective dose:** A sub-lethal dose of anticoagulant that will produce a measurable response in blood-clotting time in some proportion of a susceptible population; for example, an $ED_{50}$ will produce a measurable response in 50% of individuals in a susceptible population (Bailey *et al.* 2005, Prescott *et al.* 2007). An $ED_{50}$ is expected to be substantially less than the $LD_{50}$ for the same population.
8. **International Normalised Ratio:** INR is the ratio of post-treatment blood-clotting factor to pre-treatment blood-clotting factor, raised to the power of the international sensitivity index (ISI) value of the reagent or coagulation equipment being used (Prescott *et al.* 2007). ISI value was 1 for the blood-clotting time analyser, Coaguchek used in the present study.

# CHAPTER 1

# Introduction

## *Rodents in New Zealand*

Rodents are the largest order of mammals with around 1702 species in the world. Rodents are known to be extremely successful in adapting to every terrestrial and freshwater habitat with many species found all over the globe. There are four species of rodents in the wild in New Zealand – *Rattus exulans, Rattus rattus, Rattus norvegicus* and *Mus musculus*. All the species are invasive and were introduced accidently or deliberately, initially by Polynesians about 1000 years ago and later by Europeans in the late 18$^{th}$ and early 19$^{th}$ century (Atkinson and Moller 1990). *Rattus rattus* and *Mus musculus* will be assessed in the present study for anticoagulant susceptibility.

## *Rattus rattus*

*Rattus rattus* (also called ship rat, roof rat and black rat) is usually found in commensal and agricultural areas, but in New Zealand, it is found in abundance in the wild. Ship rats were introduced to New Zealand accidentally in the second half of the 19th century, becoming widespread in the North Island by about 1860 and in the South Island about 30 years later (Atkinson, 1973). They are now found throughout North, South and Stewart Islands and are known to occur on at least 47 offshore islands (Innes 2005).

Most ship rats live less than a year in the wild in New Zealand, though females are reported to live longer than males (Daniel 1972). This nocturnal rodent species is smaller and more agile than the Norway rat. Ship rats are very good climbers and do not build burrows; rather they nest in trees or in the voids of buildings. Adult ship rats weigh up to 215 g and have a body length of up to 230 mm. The tail is uniformly coloured and much longer than head-body length. The ears are quite long and cover the eyes when pulled forward. The number of nipples is 10-12. Breeding is polyestrous. The gestation period is usually 20-22 days long with a litter size ranging from 3-10 pups. Growth of pups is rapid, and they reach maturity in 3-4 months (Bentley and Taylor 1965). Seasonal breeding in ship rats leads to varying numbers being found at different times of the year. Ship rat numbers are low in spring and early summer, and peak in autumn (Moors 1978). There are three different colour morphs of ship rat found in New Zealand namely; *Rattus rattus rattus* which has a uniformly black back and a grey belly, *Rattus rattus alexandrinus* which has a brown back with long black guard hairs and a grey belly; and *Rattus rattus frugivorous* which has a brown back with long black guard hairs and a creamy-white belly (Cunningham and Moors 1996). These colour morphs were once given a status of three subspecies, but this was later found to be unjustified (Tomich and Kami 1966).

They are generally believed to have had a dramatic impact on the native biota during their initial spread through the North Island, which coincided with the decline of many bird species and the disappearance of others, such as saddlebacks (*Philesturnus carunculatus* and *P. rufusater*), the stitchbird (*Notiomystis cincta*) and the piopio (*Turnagra tanagra*) from the mainland (Atkinson 1973). Their impact was emphatically demonstrated more recently when they invaded the Big South Cape Islands in 1962. Within the five years of their arrival, the greater short-tailed bat (*Mystacina robusta*), a flightless weevil and five species of birds endemic to New Zealand had disappeared from the islands and other animal and plant species had declined (Bell 1978, Ramsay 1978, Innes 2005). It was found that 70% of the nests of native birds at Kowhai Bush, Kaikoura, were destroyed by mustelids and rodents, with ship rats causing the most destruction (Moors 1983).

## *Mus musculus*

*Mus musculus,* also known as house mice, are believed to be of European origin in New Zealand. House mice are smaller in size as compared to rats and are dull brown to grey in colour, usually with a grey belly. They are usually ground dwelling, but can swim and climb too. The house mouse is the most extensively distributed mammal throughout the world. In New Zealand, mice are present extensively on the North Island, South island and many offshore islands.

House mice are mostly confined to commensal conditions but they are also found in native and exotic forests in New Zealand (Taylor 1978). They can live up to 3 years in the laboratory, but the lifespan is hardly 18 months in the wild. House mice are mostly nocturnal and may have territorial or colonial social organisation. House mice usually mature in about 8 weeks in summer, but animals born in late summer do not mature until the next spring. Breeding usually ceases in winter (Badan 1979, Pickard 1984). The gestation period is 19-21 days with litter size ranging from 2-12 pups (Murphy and Pickard 1990).

Little knowledge is available on impacts of house mouse on native flora and fauna of New Zealand. House mice have been known to kill lizards (Whitaker 1978), small eggs and nestlings (Moors 1978) and prey on invertebrates such as weevils (Bull 1967). They can also have an adverse effect on regeneration of native plants (Badan 1986).

## *Anticoagulant rodenticides*

Anticoagulants are classified on the basis of their chemical structure into two main groups – hydroxycoumarine rodenticides (e.g. warfarin, brodifacoum) and indandione rodenticides (e.g.

diphacinone, chlorophacinone) (Figure 1.1). On basis of their time of origin, they can be classified as first-generation anticoagulants, which were developed between the 1940s-1960s, and second-generation anticoagulants, which were developed between the 1970s-1980s (Valchev *et al.* 2008).

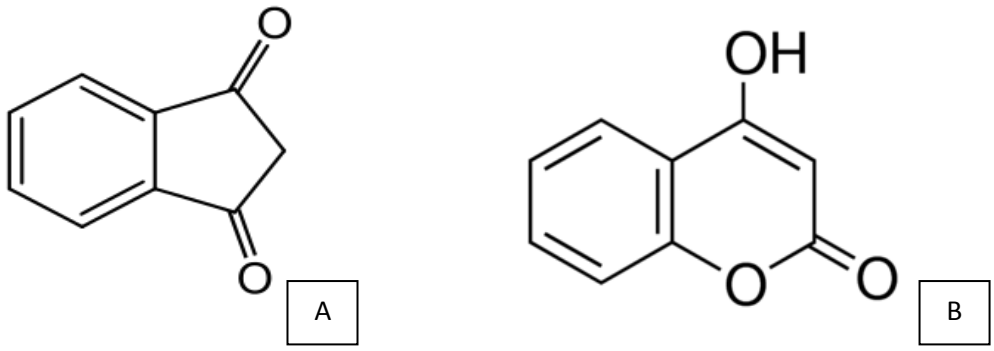



**Figure 1.1 A. The structure of indandiones; B. The structure of 4-hydroxycoumarins**

Prior to the 1950s, acute poisons such as zinc phosphide were more preferred and frequently applied (Matschke *et al.* 1982) as people were anxious to see a rapid kill of rodents and get rid of damage caused by them (Steven 2008). However, their repeated use led to bait-shy rodent populations (Prakash and Ghosh 1992, Reidinger 1995, El-Deeb *et al.* 2011). Bait-shyness, induced through conditioned taste aversion, can last more than a year, even when zinc phosphide has been removed from the baits (Shepherd and Inglis 1993). The development of chronic, first-generation anticoagulant rodenticides (warfarin and later indandione derivatives) in the early 1950s revolutionised rodent control, and for the first time, complete control of bait-shy rodent populations was possible and practical. Resistance to almost all the first-generation anticoagulants was detected in many countries across the world (Boyle 1960, Deoras 1966, Muktha Bai *et al.* 1981, Lund 1988).

In the early 1970s, second-generation anticoagulants, i.e. difenacoum and bromadiolone, were marketed and found to be effective against rodent populations resistant to first-generation anticoagulants (Hadler and Shadbolt 1975). But after a very short time, the initial success of both compounds was shaken by reports from many countries indicating rodent populations showing cross resistance either to difenacoum or bromadiolone or both (Redfern and Gill 1980, Cowan *et al.* 1995, Pelz *et al.* 2005). More potent second-generation anticoagulants like brodifacoum and flocoumafen are still effective till now, with low or no instances of resistance being reported for them.

The potent second-generation anticoagulants are more toxic than the first-generation anticoagulants (Hadler ans Shadbolt 1975). In comparison, the first-generation anticoagulants are more rapidly metabolized and excreted, thus having less risk to non-target species (Fisher *et al*. 2003). Second-generation anticoagulants like brodifacoum are effective as single-dose poisons (Singh and Saxena 1989). They are effective against rodents resistant to first-generation anticoagulant rodenticides (Greaves 1994) and rodents that are poison-shy to acute poisons like zinc phosphide (Saxena and Mathur 1996).

## *Mode of action of anticoagulant rodenticides*

All the anticoagulants have the same mode of action. Anticoagulant rodenticides act as antagonists of the enzyme Vitamin K1-epoxide reductase, causing a gradual depletion of the active form of Vitamin K resulting in its greater accumulation in the liver and kidneys (Parmar *et al.* 1987, Huckle *et al.* 1988) (Figure 1.2). This results in depletion of Vitamin K-dependent clotting factors, results in an increase in blood-clotting time until the point where no clotting occurs. For all anticoagulants, death is likely to occur within 5-7 days of the initial ingestion of a lethal dose (Buckle and Smith 1994, Littin *et al.* 2000).

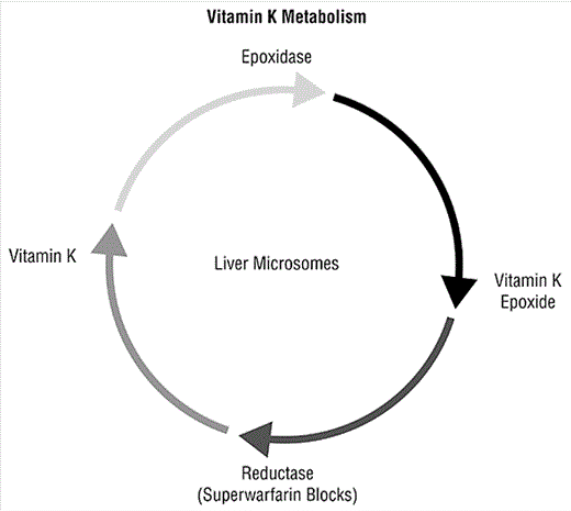



**Figure 1.2 The mode of action of an anticoagulant targeting the Vitamin K epoxide reductase complex and disrupting the Vitamin K cycle.**

Anticoagulants target Vitamin K epoxide reductase complex which helps in recycling of Vitamin K (Lowenthal and Macfarlane 1964). Vitamin K hydroquinone acts as a co-factor for the carboxylation of blood-clotting factors and other Vitamin K dependent proteins. During this carboxylation reaction, a molecule of Vitamin K hydroquinone gets oxidised to Vitamin 2, 3

epoxide. The next step in the process involves recycling of this compound back to Vitamin K hydroquinone by the Vitamin K epoxide reductase (VKOR) complex. Recycling of Vitamin K is essential to maintain the normal levels of blood-clotting factors. Suppression of the VKOR complex by anticoagulants results in inhibition of carboxylation of clotting factors and thus hindering the blood-clotting process. This depletion of blood-clotting factors can lead to fatal haemorrhages (Thijssen *et al.* 1986).

### *Anticoagulant use in New Zealand*

Anticoagulants are being used extensively in New Zealand to control rodent pests, primarily Norway rats, ship rats and house mice. Anticoagulant poisons are applied in the form of wax block or cereal- based formulations in bait stations by the Department of Conservation in one-off eradication operations, and by City and Regional Councils for ongoing control operations (Table 1.1). Regional councils are also known to subsidise these poisons occasionally to encourage farmers to use them for small scale small mammal control. Many anticoagulant formulations are available in supermarkets for residential rodent control. In addition, anticoagulants are used actively for small mammal control by more than 300 private conservation initiatives (Fisher *et al.* 2004, Cowan *et al.* 2017).

First-generation anticoagulants have very limited use in New Zealand at present. Warfarin was used for rodent control on the New Zealand mainland in the past, but its use is now restricted to offshore islands (Choquenot *et al.* 1990). Use of pindone for control of rodents and possums has declined considerably after more potent second-generation anticoagulants like brodifacoum came to the market in the 1980s. However, pindone is still used effectively to control rabbit populations (Eason *et al*. 2015). Diphacinone is used in cereal bait formulations for rodent control and in fish bait formulations to control ferrets. Diphacinone use is favoured for its low persistence. Coumatetralyl is used under the tradename Racumin®. It is less persistent than brodifacoum but more persistent than diphacinone (Fisher *et al.* 2003).

Brodifacoum, being one of the most potent rodenticides, is used extensively for rodent control in New Zealand. It is used extensively to control rodent and possum populations on the mainland and offshore islands. Other second-generation anticoagulants used in New Zealand are flocoumafen and bromadiolone. Brodifacoum and flocoumafen have very similar potency and persistence. Brodifacoum was first registered for rodents in New Zealand in 1981. It is used to target rodents along with other pest species like possums and rabbits. There are two different types of brodifacoum commonly used in New Zealand – Talon® 20P, a cereal-based

pellet containing 20 ppm of brodifacoum and Talon® 50P, cereal-based wax block comprising of 50 ppm of brodifacoum (Eason and Spurr 1995). Another trade name of brodifacoum is Pestoff®. Brodifacoum serviced in bait stations is recommended over aerially used brodifacoum, as bait stations reduce risk to non-target species (Brown 1997). However, aerial application of brodifacoum has been used extensively on New Zealand's offshore islands for rodent eradications (Veitch *et al.* 2011, Eason *et al.* 2015).

Brodifacoum is considered to be the best option for eradication of invasive pests in New Zealand. It produces delayed poisoning symptoms, thus the animal cannot relate its symptoms to the bait, meaning bait aversion is avoided. It is highly toxic to all the targeted pest species – rodents, possums and rabbits. A single bait is sufficient for the animal to consume a lethal dose. Also, brodifacoum has lower toxicity to dogs than 1080 (Hadler and Buckle 1992, Rammel *et al.* 1984).

**Table 1.1 List of anticoagulants currently registered in New Zealand for rodent control along with their active concentration used in formulated baits**.

| Anticoagulant | First or second generation | Concentration of active ingredient |
|---|---|---|
| **Coumatetralyl** | First | 0.038-0.05% |
| **Pindone** | First | 0.025-0.05% |
| **Diphacinone** | First | 0.005% |
| **Bromadiolone** | Second | 0.005-0.01% |
| **Difethialone** | Second | 0.0025% |
| **Brodifacoum** | Second | 0.002-0.005% |
| **Flocoumafen** | Second | 0.005% |

*Adapted from Cowan *et al.* 2017

### *Brodifacoum as rodenticide*

The IUPAC (International Union of Pure and Applied Chemistry) name of brodifacoum is 3-[3-[4-(4-Bromophenyl) phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-2-hydroxychromen-4-one . Brodifacoum is an off-white odourless powder with very low solubility in water (Figure 1.3).

However, it is soluble in alcohols, benzene and acetone. It is quite stable at room temperature with a melting point of 228-232°C.

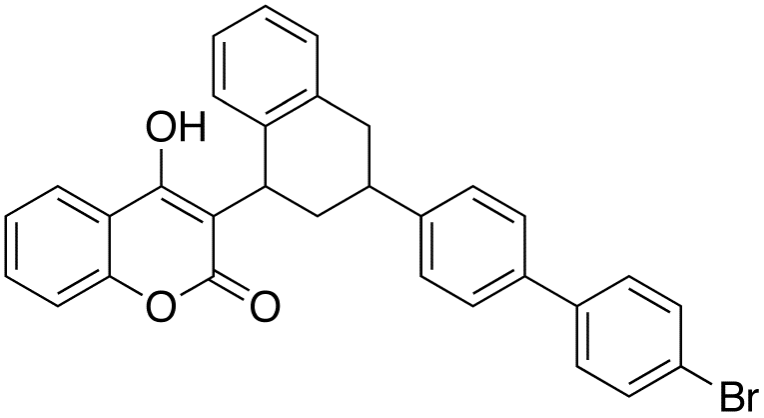



**Figure 1.3 The structure of brodifacoum.**

The rodenticidal properties of brodifacoum were described in the 1970s. Brodifacoum is a potent second-generation anticoagulant (4-hydroxy coumarin derivative), often called "super-warfarin" for its added potency and tendency to accumulate in the liver of poisoned organisms. When first introduced in the 1980s, it was effective against rodent populations that had become resistant to first-generation anticoagulants (Eason and Wickstrom 2001).

Brodifacoum, like other anticoagulant toxicants, works by increasing (or decreasing) the clotting time of blood, leading to death from haemorrhaging. Brodifacoum is absorbed through the gastrointestinal tract or the skin. After absorption, a high concentration of brodifacoum is likely to remain in the liver for a long time due to its high persistence. It is not easily metabolised and has a half-life of more than 80 days (Bachmann and Sullivan 1983). The major route of excretion of unbound brodifacoum is through faeces.

If ingested accidently, brodifacoum toxicity can be treated with the antidote Vitamin K1. Improvement can be assessed by regular checking of blood coagulation times. First signs of improvement can be seen within 12-25 hours of treatment, with human patients returning to normal within 36-48 hours. Brodifacoum baits can remain toxic in the soil for months. As baits start to disintegrate, brodifacoum is degraded slowly by soil bacteria over weeks to months. Brodifacoum contamination in water bodies is rare due to its insolubility in water. In case of direct disposal of brodifacoum into a water body, a short term contamination might occur. Brodifacoum is likely to bind to the organic matter and settle down in the sediment.

A single dose of brodifacoum is required to induce death after consumption of a lethal dose. The toxicity of brodifacoum varies among different mammals. The $LD_{50}$ values range from

0.4mg/kg in the case of house mice to 5-25 mg/kg in the case of sheep. The $LD_{50}$ value of brodifacoum for Norway rats is known to be 0.27 mg/kg (Eason *et al.* 1994, Eason and Spurr 1995). Among bird species, $LD_{50}$ values range from <0.75 mg/kg in the Canada goose to 10 mg/kg in the Australian harrier (Godfrey 1985).

Brodifacoum is among the most toxic of the anticoagulants used against rodents (Erickson and Urban 2004), so they need to ingest a relatively small amount of bait for lethal exposure. The product has become a valuable tool for island conservation because of its delayed toxicity (Kaukeinen and Rampaud 1986), high rodenticidal efficacy, and bait formulations that are highly acceptable to rodents and can be applied aerially over large areas. The greater potency of second-generation anticoagulant toxins such as brodifacoum than of first-generation anticoagulants such as warfarin and pindone is probably related to their accumulation and persistence in the liver after absorption (Huckle *et al.* 1988). However, with time, efficacy of these control measures may be lost by physiological resistance to anticoagulant rodenticides and by behavioural reactions of the target rodent population (O'Connor *et al.* 2003, Pelz and Klemann 2004).

## *Clinical signs of brodifacoum poisoning*

Brodifacoum toxicity can occur in domestic and wild animals due to oral intake of baits containing anticoagulant pesticides. The absence of odour and palatable taste due to presence of saccharose further increases chances of intoxication in humans and animals. Secondary toxicity can occur in predatory or scavenging animals and birds after feeding on poisoned rodents (Eason *et al.* 2002, Binev *et al.* 2005). Cases of toxicity due to dermal contact or from drinking contaminated water are rare (Endepols *et al.* 2003). Spontaneous intoxications have been reported in dogs, horses, cats, wild animals like deer, owls, eagles, falcons, ducks and in humans (Valchev *et al.* 2008).

The biological half-life of brodifacoum is 120 days (Steensma *et al.* 1994). Unlike first-generation anticoagulant rodenticides, a single dose of brodifacoum may cause haemorrhages. The anticoagulants inhibit carboxylation of blood-clotting factors leading to their non-functionality (Woody *et al.* 1992, Smith *et al.* 2000).

Initial symptoms of brodifacoum poisoning may include weakness, pale mucosa, decrease in appetite, increased urination, increase in thirst, rapid exhaustion with reduced locomotion (Petterino *et al.* 2001, Petterino *et al.* 2004). Increased heart rate and rapid difficult respiration or panting has also been reported in all experimental studies as well as spontaneous intoxications (Lewis *et al.* 1997). Significant intoxication of brodifacoum can lead

to hematochezia; haemorrhages on the skin, anterior eye cornea and iris, vomiting containing blood, nasal bleeding, vaginal bleeding, ear bleeding, pleural haemorrhages and blood containing nasal discharges (Valchev *et al.* 2008). Haemorrhages in internal organs may result in anaemia. Severe anemia can cause hypovolaemic shock, coma and death. The first symptoms of intoxication may be delayed for days or weeks after ingestion of substantial amounts (Watt *et al.* 2005).

### *Non-target poisoning*

Apart from resistance being the main reason for failure of anticoagulants, the broad-scale field use of such anticoagulants has also raised concern regarding their tendency to bio-accumulate in non-target species (Hosea 2000, Fisher *et al.* 2003, Giraudoux *et al.* 2006, Giorgi and Mengozzi 2010). These include both primary and secondary hazards to predatory and scavenging birds and mammals (Ebbert and Burek-Huntington 2010, Ruder *et al.* 2011, Thomas *et al.* 2011, Gabriel *et al.* 2012).

Brodifacoum has been used successfully in recent rodent eradications programmes on offshore islands worldwide to protect native birds and other species (Taylor and Thomas 1989, Courchamp *et al.* 2003, Towns and Broome 2003). Apart from the successful kill of predators, brodifacoum use has emerged as a great problem due to its characteristics of high potency, high persistence, inhumane death and non-target poisoning. Anticoagulants share a common binding site in the liver, but the second-generation anticoagulants have a greater binding affinity than the first-generation compounds (Parmar *et al.* 1987). Brodifacoum has high persistence, lasting for at least 6 months in the organs containing the VKOR enzyme such as the liver, kidney and pancreas. Wildlife contamination after the use of potent second-generation anticoagulants is reported from worldwide. There have been numerous instances of native birds being contaminated with brodifacoum directly due to consuming baits or through secondary poisoning in New Zealand (Eason *et al.* 2015).

In pest control using anticoagulants in Tawharunui Regional Park, high mortality was observed in New Zealand dotterels (Dowling *et al.* 2006). Brodifacoum-based pest control in Motuihe Island resulted in 49% and 60% mortality in pukeko and paradise shelducks respectively. 29 dead birds were found after this operation and all the carcasses contained brodifacoum residues in range of 0.12-2.31 μg/g (Dowling *et al.* 1999). In a similar pest control operation in Rangitoto and Motutapu, three out of nine dead penguins were detected with brodifacoum residues (Fisher *et al.* 2011).

In New Zealand, herbivorous and omnivorous birds like weka, pukeko and saddleback are at risk of primary poisoning from feeding directly on cereal-based baits of anticoagulants. South island robins have been spotted eating crumbs of brodifacoum dropped by the rats on Breaksea Island. Two dead robins were reported from the island (Taylor and Thomas 1993). Brodifacoum baits were reported to be consumed by western weka on Tawhitinui Island and Chetwode Island and proved to be lethal (Taylor 1984). Some bird species like shelducks and silvereyes, which were assumed to be moderately resistant to brodifacoum, were found dead by brodifacoum poisoning. Secondary poisoning is a greater risk for scavenging and predatory birds.

Five species of birds (western weka, Stewart Island weka, southern backed gull, Australian harrier and morepork) are believed to have been killed by secondary brodifacoum poisoning in New Zealand, and New Zealand falcon, brown skua and buff weka are also considered to be at high risk of secondary poisoning (Williams *et al.* 1986, Eason and Spurr 1995). Secondary poisoning is also a concern for insectivorous birds. Secondary brodifacoum poisoning was reported in Stewart Island robin on Ulva Island. Thirteen dead nestlings were collected from the Island after the application of brodifacoum bait to control rodent populations, and out these, 12 nestlings tested positive for brodifacoum residues (Masuda *et al.* 2014). Short-tailed bats are insectivorous mammals and have also been reported to consume ground laid cyanide bait used for possum control. This puts them at high risk from both primary and secondary poisoning of brodifacoum. Long-tailed bats are also at risk of secondary poisoning (Eason and Spurr 1995). Native geckos and skinks have been reported to consume brodifacoum baits but lizards and amphibians are considered to be at low risk from non-target poisoning due to distinct blood-clotting mechanism as compared to mammals (Hoare and Hare 2006).

Extent of use of brodifacoum is not the only factor that affects non-target poisoning Improving methods of brodifacoum use can help reduce the risk of non-target poisoning. Use of brodifacoum in bait stations reduces the exposure of brodifacoum to non-target species rather than placing them as easily accessible ground feeds. Additionally, lower brodifacoum residues have been reported in dead rats that were exposed to intermittent pulse baiting as compared to the rats exposed to continuous pulse baiting (Eason and Spurr 1995).

### *Pathways of resistance to anticoagulants*

Many mechanisms have been identified to explain the basis of anticoagulant resistance in rodents. The three main mechanisms hypothesised are pharmacodynamic or biochemical

resistance, pharmacokinetic-based resistance and dietary-based resistance (Suttie 1980, MacNicoll 1985 and Thijssen 1995) (Figure 1.6). Out of these, pharmacokinetic and dietary-based resistance are thought to be the secondary reasons in certain rodent strains. Pharmacodynamic resistance is believed to be the chief mechanism of anticoagulant resistance in rodents (Li *et al.* 2004, Rost *et al.* 2009). All the three mechanisms are explained below briefly.

*Pharmacodynamic or biochemical resistance*

Anticoagulants inhibit VKOR activity leading to the disruption of Vitamin K cycle. This results in undercarboxylation of Vitamin K-dependent proteins and therefore lowers the optimal levels of functional Vitamin K dependent blood-clotting proteins. Change in conformation of Vitamin K epoxide reductase complex subunit 1 (VKORC1) protein due to mutation in VKORC1 gene leads to lower susceptibility for anticoagulants in rats and mice (Thijssen *et al.* 1989) (Figure 1.4).

The biochemical basis of resistance was not known till the 1990s when Misenheimer and Suttie (1990) assessed the Vitamin K epoxide reductase enzyme activity in liver microsomes. It was established that VKOR activity was inhibited by warfarin. Meisenheimer *et al.* (1993) performed VKOR assay in a resistant strain of mice and found that the enzyme was highly insensitive to inhibition by bromadiolone and warfarin.

A molecule that contributes to VKOR activity in Norway rats was identified and named as Vitamin K epoxide reductase complex subunit 1 (Vkorc1) (Rost *et al.* 2004, Li *et al.* 2004). It was found that the C132-X-X-C135 motif in the VKORC1 gene comprises the site of redox activity that catalyses the Vitamin K epoxide reduction (Rost *et al.* 2005, Goodstadt and Ponting 2004), and mutations in this gene were found to be responsible for warfarin resistance. Pelz *et al.* (2005) also reported that several codon positions; Arrg35Pro, Ser56Pro, Leu120Gln, Leu1288Gln and Thr139Phe; in VKORC1 gene that caused resistance in rats against anticoagulants in Europe. Of these, Thr139Phe amino acid substitution was responsible for maximum resistance of VKOR activity towards warfarin when expressed in HEK 293 cells.

(Schulman and Furie 2015)

**Figure 1.4 Vitamin K cycle and its sensitivity to inhibition by anticoagulants like brodifacoum.**

Altered VKOR activity due to the genetic mutations in VKORC1 gene has also been reported in house mice. Many different amino acid substitutions, for example, W59G, R12W, R58G, R61L causing mutations in VKORC1 gene in resistant strains of mice have been described leading to reduced VKOR activity by up to 49% (Lasseur *et al.* 2006, Rost *et al.* 2009).

However, there are also instances showing mutated VKORC1 gene without any effect on VKOR enzyme activity. Rost *et al.* (2009) reported R58G mutation with no modification in VKOR enzyme activity. There is no evidence of altered VKOR enzyme activity being the cause of resistance in ship rats (Berny 2011). Recent research has proved that resistance in all the rodents is not explicable using one single model based on biochemical resistance (Kerins and MacNicoll 1999, Heiburg 2009). It is therefore evident that the presence of a particular gene mutation by individual rodents is not necessarily a "marker" for resistance of either the practical or technical type (Buckle and Prescott 2012). Technical resistance refers to cases where resistance to anticoagulants has been identified in laboratory test, but resistance has little or no practical effects. Practical resistance refers to the cases where resistance has been identified in laboratory tests and resistance factors are so high that an acceptable control level cannot be achieved (Buckle *et al.* 2010).

*Pharmacokinetic-based resistance*

Pharmacokinetics plays a vital role in the process of blood clotting as well as the reduced blood-clotting times enhanced by anticoagulants. Anticoagulant rodenticides may be effective for controlling one species but ineffective for other species. This is due to the increased ability of some animals to metabolise and excrete anticoagulant compounds from their body. The mammalian metabolic system, comprising of cytochrome P450 (CYP 450) enzymes, facilitates clearance of anticoagulants from the body. It has been found that inhibition of these CYP 450 enzymes strongly increases the effect of anticoagulants (Buckle and Prescott 2012).

Several isoforms of CYP 450, CYP2C, CYP2B, CYP1A and CYP3A catalyse the metabolism of anticoagulants by hydroxylation. The identified hydroxides of the first-generation anticoagulant warfarin are 4'-, 6-, 7-, 8-, and 10- hydroxywarfarin (Daly and King 2003, Guengerich *et al.* 1982) (Figure 1.5). The water solubility of these hydroxides is increased by UDP-glucuronyltransferase activity, and are the hydroxides are then excreted in the urine (Ishizuka *et al.* 2008).

(Ishizuka 2007)

**Figure 1.5 Metabolism of warfarin in rats by CYP 450 enzymes**

Sutcliffe *et al.* (1990) found that metabolic profile of warfarin 4'-, 6-, 7- and 8-OH was very different in susceptible and resistant mice when treated with various P450 inducers. This showed that CYP 450 played an important role in resistance of house mice. Ship rats with evidence of metabolic resistance were first described in Tokyo by Sugano *et al.* (2001). Later Ishizuka *et al.* (2006) investigated VKORC1 gene in resistant ship rats in Tokyo but failed to detect any VKORC1 mutations. On further investigation, it was found that warfarin metabolic

activities were significantly higher (nearly twice) in resistant rats as compared to susceptible rats after administration of oral dose of warfarin. It was therefore evident from these results that CYP-dependent metabolism of warfarin is a pathway of resistance in ship rats (Ishizuka *et al.* 2008). In a recent study in Tokyo, the mechanism of resistance in ship rats was investigated. It was found that mutations in the VKORC1 gene were not solely responsible for warfarin resistance. Warfarin resistance also involved high anticoagulant clearance ability due to high levels of CYP isoforms (Takeda *et al.* 2016).

Enhanced anticoagulant clearance has been thought to be particularly significant in the resistance mechanisms of house mice and ship rats (Ishizuka *et al.* 2008, Buckle and Prescott 2012). This aspect of resistance has not been evaluated much and work has only been reported for warfarin metabolism. Therefore, a lot of work still needs to be done to evaluate its role in resistance in rodents especially for second-generation anticoagulants (Berny 2011).

*Dietary-based resistance*

Anticoagulant resistance may be due to increased Vitamin K availability. Synthesis of Vitamin K from the pro-vitamin compound Vitamin $K_3$ (Menadione sodium bisulphate) has been evident in many different strains of rats (MacNicoll and Gill 1993). However, studies done on susceptible rats and both susceptible and resistant mice have shown that some animals have not been able to synthesise the compound in the same way. Vitamin $K_3$ present in many animal feeds may be the reason for levels of resistance in certain strains of rodents. Due to these reasons, dietary-based resistance is not regarded to be a significant mechanism of resistance (Buckle and Prescott 2012).

(Ishizuka 2008)

**Figure 1.6 Mechanisms of resistance in rodents against anticoagulants. More than one mechanism can be active at a time and contribute to resistance.**

### *Pleiotropic effects of resistance*

Anticoagulant resistance in rodents may be accompanied by a big cost. Some of the mutations in the VKORC1 gene can result in increased dietary requirement for Vitamin K. This may be due to structural changes in proteins caused by such mutations (Pelz and Presc6ott 2015). The dietary requirement of Vitamin K was found to be 2-3 times and nearly 20 times in Norway rats heterozygous and homozygous for mutation Tyr139Ser (Hermodson *et al.* 1969). The dietary requirement of Vitamin K may vary among anticoagulant-resistant rats depending on the mutation causing the resistance. In a study done at the University of Reading, Norway rats homozygous for mutation Tyr139Ser showed more prolonged blood-clotting times than Norway rats with mutation Leu120Gln after being fed on diet deficient in Vitamin K. Also, a greater effect was observed in males than females (Pelz and Prescott 2015).

Increase in dietary requirement of Vitamin K can act as a strong natural selection against such anticoagulant resistance in the absence of anticoagulants. Partridge (1979) observed a 33-80% decline in the number of anticoagulant resistant Norway rats over a period of 18 month in the absence of anticoagulant use. The relative fitness of resistant rats was found to be 0.47 in rats homozygous for a mutation conferring resistance and 0.77 for rats

heterozygous for resistance conferring mutation in comparison to the fitness level of 1.0 in susceptible rats. This study suggested the disappearance of resistant individuals from population in 15-25 generations. There may also be other costs to anticoagulant resistant rats. Norway rats carrying mutation Tyr139Cys were found to have arterial calcification (Kohn *et al.* 2008). Renal arteries were found to be mineralised in both sexes while aorta had significant calcification in case of males homozygous for mutation. Jacob *et al.* (2012) found minimal effect on reproduction in Norway rats carrying the Tyr139Cys mutation in Germany.

However, mutations conferring resistance in rodents are not always accompanied by such costs. Norway rats in Denmark did not show any change in dietary requirement for Vitamin K or any other selection against resistant individuals carrying the Tyr139Cys mutation. No change in proportion of resistant individuals in the population was observed over a period of two years in the absence of any anticoagulant use (Heiberg *et al.* 2006).

### *Methods to detect anticoagulant resitance in rodents*

An important step towards management of rodents using anticoagulant rodenticides on a long-term basis is monitoring their susceptibility to anticoagulant poisons. The monitoring of anticoagulant resistance becomes a real priority where anticoagulants fail to provide practical control of rodents. Such failure may be due to several reasons, like defective bait formulation, defective bait application technique, bait avoidance by rodents, ineffective bait stations, invasion of new rodents etc, and is not restricted to just resistance to anticoagulants. If resistance is assumed to be the reason for failure of anticoagulant rodenticides and no tests are run, false diagnosis may lead to wasted research efforts, inappropriate counter-measures as well as neglect of true reasons responsible for the failure (EPPO 1995, Pelz and Proscot 2015).

There are different methods used to test for resistance to anticoagulants. These include lethal feeding trial tests, blood-clotting response tests, VKOR activity analysis, CYP450 metabolism analysis and VKORC1 genotyping (Table 1.2). Lethal feeding trial tests as well as blood-clotting response tests provide information about practical resistance in the field and are used widely to study susceptibility of anticoagulants in rodents. Genetic analysis of the VKORC1 gene in rodents is used in routine tests to look for mutations associated with anticoagulant resistance. The *in vitro* assay to analyse VKOR enzyme activity in rodents has been developed mostly for identified mutations in the VKORC1 gene. Limited information is available on CYP450 metabolism and it is not usually analysed (Berny *et al.* 2018). Some methods may be more important for certain species of rodents than others. For example,

although metabolic resistance has been described in Norway rats it is a more important pathway of resistance in ship rats (Ishizuka *et al.* 2008, Buckle and Prescott 2012).

*Lethal feeding period tests*

In a lethal feeding period (LFP) test, a discriminating dose is established for a particular species of rodent for a particular anticoagulant. The discriminating dose is calculated from a ranging study where susceptible individuals of the study species are fed on anticoagulant bait in a no-choice trial. The $LD_{99}$ dose is then evaluated using logistic regression, where $LD_{99}$ is the estimated dose that kills 99% of the susceptible individuals. This dose is then used to test wild rodents for susceptibility to anticoagulant rodenticides (EPPO 1995). Many such lethal feeding trial tests have been established for Norway rats, for example, 7 days feeding on 5 ppm brodifacoum (Gill and MacNicoll 1991). An advantage of these tests is that they measure mortality as a parameter to test for resistance, and therefore the results obtained can be easily related to performance of anticoagulant rodenticide in the field. However, these tests are time-consuming and have ethical costs. For example, a house mouse needs to be fed on 0.025% warfarin for 21 days to test it for resistance. In addition, a high survival rate after intake of anticoagulant poison is not a proof of resistance but indicates further need to investigate (Pelz and Prescott 2015).

*Blood-clotting response tests*

Blood-clotting response (BCR) tests are based on measuring blood-clotting activity before-and-after administration of an anticoagulant dose called the “effective dose”. An effective dose (usually $ED_{50}$) first needs to be calculated for each species before screening animals for resistance, as it is essential to determine what level of change in blood-clotting response will be considered as significant. An $ED_{50}$ is a sub-lethal dose of anticoagulant that will not kill the animals, but will produce a critical measurable change in blood-clotting time in 50% of individuals. Each tested individual is categorized as a “responder” or “non-responder” to the administered dose depending on the delay in blood-clotting time, which reflects the decline in plasma coagulation activity (Prescott *et al.* 2007). Coagulation times can be converted into plasma percent coagulation activity by constructing calibration curves based on serial dilutions of normal plasma in saline solution (Pelz and Prescott 2015). A rodent is considered to be susceptible to a dose of anticoagulant if its plasma percent coagulation activity (PCA) is less

than 17% or 10% when blood-clotting time is measured 24 h or 96 h respectively after dosing (Martin *et al.* 1979, MacNicoll and Gill 1993, Gill *et al.* 1994, Precott and Buckle 2000). For Norway rats and house mice, 17% plasma PCA activity corresponds approximately to an International Normalised Ration (INR) of 5, meaning the blood-clotting time increases 5-fold (Prescott *et al.* 2007). For ship rats, 17% plasma PCA activity corresponds to INR of 3.6 (3.5-3.8) (Garg and Singla 2015). A "discriminating dose" is a higher multiple of the effective dose that is used to test for resistance in the field.

BCR tests are very sensitive and can detect even small differences in susceptibility of rodent species to specific anticoagulant rodenticides. This test does not rely on mortality of animals, and hence is considered more humane, and can be performed in 24 h so is time efficient (Prescott and Buckle 2000). The main disadvantage of this methodology is that resistance assessments are based on changes in coagulation time, a parameter that is difficult to relate to performance in practical rodent control (Berny *et al.* 2018). To be able to provide information about operational importance in New Zealand forests, practical resistance also needs to be evaluated. A "resistance factor" needs to be calculated for ship rats for each site of interest. The Resistance Factor is the multiple of the effective dose required to produce a blood-clotting response in a resistant animal similar to that produced by susceptible animal in response to the effective dose (Prescott *et al.* 2007, Endepols *et al.* 2015).

*VKORC1 genotyping*

The genetic basis of anticoagulant resistance was established for the first time in the 1960s in warfarin-resistant Norway rats. It was found to be present on one single autosomal gene (Rw) on chromosome number 1 (Greaves and Ayers 1967, Kohn and Pelz 2000, Lasseur *et al.* 2005). Because the Rw gene codes for subunit 1 of the Vitamin K epoxide reductase enzyme complex, this gene was named the VKORC1 (Vitamin K Epoxide Reductase Complex Subunit 1) gene. A single independent mutation referred to as a Single Nucleotide Polymorphism (SNP) leading to a change in amino acid is known to be responsible for anticoagulant resistance in rats and mice (Pelz *et al.* 2005, Rost *et al.* 2009, Grandemange *et al.* 2010, Pelz *et al.* 2012). This test does not require live animals but only a small sample of tissue and, therefore, is one of the most cost-effective and widely used resistance detection tests. If a particular mutation associated with anticoagulant resistance is found in a rodent species and established to have significant effect on practical anticoagulant resistance using laboratory tests like BCR tests and LFP trials as well

as in vitro assays, like Vitamin K epoxide reductase (VKOR) enzyme activity, rodents can be screened on a large scale for presence of the mutation using special primers to target the specific region on the VKORC1 gene known to contain the studied mutation (Prescott *et al.* 2017, Buckle *et al.* 2010).

*VKOR enzyme activity*

A VKOR activity assay is an *in vitro* assay that determines kinetic constants for the VKOR enzyme. Numerous protocols have been reported to study VKOR activity (Thijssen *et al.* 1989, Lasseur *et al.* 2005, Rost *et al.* 2009, Grandemange *et al.* 2010). This assay provides a good estimate of Vitamin K epoxide reductase (VKOR) enzyme activity and resistance status of the tested rodent population. It is a rapid and cost-effective test that provides information about all anticoagulants (Lasseur *et al.* 2006). The test requires few animals and no live animals are needed in the laboratory. However, metabolic resistance cannot be detected by VKOR activity assay.

*CYP450 Metabolism*

CYP450 metabolism activity is not used routinely to test for anticoagulant resistance in rodents. Although CYP450 metabolism activity has been described in Norway rats (Ishizuka *et al.* 2007), ship rats (Sugano *et al.* 2001, Ishizuka *et al.* 2008) and house mice (Sutcliffe *et al.* 1990), more work needs to be done to study CYP450 isoforms and standardise these tests. Preliminary study conducted at the Lyon Veterinary School reported each anticoagulant is metabolised by a different CYP450 isoform, and thus a different test needs to be done for testing different anticoagulants (Berny *et al.* 2018).

**Table 1.2 Summary of methods for detecting anticoagulant resistance in the Norway rat, ship rat and house mouse. Adapted from Berny *et al.* (2018)**

| Detection method | Norway rat | Ship rat | House mouse | References |
|---|---|---|---|---|
| Lethal feeding trial test | Standardised<br>Good estimation of practical resistance<br>Needs live animals for up to 21 days | Standardised<br>Good estimation of practical resistance<br>Needs live animals for up to 21 days | Standardised<br>Good estimation of practical resistance<br>Needs live animals for up to 21<br>Days | WHO 1982<br>EPPO 1995<br>Garg and Singla 2014 |
| Blood clotting response test | Standardised<br>Estimates technical resistance<br>Practical resistance studied using resistance factors<br>Needs live animals up to 48 hours | Not standardised for all anticoagulants<br>Estimates technical resistance<br>Practical resistance studied using resistance factors<br>Needs live animals up to 48 hours | Standardised<br>Estimates technical resistance<br>Practical resistance studied using resistance factors<br>Needs live animals up to 48 hours | Prescott *et al.* 2007<br>Endepols *et al.* 2007<br>Garg and Singla 2015<br>Present study |
| VKOR activity analysis | In vitro<br>Tests for all FGARs and SGARs<br>Limited number of animals<br>Rapid<br>Not suitable test for metabolic resistance | In vitro<br>Tests for all FGARs and SGARs<br>Limited number of animals<br>Rapid<br>Not suitable test for metabolic resistance | In vitro<br>Tests for all FGARs and SGARs<br>Limited number of animals<br>Rapid<br>Not suitable test for metabolic resistance | Thijssen *et al.* 1989<br>Meisenheimer *et al.* 1993<br>Lasseur *et al.* 2005<br>Rost *et al.* 2009 |
| Metabolism activity analysis | Suspected<br>Not standardised for routine test<br>Different for each anticoagulant | Well described<br>Not established as routine test<br>Different for each anticoagulant | Described<br>Not established as routine test<br>Different for each anticoagulant rodenticid | Sutcliffe *et al.* 1990<br>Sugano *et al.* 2001<br>Ishizuka *et al.* 2007 |

| | | | | |
|---|---|---|---|---|
| | rodenticide | rodenticide | | Takeda *et al.* 2016 |
| VKORC1 genotyping | Well adapted | Limited evidence as compared to Norway rats | Limited evidence as compared to Norway rats | Pelz *et al.* 2005 |
| | Standardised routine test widely used | Routine | Routine | Rost *et al.* 2009 |
| | Needs information on mutation/activit | Needs more study | Needs more study | Tanaka *et al.* 2012 |
| | | | | Goulois *et al.* 2016 |
| | | | | Cowan *et al.* 2017 |
| | | | | Rymer 2017 |

# Aims and objectives

Presently, the main approach for tackling rodent problems all over the world is to reduce their populations by killing them with rodenticides (Parshad 1999). Other methods such as trapping, habitat manipulation, use of repellents/attractants/pathogenic agents are used for the management of rodent population, but have never produced consistent results (Buckle and Muller 2000). Chemical control by rodenticides is the most widely used and efficient method of all the available methods for the control of rodent pests in both agricultural and commensal situations.

Among the methods of chemical control, anticoagulants are the most preferred method. Anticoagulant use patterns in New Zealand differ from those in other parts of the world, where anticoagulants are generally used for control of commensal rodents in and around buildings. The Department of Conservation has used brodifacoum extensively for island eradications but stopped using it for mainland control operations in 2002 due to concerns about its persistence in the environment. However, it continues to be used extensively by regional councils, community groups and private landowners. To date, brodifacoum baiting has been used in an estimated 71% of campaigns to eradicate introduced rodents from islands around the world (Howald *et al.* 2007). Anticoagulant-based rodent control has been shown to be effective for recovering native bird populations, both in conservation reserves (Parlato and Armstrong 2012) and in forest fragments on agricultural land (Armstrong *et al.* 2014). However, there are major concerns about the long-term sustainability of these operations.

The broad-scale field use of anticoagulants has raised concern regarding their tendency to bio-accumulate in non-target species (Fisher *et al.* 2003, Giraudoux *et al.* 2006, Giorgi and Mengozzi 2010). The effects of anticoagulants can range from acute mortality to sub-lethal effects (Hoare and Hare 2006, Dennis and Gartrell 2015). Insectivores such as the hedgehog have been detected to have significant levels of anticoagulants in their tissues, suggesting that insectivorous birds of New Zealand may also be at a high risk. Predatory and scavenging species are at alarming risk of secondary exposure to brodifacoum and other anticoagulants. Predators prey on live animals that contain residual brodifacoum due to sub lethal doses and scavengers feed on carcasses of animals killed by anticoagulant poisoning (Berny 2011, Ruder *et al.* 2011, Thomas *et al.* 2011, Gabriel *et al.* 2012).

However, another major concern, resistance, is the principal reason for failure of anticoagulant pesticides in rodent pest management. Resistance occurs when there is selection for individuals with heritable mutations allowing them to survive doses of anticoagulant that would kill normal individuals, reducing sensitivity of the population to the anticoagulant. Rodents may therefore evolve greater resistance to anticoagulants over time if they are used continuously, meaning that control will become ineffective unless application rates are substantially increased, creating even greater risks for non-target species. Such resistance is well known from agricultural research, having been demonstrated for several rodent species in countries around the world (Greaves 1986).

This PhD project assessed brodifacoum resistance in ship rat and house mouse populations in the Wellington Region and Palmerston North. These areas were chosen due to the extensive use of brodifacoum and other anticoagulants by the Wellington Regional Council and Palmerston North City Council over the last 20 years. The extended use of brodifacoum in these areas may have resulted in selection for anticoagulant resistance in rodent populations and decreased the effectiveness of pest control operations. The objectives of the research project included:

1. Determining the current effective dose of brodifacoum in the ship rat (*Rattus rattus)*
2. Testing for brodifacoum resistance in ship rat (*Rattus rattus*) and house mouse (*Mus musculus*) populations
3. If resistance is found, assess whether it is explained by mutations to the VKORC1 (Vitamin K epoxide reductase complex 1) gene.

**GAP OF KNOWLEDGE:**

Few data are available on sustainability of anticoagulants in New Zealand. No resistance to brodifacoum has been reported in New Zealand yet and brodifacoum is highly effective for rodent control at present, but it is important to regularly monitor for resistance in long-term anticoagulant use areas and establish a database for future references. There is probability of increasing tolerance of rats and mice to brodifacoum as has been reported from other parts of the world (Bailey and Eason 2000).

**THESIS OUTLINE AND STYLE**

This thesis includes five research chapters that are formatted for submission to peer reviewed journals. Therefore, there is repetition of information throughout, particularly in the introductory sections, study sites and some of the methods. This style allows for the production of several individual publishable studies while focussing on the overall aims and objectives of the thesis.

# CHAPTER 2

# Evaluating an effective dose of brodifacoum for ship rat (*Rattus rattus*) in New Zealand forests

## *2.1 Introduction*

### *Anticoagulant resistance*

Anticoagulant resistance in rodents was discovered soon after first-generation anticoagulant rodenticides (FGARs) were introduced, when there was a failure of control of Norway rats (*Rattus norvegicus*) population in Scotland after treated with warfarin (Boyle 1960). Subsequently, many reports of anticoagulant resistance were reported for warfarin as well as other first-generation anticoagulants like coumatetralyl and diphacinone throughout Europe (Lund 1966, Bentley 1968, Greaves and Renninson 1973). After the failure of FGARs, more potent second-generation anticoagulants (SGARs) were marketed in the 1970s (Hadler and Shadbolt 1975). Bromadiolone and difenacoum were first to be marketed and were initially found to be effective against rats resistant to FGARs (Halder *et al.* 1975, Marsh 1977). Soon resistance was reported to one or both of these compounds (Redfern and Gill 1978, Greaves and Cullen-Ayers 1988, Quy *et al.* 1995). Three other SGARs were introduced to the market namely; brodifacoum, flocoumafen and difethialone. These were sometimes called "resistance breakers" (Buckle *et al.* 2013) as they were found to be very effective against rodent populations resistant to FGARs and other SGARs.

Continuous long-term use of anticoagulant rodenticides may lead to selection of resistance-conferring mutations by eliminating the susceptible individuals within a population. Many studies have been done all over the world screening and confirming resistance to anticoagulants. Apart from being widely screened and thoroughly documented in European countries (Rost *et al.* 2004, Pelz *et al.* 2005), resistance to anticoagulants has been reported in many countries around the world like United States (Jackson and Ashton 1979), Indonesia (Andru *et al.* 2013), Japan (Ishizuka *et al.* 2007), and China (Liang 2005).

Although anticoagulant resistance has been reported widely in rats, most of the documented literature on resistance to anticoagulants is on Norway rats (Li *et at*. 2004, Rost *et al.* 2009, Rymer 2017). The main reason for less work having been done on ship rats (*Rattus rattus*) as compared to Norway rats is that they are rarely the main rodent pest species in other countries where extensive studies related to anticoagulant resistance have been done (Berny *et al.* 2018). Resistance to warfarin in ship rats has been reported in Denmark, France, USA, Germany, UK and Japan (Myllymaki 1995, Ishizuka *et al.* 2007). Resistance to both bromadiolone and difenacoum has been reported in France in ship rats (Desidiri *et al.* 1978, Lund 1984).

Vitamin K epoxide reductase complex subunit 1 (VKORC1) gene which is associated with resistance to anticoagulants is highly preserved in ship rats (Ishizuka *et al.* 2007, Diaz *et al.*

2010, Garg *et al.* 2017), but very little information is available on genetic resistance to anticoagulants in ship rats as compared to Norway rats and house mouse (*Mus musculus*). Genetic mutations in VKORC1 gene are known to alter the configuration of Vitamin K epoxide reductase (VKOR) enzyme leading to reduced susceptibility of the enzyme to anticoagulants. The first evidence of heritable genetic resistance was established in Japan in 2012 with multiple genetic mutations (Ala41Thr, Ala41Val, Arg61Trp, Leu76Pro, Tyr139Phe) identified in the VKORC1 gene in ship rats (Tanaka *et al.* 2012). Resistance to SGARs was documented in ship rats in India using the lethal feeding period (LFP) test and the blood-clotting response (BCR) test (Garg and Singla 2014, Garg and Singla 2015). Although BCR tests revealed presence of resistance to bromadiolone in ship rats, no genetic mutation associated with anticoagulant resistance was found (Garg *et al.* 2017). Recognition of the reduced efficiency of bromadiolone-based rodent control in Zaragoza (Spain) led to identification of a Tyr25Phe mutation causing resistance to all first-generation anticoagulants and less potent second-generation anticoagulants, namely bromadiolone and difenacoum in ship rats (Goulois *et al.* 2016). The same mutation was reported in eight out of 482 ship rats screened for anticoagulant resistance in New Zealand (Cowan *et al.* 2017). Although, resistance to less potent anticoagulants has been established in ship rats, more potent anticoagulants, namely brodifacoum and flocoumafen, are still found to be highly effective against ship rats. However, a heritable low grade resistance to brodifacoum has been found in Norway rats (Gill *et al.* 1992). In the case of house mice, reduced efficiency of brodifacoum has been reported in Canada, UK and Denmark (Siddiqi and Blaine 1982, Berny *et al.* 2018).

There is also evidence available for metabolic resistance in ship rats (Sugano *et al.* 2001). After no genetic mutation was detected in resistant ship rat population in Tokyo, cytochrome P450 (CYP) enzymes were investigated for involvement in increased metabolism of anticoagulants leading to resistance. Resistant ship rats were found to have increased numbers of CYP enzymes converting anticoagulant into their hydroxides. These hydroxides do not interfere with the coagulation process and are eliminated from the body through urine and faeces (Ishizuka *et al.* 2007, Ishizuka *et al.* 2008).

The demonstration of phenotypic changes associated with these pathways of resistance is required to conclusively document anticoagulant resistance in rodent populations. For instance, novel mutations like Ala14Val and Ala26Val have been identified in ship rats in New Zealand (Cowan *et al.* 2017), but they need to be assessed using LFP tests or BCR tests to account for degree of resistance and the resistance it confers towards individual anticoagulants used in New Zealand. For example, if the mutations confer very little degree of

practical resistance, anticoagulants can still be effectively used in the field. Similarly, if these mutations cause resistance to FGARs only, SGARs may still be an option for effective control of ship rats. The crucial information obtained from LFP and BCR tests helps in making practical decisions for control of resistant ship rat populations in the field.

*Lethal feeding period test*

One of the earliest methods developed to test for resistance of anticoagulant poison in rodents was the lethal feeding period (LFP) test, sometimes known as the laboratory feeding period test, where live animals were captured and fed anticoagulant-based bait in increasing doses until death occurs. LFP tests were common practice to test the efficacy of new rodenticides prior to marketing (Bentley *et al.* 1955, Bentley and Rowe 1956, Redfern and Gill 1978, Redfern *et al.* 1976). As resistance became established in Europe in Norway rats and house mouse, these tests began to be used for testing presence of resistance to a range of anticoagulant rodenticides. Drummond and Bentley (1965) established a lethal feeding trial where captured rodents were fed 0.005% warfarin for a period of 6 days. During these trials, 99% of the susceptible individuals were expected to die while the surviving individuals were categorized as warfarin-resistant. Similar LFP tests were also established for other anticoagulant rodenticides like difenacoum and brodifacoum (Drummond and Bentley 1965, Bentley 1968, Drummond and Wilson 1968, EPPO 1995). However, LFP tests did not provide very accurate results as individual rats responded differently to captivity and to particular bait formulations in terms of the dose ingested. Some rats feed very little on the bait while others feed at much higher levels in the absence of any other food source. Also, different rats feed at different time intervals during LFP trial making it difficult to compare bait consumption at a given point in time. Laboratory feeding tests also do not allow a precise determination of the range of susceptibility in a population as doses are used where 99% of susceptible individuals are expected to die (Rymer 2017). To make sure the minimum amount of known concentration of anticoagulant poison was ingested, a pre-calculated dose of anticoagulant may be administered to the rats via oral gavage or peritoneal injection (Nebendahl 2000). Although it is a more accurate method than feeding trials and provides better confirmation of presence of resistance the method is slow, labour intensive, and may cause a higher level of suffering in the animals (Garg and Singla 2015, Rymer 2017). For these reasons, a lethal feeding test was not preferred for the present research project.

*Blood-clotting response test*

With the development of blood-clotting response (BCR) tests, a more humane method to test resistance became available. A BCR test involves comparing blood coagulation times before and after administration of a sub-lethal dose of anticoagulant to the animals. Animals that show nominated increase in blood-clotting times after administration of a dose of anticoagulant are considered susceptible, whereas animals that show little or no increase in blood-clotting times are considered to be resistant (Kerins *et al.* 1993). This test does not rely on mortality of animals, and hence is considered to have relatively reduced animal welfare impacts, has fewer ethical constraints, and can be performed in 24 h so is time efficient. BCR tests are very sensitive and can detect even small differences in the susceptibility of rodent species to different anticoagulant compounds (Prescott and Buckle 2000). The main disadvantage of this methodology is that resistance assessments are based on changes in coagulation time not mortality. It is difficult to extrapolate the results of laboratory-based BCR tests to performance in practical rodent control, as measured by field mortality of rodents following bait application (Berny *et al.* 2018). Therefore, resistance found by BCR test is called technical resistance.

The BCR test was first used by Greaves and Ayers (1967) to study warfarin resistance in Norway rats. A dose of warfarin dissolved in dimethyl formide was administered via subcutaneous injection. A blood sample was collected from the retro-orbital sinus before and 24 hours after administration of the dose. Since then many different protocols have been developed and refined for BCR tests based on advances in technology and investigating effects or variables including type of resistance, routes of administration, Vitamin K administration, etc (Martin *et al.* 1979, MacNicoll and Gill 1993, Prescott and Buckle 2000). Due to the absence of a standard protocol, most of these tests could not be compared accurately between studies as they were based on different administrated doses. Further, the results of a BCR test in rodents from one population may not be applicable to the same species of rodent in other regions because of regional variability in rodent populations.

For my research, I followed the BCR test protocol developed by Prescott *et al.* (2007). This protocol provides a standardised BCR test for Norway rats and house mice for all the anticoagulant rodenticides. This method enables a system where all the future data generated from different anticoagulant based tests involving different rodent species can be compared if the protocol is followed precisely and same dose of the specific anticoagulant for a specific species of rodent is administered (Rymer 2017). This dose is called effective dose ($ED_{50}$). An $ED_{50}$ is a sub-lethal dose of anticoagulant that will not kill the animal but will produce a critical

measurable change in blood-clotting time. An effective dose ($ED_{50}$) first needs to be calculated for each species before screening animals for resistance, as it is essential to determine what level of change in blood-clotting response will be considered as significant for a susceptible individuals of a species of rodent to the administered anticoagulant. Effective dose is known to be influenced by sex of rodents however; the difference in $ED_{50}$ value may or may not differ significantly between male and female individuals of a species. In case of Norway rats, the $ED_{50}$ value of brodifacoum has been estimated to be 0.217 mg/kg for males and 0.222 mg/kg for females and this difference was not statistically significant. On the other hand, the $ED_{50}$ value of difenacoum, has been found to be significantly different for male and female Norway rats, i.e. 0.65 mg/kg for males and 0.79 mg/kg for females. In house mice, $ED_{50}$ value of brodifacoum for male and female mice is known to be 0.39 (0.37-0.40) mg/kg and 0.35 (0.33-0.36) mg/kg respectively (Prescott *et al.* 2007).

This protocol by Prescott *et al.* 2007 is based on $ED_{50}$ rather than $ED_{99}$ as it is more statistically robust and can be used to calculate the resistance (Berny *et al.* 2018). For a particular anticoagulant and species of rodent, using twice the effective dose as the discriminating dose provides a better conservative assessment of resistance in rodent populations and has been successfully used to quantify resistance in wild Norway rats before conducting full field trials (Endepols *et al.* 2007).

From the published literature, the $LD_{50}$ value of brodifacoum for ship rats has been found to range from 0.46 mg/kg (O'Connor and Booth 2001) to 0.77 mg/kg (Mathur and Prakash 1981). The highest published $LD_{50}$ dose of brodifacoum for ship rat is 0.77 mg/kg and the effective dose of an anticoagulant poison should be smaller than the $LD_{50}$ dose for the ship rat population. These published $LD_{50}$ doses can therefore used to calculate the starting point for BCR tests.

### *International normalised ratio*

There are different thromboplastin reagents like rabbit brain thromboplastin reagent and recombinant human thromboplastin available in the market that used for assessment of blood-clotting time. These reagents may have different levels of sensitivity resulting in different blood-clotting times even when used to assess the same sample. For measuring human blood-clotting times, various methods and reagents have a standardised calibration reference system based on World Health Organisation recommendations (Denson 1998). The International Normalised Ratio (INR) is used to standardise these results (Hirsh *et al.* 1994, Sheth *et al.* 2001). INR is the ratio of post treatment blood-clotting factor to pre-treatment blood-clotting factor, raised to the power of the international sensitivity index (ISI) value of the

reagent or coagulation equipment being used. Every reagent or equipment manufactured to calculate blood-clotting time or prothrombin time is assigned an ISI value. ISI is indicative of a particular reagent or equipment compared to international reference tissue factor. An ISI ranging between 0.9-1.4 indicates highly sensitive reagents.

A rodent is considered to be susceptible to a dose of anticoagulant, if its plasma percent coagulation activity (PCA) is less than 17% after a 24h period (Martin *et al.* 1979, MacNicoll and Gill 1993, Precott and Buckle 2000, Garg and Singla 2015). In case of Norway rat and house mouse, 17% plasma PCA activity corresponds approximately to an INR of 5 (Prescott *et al.* 2007). In ship rats, 17% plasma PCA activity corresponds to INR of 3.6 (3.5-3.8).Therefore, a ship rat is considered to be a responder (susceptible) to a given dose of anticoagulant when it has an INR ≥ 3.6 (Garg and Singla 2015).

*Gap of knowledge*

Anticoagulants have been used in New Zealand for decades now and very little research has been done on anticoagulant resistance in wild rodents in New Zealand. Anticoagulant resistance was suspected in parts of Auckland and on Raoul Island in 1978; however, no formal investigation took place. Rats were eradicated successfully in Raoul island using brodifacoum by aerial application in 2002 (Wodzicki 1978). Recently, pest control operations in New Zealand have come under scrutiny for their animal welfare impacts (Beausoleil *et al.*, 2016), and anticoagulant poisons were highlighted as being the least favourable option from this perspective. In order to evaluate the future role of anticoagulant poisons in pest control in New Zealand, it is necessary to screen for the prevalence and mechanisms of anticoagulant resistance in ship rat populations with a history of long term exposure to anticoagulants. BCR tests for ship rats need to be established for the various anticoagulants used in New Zealand. These tests may provide information regarding the efficacy of rodent pest control using anticoagulant poisons in New Zealand. An additional benefit would be to establish the prevalence of resistance in wild populations as a baseline for future changes in wild rodent populations. This present study is being carried out to determine effective dose ($ED_{50}$) of brodifacoum for ship rats for BCR tests. Once $ED_{50}$ value has been established, a multiple of this effective dose ($ED_{99}$) called the discriminating dose can be used to test resistance of brodifacoum in wild ship rats in New Zealand (Chapter 3).

## *2.2 Materials and methods*

*Study site*

To study resistance of brodifacoum in ship rats in New Zealand, an effective dose must be calculated from a population of rats that have never been exposed to any kind of anticoagulant. Since resistance to anticoagulants is a heritable character in rats and mice (Pelz *et al.* 2005), anticoagulant exposure history of immediate predecessors in a population is important. Therefore, laboratory rats are usually preferred for similar experiments as one can be sure of the complete history regarding exposure to any kind of poison for the respective rat or its ancestors. Unfortunately, the only laboratory-strain rats available in New Zealand are Norway rats, so any studies of New Zealand ship rats will rely on using animals captured from wild populations.

I therefore captured wild ship rats from the north-west region (Latitude 40°58'34.66"S, Longitude 175° 1'35.45"E) of Akatarawa forest (Figure 2.1, 2.2), a site where there was no known prior anticoagulant history and the small mammal pest control history was well documented (Table 2.1). Small mammal pest control in New Zealand targets possums (*Trichosurus vulpecula*), rabbits (*Oryctolagus cuniculus*), ferrets (*Mustela furo*), weasels (*Mustela nivalis*), stoats (*Mustela ermina*), hedgehogs (*Erinaceus europaeus*), Norway rats, ship rats, kiore (*Rattus exulans*) and house mice. Akatarawa Forest is located in the steep hill country of the Akatarawa Ranges, covering 15,500 ha and is managed by Greater Wellington Regional Council (GWRC). It is mainly covered by regenerating lowland podocarp forest, red beech forest and exotic plantations along with numerous wetlands in Akatarawa and Whakatikei river catchment areas. This makes Akatarawa forest a great home to high diversities of birds and insects (Parks Network Plan July 2011).

Trapping was carried out in the north-west region of Akatarawa because there was temporary use of anticoagulants in the south-east region (18 km away) in 2000-01 (Table 2.1). Brodifacoum has also been used in the neighbouring private lands by GWRC (Figure 2.3a). To avoid catching rats from these surrounding areas, no traps were placed within 1 km from the boundary of the forest. The north-western region is mainly comprised of exotic plantations.

**Table 2.1 Small mammal pest management history at Akatarawa forest as provided by the Greater Wellington Regional Council.**

| YEAR | POISON USED | TARGETED AREA |
|---|---|---|
| 1995-97 | Sodium fluoroacetate (1080) | Selected parts of the forest |
| 2000-01 | Cholecalciferol and Brodifacoum | An initial knockdown using cholecalciferol and brodifacoum followed by brodifacoum used in bait stations every 3 months in south eastern parts of the forest around the Cannon point walkway, Birchville dam and Totara Park |
| 2007 | 1080 | Entire forest |
| 2013 | 1080 | Entire forest |

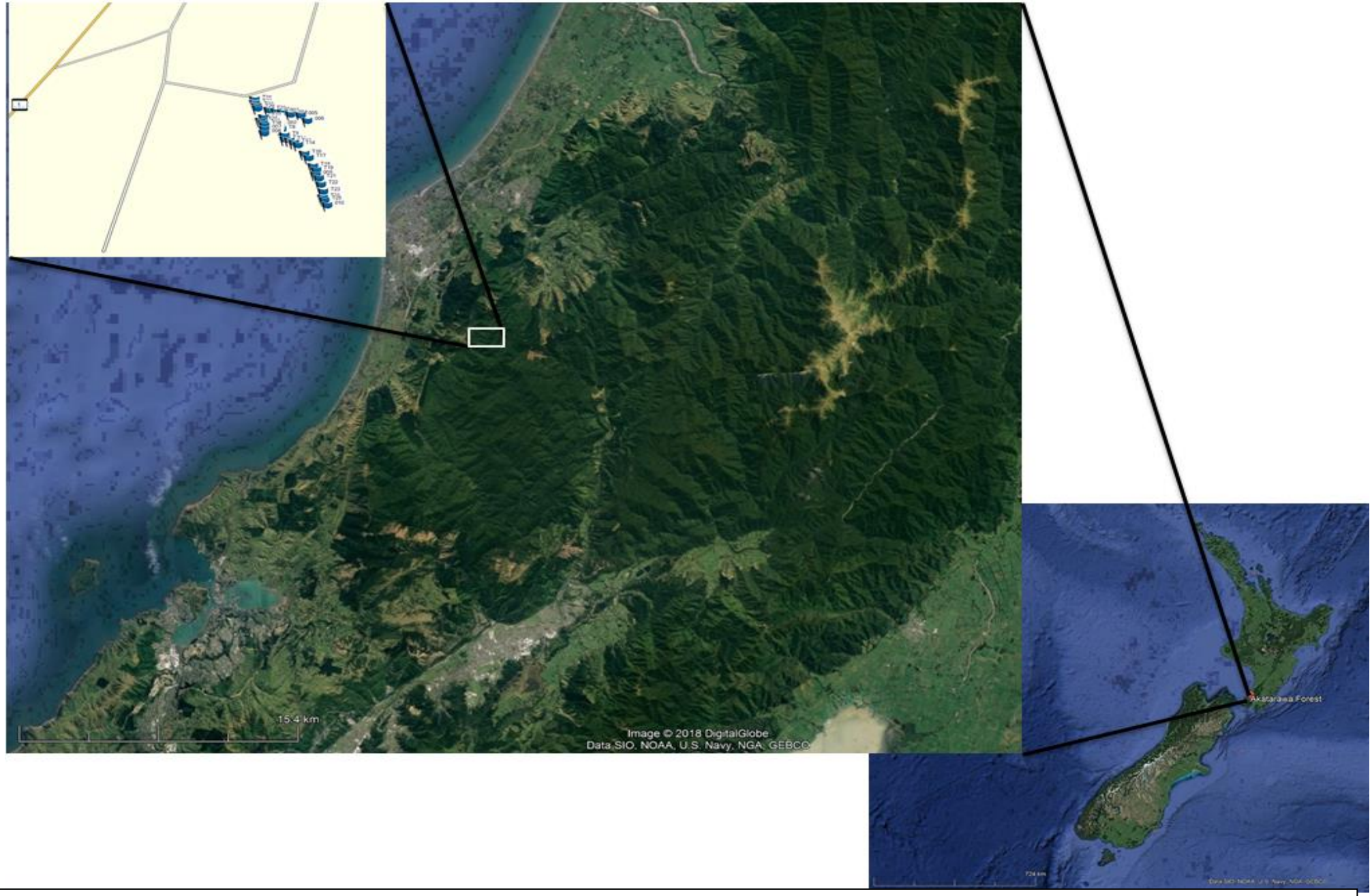



Figure 2.1 Map of New Zealand s**howing location of Akatarawa forest along with expanded map of north-west region depicting location of individual traps placed about 100 m** from each other.

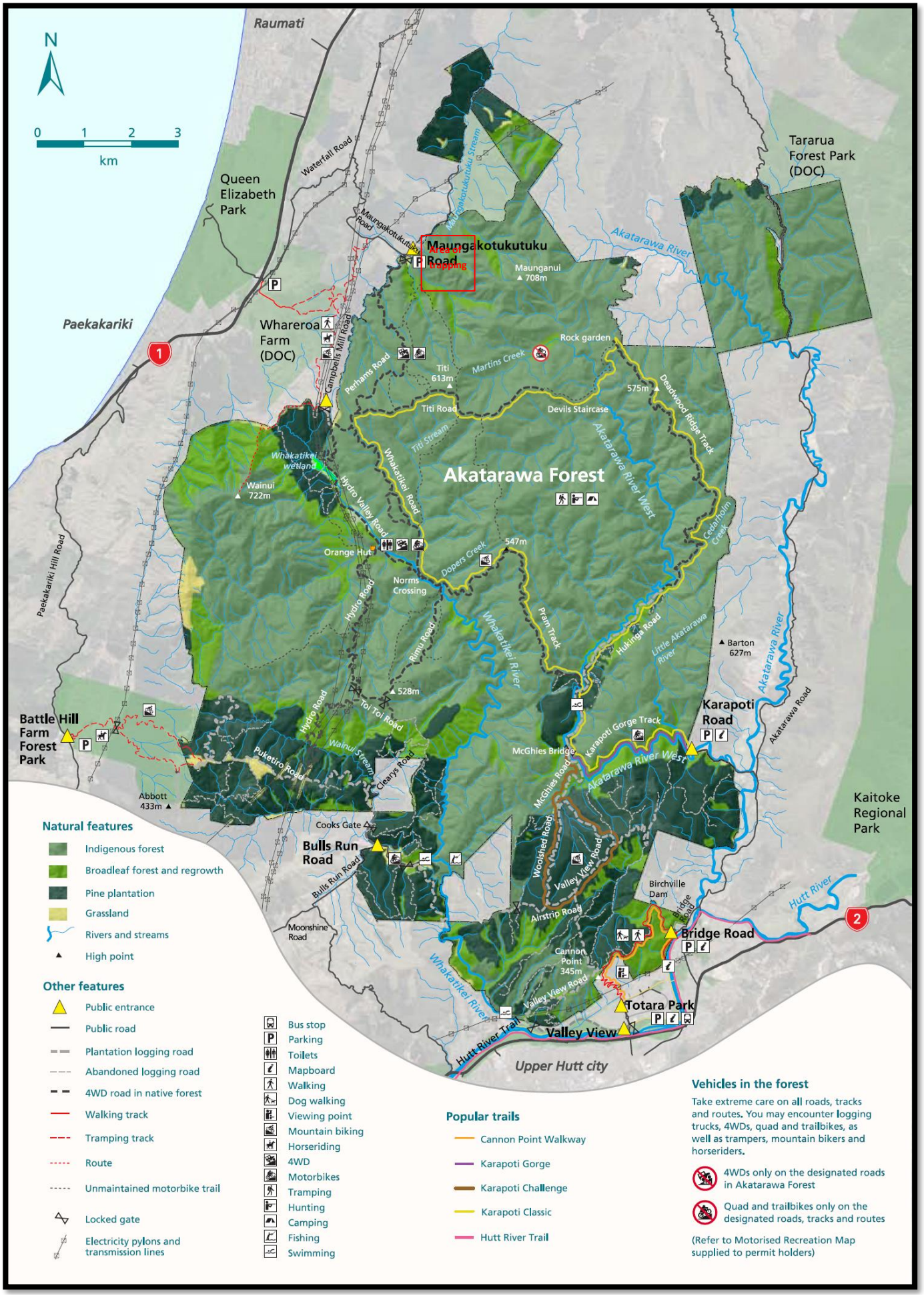



**Figure 2.2 A map of Akatarawa forest by Greater Wellington Regional Council showing boundaries and important locations**

general anaesthesia (isoflurane) as per the request of the GWRC. In the laboratory, rats were acclimatised individually in 30 cm x 30 cm x 25 cm cages for 15 days before the commencement of the experiment. Every cage

*Trapping*

Forty mesh cage rat traps measuring 11.8" x 4.7" x 4.3" were supplied by Massey University and set up in the north-western area of Akatarawa forest close to Maungatotukutuku access point on 4 July 2016 (Figures 2.1, 2.3). Traps were placed 100 m apart and baited with cheese and peanut butter. A small plastic bottle of water was also attached inside the trap. Traps were cleaned every alternate day to replace cheese, peanut butter and fresh water. Traps were checked every morning and captured animals were transported in their traps to Laboratory 4 in the Ecology Department at Massey University, Palmerston North, within 24 h. Animals were provided with cheese, peanut butter, a slice of apple, and water before transportation. On arrival at Massey University, each animal was transferred from its trap to an individual rat cage.

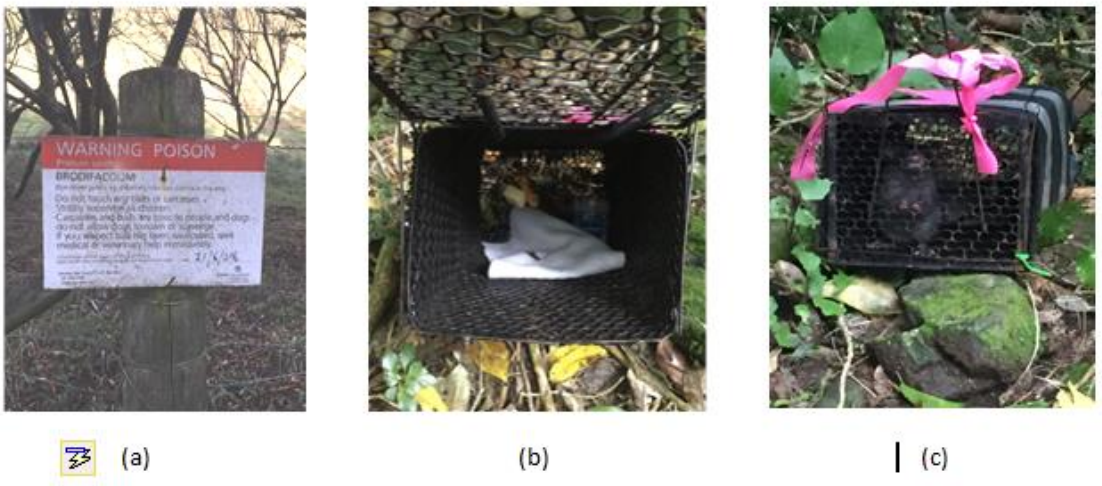



**Figure 2.3(a) A sign board warning of brodifacoum use at a private field adjoining boundary of Akatarawa. (b) A single catch rat trap set with cheese and peanut butter as bait and provided with a water filled plastic bottle and a piece of insulation/bedding. (c) A ship rat caught live in a trap.**

*Maintenance of captive rats*

All rats captured were visually confirmed to be ship rats based on their morphology and appearance (Cunningham and Moors 1996). Only mature and healthy rats were used for the experiment. Any pregnant females and immature rats caught were euthanased in the laboratory by cervical dislocation while under general anaesthesia (isoflurane) as per the

request of the GWRC. In the laboratory, rats were acclimatised individually in 30 cm x 30 cm x 25 cm cages for 15 days before the commencement of the experiment. Every cage included a 30 cm (l) x 15 cm (w) x 25 cm (h) nest box. Cages were provided with sawdust for bedding and shredded newspaper for nesting material. Cages were marked with a unique code for each rat along with information about its weight and sex. Cages were cleaned weekly and food and water provided *ad libitum*. Rats were provided with rat cereal feed pellets named Diet 86 manufactured at the feed mill at Massey University. Diet 86 contains all the components necessary for rats (Table 2.2). A slice of apple or carrot was provided each day for variety as well as an acorn or walnut every few days. The laboratory was maintained at 20±2°C on a 12-hour light/dark cycle.

**Table 2.2 Composition of Diet 86 rat cereal feed pellets**

| INGREDIENT | % |
|---|---|
| Wheat | 40.35 |
| Barley | 30.00 |
| Broll | 5.00 |
| Lucerne | 5.00 |
| Meat & Bone | 6.00 |
| Fishmeal | 7.00 |
| Skim Milk Powder | 5.00 |
| Soyabean Oil | 1.00 |
| Methionine | 0.10 |
| Salt | 0.05 |
| Premix | 0.50 |

Vitamin K is an important component in formation of blood-clotting factors in mammals. Deficiency of Vitamin K can lead to prolonged blood-clotting times. Similarly, excess of Vitamin K in the body from the diet can act as an antidote to an administrated brodifacoum dose (Prescott *et al.* 2007). The levels of Vitamin K in wild ship rats captured from the wild is unknown and, therefore, it becomes very important that ship rats have adequate amounts of Vitamin K before experimentation.

The diet provided to the rats contained a minimum amount of Vitamin K required by a rat i.e. 1 mg/kg of diet (Fu *et al.* 2007) to avoid Vitamin K deficiency. Any initial excesses of Vitamin K present in their bodies was expected to have been utilized during the 15 day acclimatization period.

*Brodifacoum dose-preparation*

Brodifacoum and Polyethylene glycol (PEG200) were obtained from Sigma Aldrich Company Ltd, Auckland, New Zealand. Successive doses of brodifacoum were formed individually for a

group by dissolving the required amount of brodifacoum in PEG200 in a beaker to make a total volume of 0.08 ml per 10 g body weight of rat. The solution was shaken and heated to 60°C in a laboratory incubator overnight to aid dissolution. The beaker was covered with aluminium foil to avoid light throughout the procedure.

*Experimental design*

The experiment involved a ranging study whereby ship rats were divided into groups of six (three males, three females), and the doses for each successive group were adjusted up or down until the effective dose was found. Such procedures reduce the number of animals used (Bruce 1985). The first group of rats (3 of each sex) was administered a dose of 0.72 mg/kg brodifacoum dissolved in PEG, and doses for each successive group were then expected to be decreased progressively until an International Normalised Ratio (INR) ≥ 3.6 was reached. INR is the ratio of post-treatment blood-clotting time to the pre-treatment blood-clotting time, and a ship rat is considered to be a responder (susceptible) to a given dose of anticoagulant when it has an INR ≥ 3.6 (3.5-3.8) (Garg and Singla 2015). A control group comprising of six rats (3 males and 3 females) was administered with vehicle only (PEG 200) to ensure that the vehicle did not have any effects on blood-clotting times and health of rats. A CoaguChek® XS Plus analyser (Roche Diagnostics International Ltd) was used to determine blood-clotting time of tested animals before and after treatment. The ISI value of the CoaguChek analyser is 1 (CoaguChek XP Plus Manual).

PRE-TREATMENT: After the acclimatisation period of at least 15 days, a baseline blood sample was taken from each rat before treatment. The rat was placed under general anaesthesia using inhalational isoflurane gas in oxygen, delivered via an induction anaesthetic chamber initially and then via a facemask for blood sampling. Blood was collected from the lateral saphenous vein using a 25 gauge needle. Up to 20 µl of blood were collected, and blood-clotting time was measured immediately using the blood-clotting time analyser. The analyser measures clotting times within the range of of 9.6 – 96 secs; therefore for some rats with clotting times less than 9.6 secs we were only able to determine that this was the maximum clotting time.

TREATMENT: Immediately following the pre-treatment blood sample, the rats in each group were provided with the appropriate subcutaneous dose of brodifacoum dissolved in PEG200. The control group of rats was administered with PEG200 only by subcutaneous injection. The rats were returned to their cages while they were still under the influence of anaesthesia and recovered 2-3 minutes later.

POST-TREATMENT: 24 h after administering the dose of brodifacoum, up to 20 µl of blood was collected from each rat. Rats were anaesthetised as described above, and then the post-treatment blood sample was collected via cardiac puncture by 22 gauge needle. After blood collection, the rats were euthanased by cervical dislocation while still under general anaesthesia. Blood-clotting time was measured immediately as for the pre-treatment sample.

The control group was also sampled 24 h after the PEG was administered, but at this stage 20 µl of blood were collected from each rat in same manner as described for pre-treatment blood sampling. These rats were monitored twice daily for at least 7 days to study any ill effects of PEG on them. They were then anaesthetised, and euthanased by cervical dislocation.

*Tissue conservation*

After euthanisation, all rats were dissected to remove a section of liver after perfusion through hepatic vein as per the method described by Thijssen (1987). Section of distal end of tail up to 2cm was preserved in ethyl alcohol for genetic analysis.

*Statistical analysis*

Probit analysis is used commonly to calculate the effective doses of different rodenticides in rodents (Prescott *et al.* 2007, Singla and Kaur 2015, Akcay 2013). In probit analysis each animal's blood-clotting times are reduced to a binary variable indicating whether or not it is considered to be a responder. The approach is therefore wasteful of data that have a range of values, and inappropriate for a sample of 36 animals. I therefore used linear regression to model the relationship between dose and INR and used the resulting model to estimate the $ED_{50}$ dose, i.e. the dose yielding a median INR of 3.6. We tested whether the slope of the relationship differed between males and females. Because we only had maximum pre-treatment clotting times for some animals, we used multiple imputation to model these missing values (Carpenter & Kenward 2013), with the maximum set as the upper limit and assuming that the pre-treatment clotting times were normally distributed. We used OpenBUGS (Spiegelhalter *et al.* 2014) to model the data, both because this approach facilitated use of multiple imputation, and because it allowed the effective dose to be easily calculated as a derived parameter. The code is provided in Appendix 1. Parameter estimates are reported ± 1 standard error.

## *2.3 Results*

*Trapping*

A total of 36 ship rats were treated with brodifacoum for the present experiment (Table 2.3). Two of the captured rats were and euthanized as per guidelines from Animal Ethics Committee, Massey University. One of the females was found dead after 8 days of acclimatization. Post-mortem of dead female revealed the cause of death to be pneumonia. All the rats used in the experiment were healthy mature adults. The average weight of males was 169g (142-219) and the average weight of females was 148g (130-162).

**Table 2.3 Live rat trapping at Akatarawa forest with trapping dates and number of ship rats caught.**

| Traps set | Traps removed | Trapping nights | No. rats | No. male | No. female | No. juveniles |
|---|---|---|---|---|---|---|
| 4 July 2016 | 8 July 2016 | 4 | 9 | 4 | 4 | 1 |
| 11 July 2016 | 14 July 2016 | 3 | 8 | 5 | 2 | 1 |
| 24 Nov 2016 | 29 Nov 2016 | 5 | 19 | 12 | 10 | 0 |
| | Total | 12 | 39 | 21 | 16 | 2 |

*BCR test*

The average normal blood-clotting time in ship rats was estimated to be 9.6 s ± 0.1, including modelling of the missing values, and this was consistent for males and females (Table 2.4). No clinical signs of lethargy, distress or haemorrhage were identified before the end of the post-dosing 24 h period in any of the rats. BCR tests for the control group showed no difference in clotting times of blood collected before and after the administration of PEG (Table 2.4). No ill effects of PEG were observed in the 7 day period after the dose of PEG was administered.

Male and female rats administered with the initial dose of 0.72 mg/kg produced an INR ranging between 1.86-2.38 and 1.86-2.13 respectively, so were all below the value of 3.6 that was considered a critical response for susceptible ship rats to a given anticoagulant dose (Figure 2.4). The dose of brodifacoum was increased successively to 0.8, 1.0 and 2 mg/kg for subsequent male ship rat groups, and still no rats had INR values ≥ 3.6. The dose was further increased to 3 mg/kg to which all rats responded with an INR ≥ 3.6, with values ranged between 3.63-4 mg/kg. A subsequent reduction to 2.5 mg/kg in the final group again resulted in none of the male rats having INR values ≥ 3.6.

The brodifacoum dose was increased directly to 3 mg/kg in the second group of female rats because all the male rats in the group responded to this dose with INR ≥ 3.6. However, none of female rats responded to this dose. An increase to 4 mg/kg resulted in all three

females responding with INR values ranging from 3.84-4.25. A reduction to 3.5 mg/kg for the final group resulted in one of the three females having an INR ≥ 3.6.

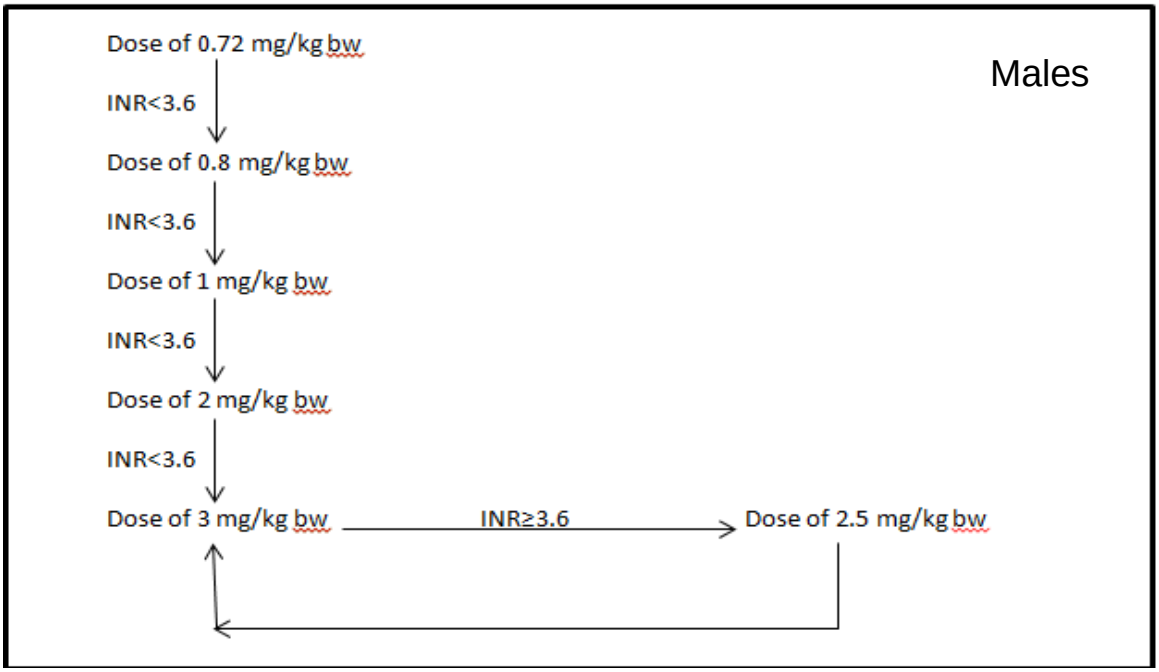



**Figure 2.4. Flowcharts depicting the ranging study conducted for calculating the effective dose of brodifacoum for male (top) and female (bottom) ship rats collected from Akatarawa forest. The dose was increased until the INR value ≥ 3.6 was achieved, and then decreased. All the groups administered different doses of brodfiacoum consisted of three rats.**

**Table 2.4 Blood-clotting responses of ship rats from Akatarawa forest to different doses of brodifacoum (shown as mg brodifacoum per kg of rat body weight). INR is the post-treatment blood-clotting time (BCR) divided by the pre-treatment blood-clotting time. The blood-clotting time analyser only provided a maximum clotting time if the value was < 9.6 s. In these cases the clotting time was modelled as a constrained missing value, hence there is a standard error (SE) around the INR.**

| Sex | Body weight (g) | Dose (mg/kg) | Pre-Treat. BCR (s) | Post-Treat. BCR (s) | INR (SE) |
|---|---|---|---|---|---|
| Male | 197.0 | 0.00 | < 9.6 | < 9.6 | 0.97 (0.06) |
| | 160.0 | | < 9.6 | < 9.6 | 0.97 (0.06) |
| | 145.0 | | 9.6 | 9.6 | 1.00 |
| | 202.0 | 0.72 | < 9.6 | 16.8 | 1.86 (0.09) |
| | 187.5 | | < 9.6 | 21.6 | 2.38 (0.11) |
| | 164.5 | | 9.6 | 21.6 | 2.25 |
| | 144.9 | 0.80 | < 9.6 | 18.0 | 1.99 (0.10) |
| | 148.7 | | < 9.6 | 16.8 | 1.86 (0.09) |
| | 144.2 | | 9.6 | 18.0 | 1.88 |
| | 147.9 | 1.00 | < 9.6 | 18.0 | 1.99 (0.10) |
| | 196.0 | | < 9.6 | 26.4 | 2.90 (0.13) |
| | 147.6 | | 9.6 | 34.8 | 3.63 |
| | 219.3 | 2.00 | < 9.6 | 25.2 | 2.79 (0.14) |
| | 149.0 | | 9.6 | 26.4 | 2.75 |
| | 170.3 | | 10.8 | 27.6 | 2.56 |
| | 178.0 | 2.50 | < 9.6 | 22.8 | 2.57 (0.15) |
| | 181.0 | | 9.6 | 31.2 | 3.25 |
| | 162.5 | | 10.8 | 26.4 | 2.44 |
| | 142.0 | 3.00 | 9.6 | 34.8 | 3.63 |
| | 197.0 | | 10.8 | 40.8 | 3.78 |
| | 160.8 | | 10.8 | 43.2 | 4.00 |
| Female | 145.0 | 0.00 | < 9.6 | < 9.6 | 0.97 (0.06) |
| | 144.5 | | < 9.6 | < 9.6 | 0.97 (0.06) |
| | 139.0 | | 9.6 | 9.6 | 1.00 |
| | 152.0 | 0.72 | 9.6 | 20.4 | 2.13 |
| | 144.0 | | < 9.6 | 16.8 | 1.86 (0.09) |
| | 162.0 | | 9.6 | 18.0 | 1.88 |
| | 155.1 | 3.00 | 9.6 | 31.2 | 3.25 |
| | 130.0 | | 10.8 | 30.0 | 2.78 |
| | 153.4 | | < 9.6 | 28.8 | 3.18 (0.15 |
| | 157.0 | 3.50 | 10.8 | 33.6 | 3.11 |
| | 133.5 | | 9.6 | 36.0 | 3.75 |
| | 157.0 | | 9.6 | 21.6 | 2.25 |
| | 158.4 | 4.00 | < 9.6 | 37.2 | 4.09 (0.18) |
| | 140.0 | | < 9.6 | 34.8 | 3.84 (0.18) |
| | 152.0 | | 9.6 | 40.8 | 4.25 |

*Estimation of effective dose*

The data indicated that it was reasonable to assume a linear relationship between INR and dose (Figure 2.5). Because there was no apparent effect of the PEG200, it was also assumed in the model that INR = 1 when the dose = 0. There was a significant difference in the slope of the relationship for male and female ship rats (P = 0.0091; Fig. 2.5). The $ED_{50}$ of brodifacoum was estimated to be 2.88 ± 0.23 mg/kg for males and 3.81 ±0.32 mg/kg for females.

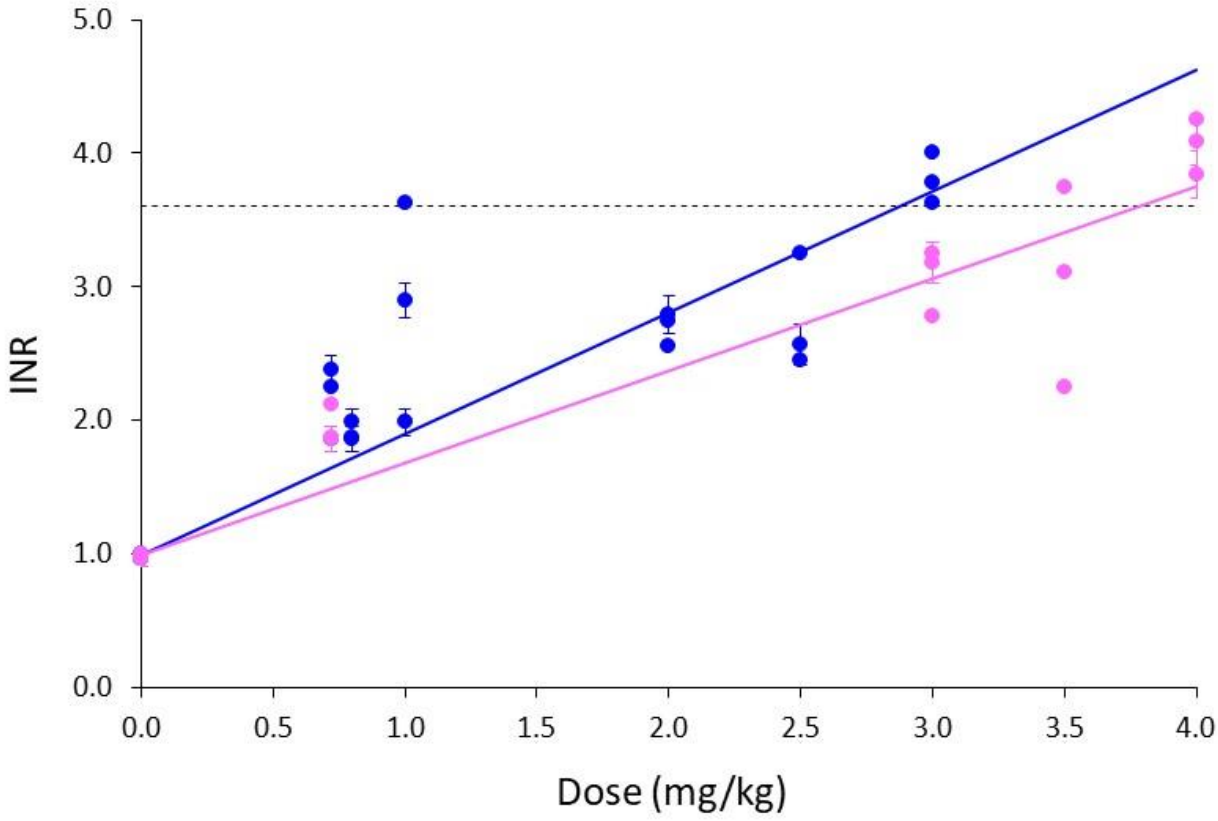



**Figure 2.5 Linear regressions for the relationship between brodifacoum dose (mg per kg of body weight) and proportionate increase in blood-clotting time (INR) for male (blue) and female (pink) ship rats from Akatarawa forest. The dotted line shows the INR level at which a ship rat is considered to a responder (Garg and Singla 2015). The bars on the data points indicate Standard Errors, which reflect uncertainty in INR values when only the maximum pre-dose blood-clotting time could be measured.**

## *2.4 Discussion*

In the present study, successive doses of brodifacoum were administered to each group ranging from 0.72 – 3 mg/kg in male ship rats and 0.72 – 4 mg/kg in female ship rats until an INR ≥ 3.6 was obtained. Each dose group in this experiment included only three individuals.

This small sample size is justified on animal welfare grounds, but taking into account the unknown population variability of ship rats, larger sample sizes per group would likely have provided a more accurate estimate of average blood-clotting response at a specific concentration of brodifacoum.

$ED_{50}$ dose of brodifacoum was estimated to be 2.88 mg/kg in case of males and 3.81 mg/kg in case of females. Effective dose is a sub-lethal dose that produces a critical measurable response to an administered dose of anticoagulant. Therefore, it is always less than lethal dose ($LD_{50}$) of brodifacoum. $LD_{50}$ value refers to a dose of a toxin that kills 50% of individuals of the susceptible population. The $LD_{50}$ dose of brodifacoum is known to vary in rodents around the world and may be affected by a number of factors including historic exposure of poison in ancestors, age, sex, nutritional status and different experimental approaches to calculate lethal dose (Kaukeinen and Rampaud 1986, Moussa 2005). The published data suggest a range of 0.46-0.77 mg/kg for $LD_{50}$ values of brodifacoum (Dubock and Kaukeinen 1978, Fisher 2005).

The highest published $LD_{50}$ value of brodifacoum, 0.77 mg/kg, was evaluated from India (Mathur and Prakash 1981). In New Zealand, the $LD_{50}$ value of ship rats was found to be 0.46 mg/kg in a study carried out using rats captured from a brodifacoum-free area on the west coast of the South Island (O'Connor and Booth 1991). The latter location is a more remote place than Akatarawa forest, from which rats were captured for the present study. Therefore, the chances of ship rat populations being exposed to any anticoagulants from surrounding areas are far less in the West coast of the South Island as compared to Akatarawa forest. The effective dose estimated for male ship rats in this study was 2.88 mg/kg, which is nearly 3.7 times the highest published $LD_{50}$ value of brodifacoum and 6.2 times the published $LD_{50}$ value in New Zealand. In females it was 3.81 mg/kg, which is nearly five times the highest published lethal dose in India and 8.2 times the published lethal dose in New Zealand. Despite the variation in the estimated $LD_{50}$ values of brodifacoum, the calculated effective doses in this study are very high compared to all known $LD_{50}$ values.

In a study done to evaluate brodifacoum resistance in Norway rats in New Zealand, $LD_{15}$ value was used as the effective dose in blood-clotting response tests (Bailey *et al.* 2005). No resistant rats were reported in the study. Comparing it to the present results where the effective dose of brodifacoum is 3-6 times its highest published lethal dose, the effective dose estimated in the current study seems remarkably high.

Most of the studies involving blood-clotting response tests have been focused on Norway rats and house mice. There is no BCR test standardised for ship rats providing us with

an $ED_{50}$ value for any known anticoagulant (Berny *et al.* 2018). The only previous study for BCR test was for detecting resistance to bromadiolone in ship rats in India (Garg and Singla 2015). However, the BCR test was based on calculated $LD_{50}$ value rather than $ED_{50}$ value. Therefore, we do not have any data to compare the effective dose and lethal dose for any anticoagulant in ship rats, but can make comparisons with published data for $LD_{50}$ and $ED_{50}$ value of brodifacoum in Norway rats and house mice (Table 2.5). Norway rats and house mice are major pests in most parts of the world where anticoagulant resistance has been studied extensively. There are published lethal doses of brodifacoum available for Norway rats from studies around the world, and the calculated effective dose is far less than highest published lethal dose. Similarly for house mice, published lethal doses are always higher than the published effective doses.

**Table 2.5 Lethal and effective doses of brodifacoum for Norway rat, ship rat and house mouse**

| Species of rodent | $LD_{50}$ value (mg/kg) | $ED_{50}$ value (mg/kg) |
|---|---|---|
| Wild Norway rat | 0.17-0.56 (Redfern *et al.* 1976, O'Connor and Booth 2001) | 0.22 (Prescott *et al.* 2007) |
| Wild house mouse | 0.40-0.52 (Redfern *et al.* 1976, O'Connor and Booth 2001) | 0.35-0.39 (Prescott *et al.* 2007) |
| Ship rat | 0.46-0.77 (O'Connor and Booth 2001, Prakash and Mathur 1981) | 2.88-3.81 (Present Study) |

The second dose administered to the rats, 0.8 mg/kg was higher than the highest published $LD_{50}$ value of 0.77mg/kg. Therefore, all the animals were expected to respond to this dose and produce an INR ≥ 3.6. A recent study was done on Howe Island in Australia before planning a rodent eradication programme. A dose of 0.8 mg/kg of brodifacoum administered to ship rats achieved 100% mortality rate irrespective of a long history of use of FGARs and SGARs since the 1960s. There is no formal record of brodifacoum use by residents on the island, but it has exceeded 400 kg per annum since 2000 (Wheeler *et al.* 2018). Contrary to these results, none of the ship rats from Akatarawa forest with no recent history of

anticoagulant use responded to 0.8 mg/kg brodifacoum dose with an INR ≥ 3.6. This may represent general lower susceptibility of captured ship rats to brodifacoum.

Susceptibility may vary in different populations of ship rats due to factors such as general variability in the population and historic exposure to the concerned. Also, susceptibility is a relative trait and in a broader sense, a population is known to be tolerant or resistant to an anticoagulant when another population is comparatively more susceptible to the anticoagulant at a given dose (EPPO 1995, Buckle *et al.* 2007). Although wild rodents are direct representatives of field populations, their true susceptibility may be unknown and their responses are likely to have more variance than laboratory strain of ship rats (Prescott *et al.* 2007). Animals used to calculate effective doses need to be fully susceptible to all the known anticoagulants. Therefore, laboratory rats are mostly preferred for evaluation of effective dose for a poison. The effective dose for the present study has been estimated using wild ship rat populations due to non-availability of laboratory ship rats in New Zealand and has been complicated by a high level of anticoagulant resistance inherent in the sampled population.

Another less likely reason for reduced susceptibility in ship rats may be high intake of Vitamin K in their diet in Akatarawa forest. Vitamin K is an antidote of anticoagulants and would result in no or low response to administered brodifacoum dose. However, the acclimatization period of 15 days should have led to utilisation of any extra Vitamin K in the body. The diet provided to ship rats during this period only contained the minimum amount of Vitamin K required by rats. Therefore, high intake of Vitamin K can be ruled out as a cause for low susceptibility to brodifacoum in tested ship rats.

The response of ship rats to lower doses like 0.72-1.0 mg/kg can be explained based on general low susceptibility to brodifacoum but it becomes difficult to believe that general low susceptibility may cause rats to not respond to higher doses like 2.5 mg/kg in males and 3 mg/kg in females. The lack of blood-clotting inhibition of ship rats with these doses as shown by an INR <3.6 is most plausibly due to a high prevalence of resistance to brodifacoum within the ship rat population at Akatarawa forest. Such resistance generally emerges with continuous use of the same anticoagulant for many generations leading to selection and survival of resistant individuals in the population while a majority of the susceptible population dies of anticoagulant poisoning.

Although brodifacoum or any other anticoagulant was never used in the part of the forest from where rats were captured, it was found that brodifacoum is used extensively in the neighbouring areas by private landowners as well as by the Greater Wellington Regional Council. Ship rats exposed to brodifacoum from these areas may have been invading the forest

and may be the cause of this significantly low susceptibility. Sodium fluoroacetate (1080) has been the only poison used in pest control operations in north-western Akatarawa forest. 1080 is an acute metabolic poison and has a different mode of action as compared to anticoagulants. It has been used every 4-6 years since 2001. 1080 is a very effective rodenticide and a sharp decline in rat population has been observed in targeted areas each time it is used (Innes and Williams 1991, Kemp 2008). This may be facilitating a more rapid invasion of ship rats into the forest. The rat population in the area is known to recover quickly after the use of 1080 pellets. Ship rat populations are known to recover in about 6-12 months post control (Barron *et al.* 2014). This time window provides great opportunity for ship rats to move to the forest with abundant food and space with little competition. Both male and female ship rats have a home range of 0.5-1.0 ha, but males increase their home ranges during the breeding season (Dowding and Murphy 1994, Harper and Rutherford 2016). In beech forests, ship rats have been found to have a home range of up to 11.4 ha (Pryde *et al.* 2005). Breeding in ship rats is polyoestrous. The gestation period is usually 20-22 days long with a litter size ranging from 3-10 pups. Growth of pups is rapid, and they reach maturity in 3-4 months (Bentley and Taylor 1965). Taking into consideration the fast generation time, high mobility of ship rats and high effective dose evaluated from an area of historically negligible anticoagulant use suggests that anticoagulant resistance may be well spread in ship rats in Wellington region. It also indicates that the ship rats from areas of long-term brodifacoum use (20+ years) may have even lower susceptibility to brodifacoum. However, the results of this study suggest lack of a pattern and high chances of presence of brodifacoum resistance in areas of even negligible or low brodifacoum use that are spatially connected to other areas where these poisons are used.

Genetic analysis of the rat tail samples preserved from this experiment (Chapter 5) may provide further clarification if the reduced susceptibility in these ship rats is due to heritable resistance. If this is the case, rodent pest management by anticoagulants may become a challenge in the near future. Brodifacoum is a very potent second-generation anticoagulant and is one of the most cost effective and toxic rat poisons on the market. A rodent resistant to a more toxic anticoagulant is known to develop resistance to less toxic anticoagulants to which it may have never been exposed (Pelz and Prescott 2015). This removes the option of using alternative anticoagulants to control rodent pest populations with lowered susceptibility to brodifacoum. In this situation, effective rodent control may be achieved only by increasing the concentration of brodifacoum in the baits leading to an increase in secondary and non-target

poisoning or using alternative poisons like cholecalciferol or 1080. Continual use of brodifacoum may also encourage further development of resistance in ship rat populations.

Another factor affecting pest control operation may be higher effective dose found in females as compared to males. This may be due to two main reasons. Firstly, basal activity of vitamin–K dependent blood-clotting factors (II, VII and X) is higher in females as compared to males, and there is also higher plasmic half-life of prothrombin and blood factor X in females (Lefebvre *et al.* 2016). Anticoagulants are known to inhibit Vitamin K recycling leading to reduced blood clotting which may prove to be fatal. Higher activity of vitamin-K dependent blood-clotting factors would reduce susceptibility of females to brodifacoum. Secondly, there is sexually dimorphic expression of cytochrome P450 complex in rats. Cytochrome P450 plays a crucial role in metabolism of anticoagulants in the body. In a study done on bromadiolone resistant rats, CYP2c13, Cyp3a2, CYP2e1 and CYP3a3 have been known to express 80, 25, 3 and 5 fold respectively, in bromadiolone resistant female rats as compared to susceptible female rats. On the other hand, these enzymes overexpressed only 1.7-3 fold in resistant male rats (Markussen *et al.* 2008).

If brodifacoum resistance in female ship rats is accompanied by one or more heritable mutations, it may pose a real threat to effective rodent control by brodifacoum in the future in New Zealand forests, because resistant female rats are potentially less likely to succumb following ingestion of toxic bait than are resistant males (Rymer 2017). This is because only one male is required to mate with several females, and therefore a reduction in male density alone is expected to have little effect on population dynamics. Female ship rats can reach maturity at an age of 3-4 months and are polyoestrous, producing up to 10 pups in a litter (Bentley and Taylor 1965). So with brodifacoum resistant females passing resistance to the next generations, resistance can spread at faster rates and ship rat populations may recover rapidly after application of an anticoagulant.

At present, there is a need to consider alternative poisons other than anticoagulants that can be used for rodent management in New Zealand. Also, regular monitoring of susceptibility of each anticoagulant poison used for rodent species in New Zealand needs to be done. Assessment of susceptibility in areas with long-term use of anticoagulant poisons must be a priority to ensure effective rodent control in the future.

The estimated effective dose in this study will be used to access the susceptibility of ship rats collected from areas of low, medium and high brodifacoum use (Chapter 3). This will provide further information about the status of susceptibility of ship rats to brodifacoum in

New Zealand. It is recommended to evaluate the effective dose by using ship rats captured from isolated areas with no history of brodifacoum use, such as islands, so that an accurate effective dose can be estimated and compared to the current effective dose. Ship rats collected from such islands with no past anticoagulant history may show an increased susceptibility to anticoagulants that can be compared to data collected from ship rat populations on mainland.

# CHAPTER 3

# Comparison of blood-clotting responses to brodifacoum between ship rat (*Rattus rattus*) populations captured from areas of low, medium and high brodifacoum use

## *3.1 Introduction*

Anticoagulants were introduced as rodenticides in the 1950s and revolutionised rodent control. The reason for these poisons' success was the effectiveness on rodents that became bait shy due to other acute poisons. Anticoagulants are slow acting poisons, so rodents cannot relate the symptoms of anticoagulant poisoning to their source, making them the best choice for rodent control worldwide. Anticoagulants are cheap, easy to use and have been known to achieve 100% mortality during rodent control operations if best practice is followed (Jacob and Buckle 2018).

Despite the advantages, there are major concerns about long-term use of anticoagulants. Anticoagulants have been known to cause non-target poisoning including both primary and secondary poisoning (Fisher *et al.* 2004, Guitart *et al.* 2010, Masuda *et al.* 2014, Gaduhn *et al.* 2016) and there are ethical concerns about the suffering they cause (Littin *et al.* 2004, Beausoleil *et al.* 2016). Another concern about anticoagulant use is the potential emergence of resistance due to continuous long-term use. Resistance is a heritable trait developed in rodents that causes resistant individuals to survive a dose of anticoagulant poison that will kill 99% of susceptible individuals (Bailey *et al.* 2005). Anticoagulant resistance is a worldwide known phenomenon. Resistance has been reported against almost all first-generation anticoagulants and less potent second-generation anticoagulants like difenacoum and bromadiolone (Lund 1964, Greaves *et al.* 1976, Buckle 2013, Meerburg *et al.* 2014). More potent second-generation anticoagulants like brodifacoum and flocoumafen are still used effectively to control rodent pest populations although low-grade resistance has been found to brodifacoum in Norway rats (Gill *et al.* 1992).

Rodent pest management in New Zealand relies greatly on brodifacoum for both rodent control and rodent eradication programmes on mainland and offshore islands (Veitch *et al.* 2011, Eason *et al.* 2015, Howald *et al.* 2015). After more than a decade of long-term continuous use of brodifacoum on both main islands in New Zealand by regional councils, private contractors and landowners, concern of development of resistance to brodifacoum in rat and mice populations has increased.

Very few studies are available documenting the susceptibility of rodents to anticoagulants in New Zealand. Anticoagulant resistance was suspected in parts of Auckland and on Raoul Island in 1978 (Wdzicki 1978); however no investigation took place. Later in 2002, rats were eradicated successfully in Raoul Island using brodifacoum by aerial application (Cowan *et al.* 2017). A preliminary study done on Norway rats (*Rattus norvegicus*) captured

from different parts of New Zealand suggested no resistance to brodifacoum (Bailey *et al.* 2005). However, considering the small sample size of animals tested, it was suggested that resistance may still be present at low density among individuals of the population. Also, ship rats (*Rattus rattus*) are the most common rats in New Zealand forests so screening of ship rats for resistance was needed. In 2012, tail samples were collected from all three species of rats found in New Zealand, i.e. Norway rat, ship rat and kiore (*Rattus exulans*). A well-established mutation, Tyr25Phe, was found to be present in ship rats along with two naive mutations, Ala14Val and Ala26Val, which still need to be assessed (Cowan *et al.* 2017). The Tyr25Phe mutation in ship rats has been associated with resistance to both first-generation anticoagulants and second-generation anticoagulants like bromadiolone in Spain (Goulois *et al.* 2016). This further indicates the importance of assessing status of resistance to brodifacoum in ship rats in New Zealand as cross-resistance may lead ship rats that are resistant to one anticoagulant to develop resistance to another anticoagulant over time. Cross-resistance is a very well-known phenomenon in anticoagulants whereby a rodent resistant to one anticoagulant may develop resistance to another anticoagulant even if it has not been exposed to it earlier (Pelz and Prescott 2015).

The present study was proposed to assess presence of brodifacoum resistance in ship rats captured from the Wellington Region and Palmerston North. The Wellington Region was chosen due to the extensive use of brodifacoum and other anticoagulants by the Greater Wellington Regional Council (GWRC) over the last 20 years. I planned to assess the frequency of brodifacoum resistance in rodent populations between areas of high, moderate and low known anticoagulant use. The extended use of anticoagulants in these areas may have resulted in selection for anticoagulant resistance in rodent populations.

Blood-clotting response (BCR) tests were chosen for assessing susceptibility of ship rats against brodifacoum. A BCR test involves collection of blood sample and evaluating coagulation time of captured wild animals both before and after administration of a known anticoagulant dose. Animals that show considerably increased blood-clotting times after administration of a dose of anticoagulant are considered susceptible, whereas animals that show little or no increase in blood-clotting times are considered to be resistant (Kerins *et al.* 1993). This test does not rely on mortality of animals, unlike the lethal feeding period test (LFP) (see Chapter 2), and hence is considered more humane, has fewer ethical constraints, and can be performed in 24 h so is time efficient. BCR tests are very sensitive and can detect even small

differences in susceptibility of rodent species to specific anticoagulant rodenticides (Prescott and Buckle 2000).

The International Normalised Ratio (INR) is used to analyse blood-clotting times in BCR test (see Chapter 2). INR is the ratio of post treatment blood-clotting factor to pre-treatment blood-clotting factor. A rodent is considered to be susceptible to a dose of anticoagulant, if its plasma percent coagulation activity (PCA) is less than 17% after administration of that dose after 24 h period (Martin *et al.* 1979, MacNicoll and Gill 1993, Precott and Buckle 2000). In case of Norway rat and house mouse, 17% plasma PCA activity corresponds approximately to an INR of 5 (Prescott *et al.* 2007). In ship rats, 17% plasma PCA activity corresponds to an INR of 3.6 (3.5-3.8). Therefore, a ship rat is considered to be a responder (susceptible) to a given dose of anticoagulant when it has an INR ≥ 3.6 (Garg and Singla 2015).

For my research, I followed the BCR test protocol developed by Prescott *et al.* (2007). This protocol provides a standardised BCR test for Norway rats and house mice (*Mus musculus*) for all the anticoagulant rodenticides. This method enables a system where all the future data generated from different anticoagulant based tests involving different rodent species can be compared if the protocol is followed precisely and the same dose of the specific anticoagulant for a specific species of rodent is administered (Rymer 2017). This administered dose is called the effective dose ($ED_{50}$). An $ED_{50}$ dose is a sub-lethal dose of anticoagulant that will not kill the animal, but will produce a critical measurable change in blood-clotting time in response to an administered dose. An effective dose ($ED_{50}$) first needs to be calculated for each species before screening animals for resistance, as it is essential to determine the dose that will achieve the critical level of change in blood-clotting response (INR ≥ 3.6 in the case of ship rats) considered as significant for a susceptible individuals of a specific species of rodent. This method is based on $ED_{50}$ rather than $ED_{99}$ as its statistical threshold (Berny *et al.* 2018). For a particular anticoagulant and species of rodent, using twice the effective dose as the discriminating dose provides a better conservative assessment of resistance in rodent populations and has been successfully used to quantify resistance in wild Norway rats before conducting full field trials (Gill *et al.* 1992, Endepols *et al.* 2007).

The effective dose is known to be sex-specific although the $ED_{50}$ values may or may not differ significantly between male and female individuals of a species. For the purposes of this study, the effective dose ($ED_{50}$) was calculated for male ship rats at 2.9 mg/kg and for female rats at 3.8 mg/kg (see Chapter 2). In many studies, blood-clotting response tests are conducted based on lethal dose as compared to effective dose. 2X $LD_{50}$ dose is generally used to differentiate between susceptible and resistant individuals of a population (Garg *et al.* 2017).

The lethal dose ($LD_{50}$) of brodifacoum is known to be within the range of 0.46-0.77 mg/kg (Mathur and Prakash 1981, O'Connor and Booth 2001, Fisher 2005). The $ED_{50}$ dose estimated for male ship rats is approximately 4-6 times the lethal dose whereas $ED_{50}$ dose for females is 5-8 times published lethal dose of brodifacoum. In general, $ED_{50}$ dose should be less than the $LD_{50}$ dose because lethal dose is a dose that would kill 50% of the susceptible population whereas effective dose is a sub-lethal dose that does not kill an animal but produces a critical measurable change in blood-clotting time (Prescott *et al.* 2007).

Also, animals that are used to calculate the effective dose need to be fully susceptible to all the known anticoagulants. Although wild strains are direct representatives of a field population, their true susceptibility may be unknown and their responses are likely to have more variance than laboratory strain of ship rats (Prescott *et al.* 2007). Therefore, laboratory rats are mostly preferred for evaluation of effective dose for a poison. However, wild ship rats were used for the evaluation of effective dose of brodifacoum for ship rats due to the unavailability of laboratory bred ship rats in New Zealand (see Chapter 2).

Due to the significantly high effective dose with respect to lethal dose and unknown susceptibility of ship rats used for the experiment to calculate effective dose, the $ED_{50}$ dose was chosen to screen wild ship rat populations for potential for resistance to brodifacoum.

## *3.2 Material and methods*

*Study Sites*

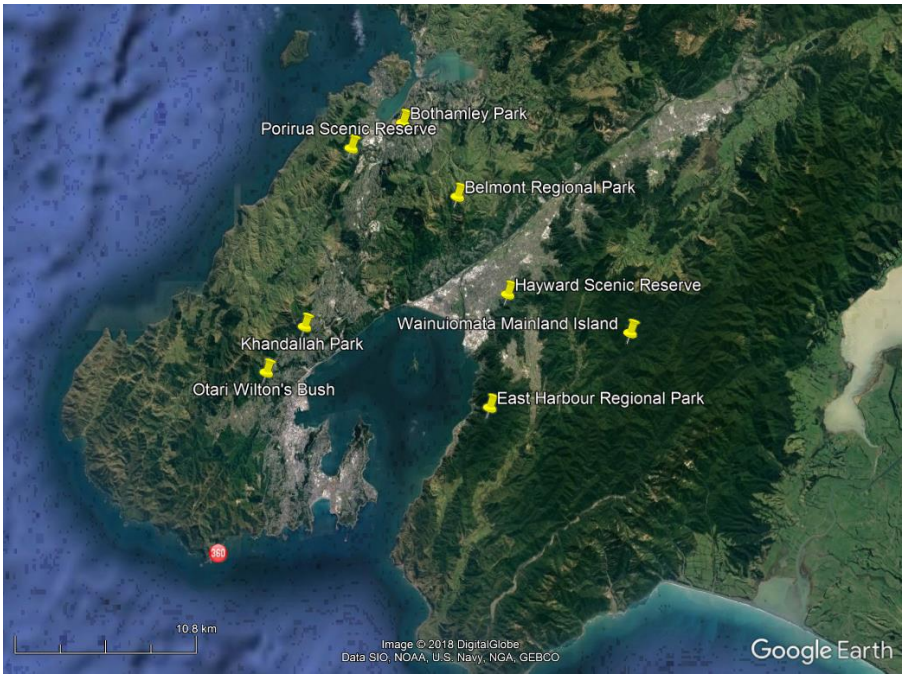
Eight different sites in the Wellington region were selected and were categorised as having high, moderate or low brodifacoum use areas based on their recorded management histories (Table 3.1; Figure 3.1). All the sites have had continuous brodifacoum use with bait stations serviced 4-6 times a year and bait is available in bait stations around the year (Uys 2017). Another site, Turitea reserve in Palmerston North, was added later due to its history of long continuous use of brodifacoum. A detailed small mammal pest control history for each site for the past 25 years is provided in Appendix 2. It was very difficult to find sites with low brodifacoum use (< 5 years) and therefore only one of the eight sites falls tothis category.

**Table 3.1 Selected sites in Wellington and Palmerston North categorised into high, moderate and low brodifacoum use.**

| Brodifacoum use | Site | Brodifacoum Use History (Years) |
|---|---|---|
| High | Otari-Wilton's Bush | 23 |
| | Porirua Scenic Reserve | 20 |
| | Khandallah Park | 19 |
| Moderate | Belmont regional Park | 13 |
| | Turitea Reserve | 12 |
| | Wainui Mainland Island | 12 |
| | East Harbor Regional Park | 12 |
| | Bothamley Park | 9 |
| Low | Hayward Scenic Reserve | 2 |

*Trapping*

Forty mesh cage rat traps measuring 11.8" x 4.7" x 4.3" were supplied by Massey University and set up at each selected site successively for a period of 3 weeks in February-December 2017. Traps were placed 100 m apart and baited with cheese and peanut butter. A small plastic bottle of water was also attached inside the trap. Traps were cleaned every alternate day to replace the cheese, peanut butter and water. Traps were checked every morning and captured animals were transported in their traps to the Ecology Department at Massey University, Palmerston North, within 24 h. Animals were provided with cheese, peanut butter, a slice of apple and water before transportation. On arrival at Massey University, each animal was transferred from its trap to an individual rat cage.




(a)


(b)

**Figure 3.1 (a) Sites used for rat trapping in the Wellington region, and (b) Location of Turitea Reserve in Palmerston North in relation to other sites of study in Wellington.**

*Maintenance of captive rats*

All rats captured were confirmed to be ship rats based on their morphology (Cunningham and Moors 1996). Only mature and healthy rats were used for the experiment. Any pregnant females and immature rats caught were not used in the experiment and euthanased by cervical dislocation while under general anaesthesia (isoflurane). In the laboratory, rats were acclimatised individually in 30 cm x 30 cm x 25 cm cages for 15 days before the commencement of the experiment. Every cage included a 30 cm (l) x 15 cm (w) x 25 cm (h) nest box. Cages were provided with sawdust for bedding and shredded newspaper for nesting material. Cages were marked with a unique code for each rat along with information about its weight and sex. Cages were cleaned weekly and food and water provided *ad libitum*. Rats were provided with rat cereal feed pellets named Diet 86 manufactured at the feed mill at Massey University. Diet 86 contains all the components necessary for rat nutrition (National Research Council (US) Subcommittee on Laboratory Animal Nutrition 1995). A slice of apple or carrot was provided each day for variety as well as an acorn or walnut every few days. The laboratory was maintained at 20±2°C on a 12-hour light/dark cycle.

Vitamin K is an important component of blood-clotting system in mammals. Deficiency of Vitamin K can lead to prolonged blood-clotting time while an excess of Vitamin K in the body from the diet can act as an antidote to an administrated brodifacoum dose (Prescott *et al.* 2007). The level of Vitamin K in wild ship rats captured from wild is unknown and, therefore, it becomes very important that ship rats have an adequate amount of Vitamin K before experiment. The diet provided to the rats contained a minimum amount of Vitamin K required by a rat i.e. 1 mg/kg/diet (Fu *et al.* 2007) to avoid Vitamin K deficiency. Also, any excess Vitamin K present in the rat body may have been utilized during the 15 day acclimatization period.

*Brodifacoum dose-preparation*

Brodifacoum and polyethylene glycol (PEG) 200 was obtained from Sigma Aldrich Company Ltd, Auckland, New Zealand. Successive doses of brodifacoum were formed individually for a group by dissolving the required amount of brodifacoum in PEG200 in a beaker to make a total volume of 0.08 ml per 10 g body weight of rat. The solution was shaken and heated to 60°C in a laboratory incubator overnight to aid dissolution. The beaker was covered with aluminum foil to avoid light exposure throughout the procedure.

*Experimental Design*

The aim of this experiment was to assess brodifacoum resistance in ship rats captured from selected sites with different periods of brodifacoum exposure. Phenotypic anticoagulant resistance was assessed by their blood-clotting response (prothrombin time) using a Coaguchek analyser. Blood samples were collected and blood-clotting times measured both before and 24 h after the treatment with the effective dose of brodifacoum. These pre- and post-treatment blood-clotting times were converted into INR (International Normalised Ratio) values, where INR is the ratio of the post-treatment blood-clotting time to the pre-treatment blood-clotting time. A ship rat was considered a responder (susceptible) to an anticoagulant dose when it has an INR ≥ 3.6 after administration of the concerned dose (Garg and Singla 2015). Failure to respond to administered effective dose of brodifacoum will be regarded as evidence of resistance.

Pre-treatment: After the acclimatisation period of at least 15 days, a baseline blood sample was taken from each rat before treatment. Each rat was placed under general anaesthesia using inhalational isoflurane gas in oxygen, delivered via induction anaesthetic chamber initially and then via facemask for blood sampling. Blood was collected from the lateral saphenous vein using a 25 gauge needle. Up to 20 µl of blood were collected, and blood-clotting time was measured immediately using the blood-clotting time analyser. The analyser can measure clotting times ranging from ≥ 9.6 s -96 s; therefore for some rats we were only able to determine that 9.6 s was the maximum clotting time.

Treatment: Immediately following the pre-treatment blood sample, the rats were provided with the the effective dose of brodifacoum (2.9 mg/kg for male ship rats and 3.8 mg/kg for female rats) subcutaneously dissolved in PEG by subcutaneous injection using 22 gauge needle. The rats were returned to their cages while they were still under influence of anaesthesia and woke up in 2-3 minutes.

Post-treatment: 24 h after administering the dose of brodifacoum, up to 20 µl of blood was again collected from each rat. Rats were anaethestised as described above, then the post-treatment blood sample was collected via cardiac puncture using a 25 gauge needle. After blood collection, the rats were euthanased by cervical dislocation while still under general anaesthesia. Blood-clotting time was measured immediately as for the pre-treatment sample.

*Tissue conservation*

After euthanisation, all rats were dissected to remove a section of liver after perfusion through hepatic vein as per the method described by Thijssen (1987). A section of the distal end of the tail measuring up to 2 cm was preserved in ethyl alcohol for genetic analysis.

*Statistical Analysis*

Animals with INR of 3.6 and higher were regarded as responders (susceptible) to the administered dose of brodifacoum while animals with INR less than 3.6 were regarded as non-responders (resistant). Failure to respond to the effective dose was taken as an evidence of resistance. We assumed a linear relationship and a normal error distribution between years of exposure to brodifacoum and INR obtained for each ship rat. Effects of gender of rat and random site effects were included in the model. There were only maximum pre-treatment clotting times for some animals, we used multiple imputation to model these missing values (Carpenter & Kenward 2013), with the maximum set as the upper limit and the pre-treatment clotting times were normally distributed. We used OpenBUGS (Spiegelhalter *et al.* 2014) to model the data and the code is provided in Appendix 3. Parameter estimates are reported ± standard error.

## *3.3 Results*

*Trapping*

After three weeks of trapping at each selected study site, a total of 71 rats were captured, including 57 mature ship rats, 2 immature ship rats and 12 Norway rats (Table 3.2). A total of 5 ship rats were captured from high brodifacoum use area, 51 from moderate brodifacoum use area whereas only 1 ship rat was captured from the low brodifacoum use area. Out of these 57 ship rats, 2 female ship rats (Moderate brodifacoum use area) were found to be pregnant and were euthanized. All captured Norway rats as well as any immature rats were not used in the experiment and were also euthanized. All 55 ship rats used for the experiment, including 30 males and 25 females, were mature and healthy. The average weight of males 156.99g (91.3-205) was more than the average weight of females 141.47g (83.5-189).

**Table 3.2 Trapping results at selected study sites with trapping period and number of ship rats and Norway rats captured from each site**

| Brodifacoum use | Site | Trapping period | No. ship rat | No. Norway rat |
|---|---|---|---|---|
| High | Otari Wilton's Bush | Feb-May 2017 | 4 | 1 |
| | Porirua Scenic Reserve | June 2017 | 1 | 1 |
| | Khandallah Park | June-July 2017 | 0 | 0 |
| Moderate | Bothamley Park | July- August 2017 | 16 | 7 |
| | Wainuiomata Mainland Island | Sept-Oct 2017 | 7* | 0 |
| | Turitea Reserve | Oct 2017 | 14 | 0 |
| | East Harbour Regional Park | Nov 2017 | 7** | 1 |
| | Belmont Regional Park | Dec 2017 | 7 | 1 |
| Low | Hayward Scenic Reserve | Nov-Dec 2017 | 1 | 1 |
| Total | | | 57 | 12 |

*2 ship rats were captured from treatment area while 5 ship rats were captured from control area of Wainuiomata Mainland Island.

**A total of 7 ship rats were captured from East Harbour Regional Park included 2 immature rats which were euthanized and not used for BCR test.

*BCR test*

The average baseline blood-clotting time in ship rats was estimated to be 9.6s, including modelling of the missing values, and this was consistent for males and females. INR value was calculated for each individual rat and was used to distinguish between resistant and non-resistant rats. A ship rat was considered a responder (susceptible) to the administered effective dose of brodifacoum if it had an INR value of ≥3.6. Male rats were administered with a dose of brodifacoum at 2.9 mg/kg and produced an overall average INR of 3.94 (1.96-6.63), while female rats were administered with a dose of brodifacoum at 3.8 mg/kg and produced an average INR of 3.17 (1.63-8.5) (Table 3.3). No significant difference based on gender was observed in INR values produced in response to administered brodifacoum dose in male and female ship rats. No signs of lethargy, distress or haemorrhages were identified before the end of the 24 h period after administration of the effective dose of brodifacoum.

Out of 55 ship rats, 54 rats were successfully tested for resistance to brodifacoum using the blood-clotting response test and 57% (31/54) of the individuals were found to be resistant to the administered dose of brodifacoum (Table 3.3). Post-treatment blood-clotting time was not obtained for one of the male rats captured from Turitea reserve due to a technical error.

**Table 3.3 Blood-clotting responses of ship rats captured from different selected study sites to calculated effective dose of brodifacoum (shown as mg brodifacoum per kg of rat body weight). INR is the post-treatment blood-clotting time (BCR) divided by the pre-treatment blood-clotting time. The blood-clotting time analyser only provided a maximum clotting time if the value was < 9.6 s. In these cases the clotting time was modelled as a constrained missing value, hence there is a standard error (SE) around the INR for the respective individuals.**

| Brodifacoum Use site | No of rats trapped | Weight (g) | Sex | Dose (mg/kg) | Pre-treat. BCR (s) | Post-treat. BCR (s) | INR | SD |
|---|---|---|---|---|---|---|---|---|
| Otari Wilton's Bush | 4 | 120.0 | M | 2.9 | 9.6 | 26.4 | 2.75 | |
| | | 157.2 | M | 2.9 | <9.6 | 18.0 | 1.99 | 0.10 |
| | | 145.0 | F | 3.8 | 10.8 | 28.8 | 2.66 | |
| | | 184.0 | M | 2.9 | <9.6 | 21.6 | 2.39 | 0.11 |
| Porirua Scenic Reserve | 1 | 141.6 | F | 3.8 | 9.6 | 26.4 | 2.75 | |
| Bothamley Park | 16 | 83.50 | F | 3.8 | <9.6 | 81.6 | 8.85 | 0.32 |
| | | 156.5 | M | 2.9 | 10.8 | 50.4 | 4.60 | |
| | | 119.2 | F | 3.8 | <9.6 | 28.8 | 3.19 | 0.16 |
| | | 114.0 | M | 2.9 | <9.6 | 63.6 | 6.94 | 0.28 |
| | | 174.5 | M | 2.9 | 9.6 | 56.4 | 5.87 | |
| | | 153.8 | F | 3.8 | 9.6 | 52.8 | 5.50 | |
| | | 123.1 | F | 3.8 | 9.6 | 34.8 | 3.63 | |
| | | 185.0 | M | 2.9 | <9.6 | 58.8 | 6.43 | 0.27 |
| | | 152.0 | M | 2.9 | 9.6 | 31.2 | 3.25 | |
| | | 112.4 | F | 3.8 | <9.6 | 44.4 | 4.88 | 0.21 |
| | | 181.0 | M | 2.9 | 9.6 | 39.6 | 4.10 | |
| | | 131.0 | M | 2.9 | <9.6 | 30.0 | 3.34 | 0.17 |
| | | 132.2 | M | 2.9 | 9.6 | 52.8 | 5.50 | |
| | | 171.0 | M | 2.9 | 9.6 | 38.4 | 4.00 | |
| | | 165.0 | M | 2.9 | <9.6 | 31.2 | 3.47 | 0.18 |
| | | 164.0 | M | 2.9 | <9.6 | 42.0 | 4.64 | 0.22 |
| Wainuiomata Mainland Island | 7 | 91.30 | M | 2.9 | 9.6 | 33.6 | 3.50 | |
| | | 108.3 | F | 3.8 | <9.6 | 25.2 | 2.79 | 0.14 |
| | | 139.0 | M | 2.9 | 10.8 | 43.2 | 4.00 | |
| | | 147.0 | M | 2.9 | 9.6 | 30.0 | 3.12 | |
| | | 123.0 | M | 2.9 | 9.6 | 34.8 | 3.62 | |
| | | 117.0 | F | 3.8 | <9.6 | 15.6 | 1.73 | 0.09 |
| | | 189.0 | F | 3.8 | 9.6 | 21.6 | 2.25 | |
| Turitea Reserve | 14 | 219.0 | M | 2.9 | 10.8 | 40.8 | 3.80 | |
| | | 125.6 | F | 3.8 | 9.6 | 28.8 | 3.00 | |
| | | 157.5 | F | 3.8 | <9.6 | 18.0 | 2.00 | 0.10 |
| | | 151.5 | F | 3.8 | 9.6 | 22.8 | 2.37 | |
| | | 217.0 | M | 2.9 | NS | 38.4 | 3.99 | 1.13 |
| | | 205.0 | M | 2.9 | 10.8 | NS | - | |
| | | 189.0 | F | 3.8 | 9.6 | 21.6 | 2.25 | |
| | | 156.6 | F | 3.8 | 10.8 | 24.0 | 2.22 | |
| | | 142.0 | F | 3.8 | 9.6 | 18.0 | 1.87 | |
| | | 157.4 | M | 2.9 | 9.6 | 39.6 | 4.12 | |
| | | 139.5 | F | 3.8 | 9.6 | 21.6 | 2.25 | |
| | | 146.0 | F | 3.8 | <9.6 | 19.2 | 2.13 | 0.11 |
| | | 175.5 | F | 3.8 | 10.8 | 24.0 | 2.22 | |
| | | 166.0 | M | 2.9 | 1 | 38.4 | 3.20 | |
| East Harbour Regional Park | 5 | 150.7 | F | 3.8 | <9.6 | 20.4 | 2.26 | 0.11 |
| | | 113.2 | M | 2.9 | 9.6 | 36.0 | 3.75 | |
| | | 140.0 | M | 2.9 | <9.6 | 33.6 | 3.72 | 0.19 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 147.5 | M | 2.9 | 9.6 | 26.4 | 2.75 | |
| | | 128.5 | M | 2.9 | 9.6 | 40.8 | 4.25 | |
| Belmont Regional Park | 7 | 158.1 | F | 3.8 | 9.6 | 26.4 | 2.75 | |
| | | 130.0 | F | 3.8 | <9.6 | 49.2 | 5.39 | 0.23 |
| | | 135.2 | F | 3.8 | 9.6 | 32.4 | 3.37 | |
| | | 144.0 | F | 3.8 | 9.6 | 43.2 | 4.50 | |
| | | 201.0 | M | 2.9 | 9.6 | 36.0 | 3.75 | |
| | | 174.6 | M | 2.9 | <9.6 | 52.8 | 5.78 | 0.24 |
| | | 200.9 | M | 2.9 | 10.8 | 38.4 | 3.55 | |
| Hayward scenic reserve | 1 | 142.8 | F | 3.8 | 9.6 | 46.8 | 4.87 | |

Note: NS means blood-clotting time was not obtained due to technical error.

There was a significant decrease in INR value produced by ship rats in response to an administered brodifacoum dose of 2.99 mg/kg and 3.8 mg/kg in males and females respectively associated with increased number of years of brodifacoum exposure (Figure 3.2). All five rats from high brodifacoum use areas had an INR <3.6 (1.86-2.75) after 24 h of administration of effective dose of brodifacoum and hence were categorised as brodifacoum-resistant rats. Out of 48 ship rats captured from moderate brodifacoum use areas, 26 ship rats had an INR <3.6 and were categorised as resistant to the administered dose of brodifacoum. These 26 resistant rats included 10/26 tested male ship rats and 16/22 tested female rats captured from moderate brodifacoum use areas. Only one female ship rat was captured from a low brodifacoum use sites and it produced an INR > 3.6 (4.87) after administration of effective dose of brodifacoum and hence, was considered susceptible.

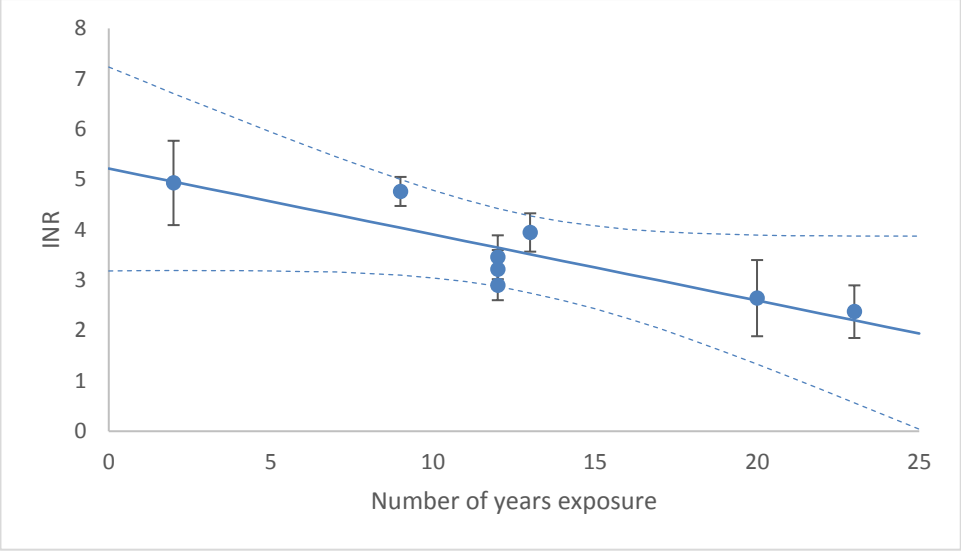



**Figure 3.2 A graph depicting the linear relationship between number of years of brodifacoum exposure and average INR value for each site. The error bars represent standard deviations for each site and the dotted lines represent 95% confidence intervals for regression line.**

## *3.4 Discussion*

*BCR test*

A total of 54 ship rats were successfully tested for resistance to brodifacoum using blood-clotting response test and 57% of the individuals were found to be resistant to the administered dose of brodifacoum. 41% of the tested male rats and 72 % of the female rats were found to be resistant to a relatively very high effective dose of brodifacoum. There was a significant decrease in INR value in response to administered brodifacoum dose associated with increasing number of years of brodifacoum use in an area.

However, it should be noted that all the conclusions derived from the results are limited by small sample sizes. Larger sample sizes would have provided a more precise assessment of prevalence and frequency of brodifacoum resistance in ship rat population in each specific site. This is a common drawback of working with live wild animals and is unavoidable and unpredictable under practical conditions. Another important reason for the small sample sizes obtained in this study is the extensive predator control regimes including different types of kill traps like D0C200 traps and Goodnature® A24 traps at each site used by Regional Councils, private landowners, contractors, and community groups.

*Male vs Female Responses to Brodifacoum*

Fifty-four captured ship rats used in blood-clotting response tests included 29 males and 25 females. Although there was no significant difference based on gender in INR values obtained after administration of effective dose of brodifacoum, 72% of the tested females were found to be resistant in comparison to 41% of the tested males that were resistant. It also needs to be considered that females are generally less susceptible to anticoagulants than males and the effective dose for females (3.8 mg/kg) was higher than effective dose for males (2.9 mg/kg) (see Chapter 2).

If brodifacoum resistance in female ship rats is accompanied by one or more heritable mutations, it may pose a real threat to effective rodent control by brodifacoum in New Zealand forests in the future because the resistant female rats are likely harder to control as compared to resistant males (Rymer 2017). Only one male is required to mate with several females, and therefore a reduction in male density alone is expected to have little effect on population dynamics. Female ship rats can reach maturity at an age of 3-4 months and are polyoestrous producing up to 10 pups in a litter (Bently and Taylor 1965). So with a high number of brodifacoum resistant females passing resistance to the next generations,

resistance can spread at alarming rates and ship rat populations may recover rapidly after application of an anticoagulant.

*Technical vs Practical resistance*

BCR tests are very sensitive and can detect even low-grade resistance. However, resistance suggested by BCR tests may or may not have any practical importance and therefore, is called "technical resistance". Resistance is a relative term and is quantitative rather than qualitative (Buckle and Prescott 2012). In a broader ecological sense, a population is known to be resistant to an anticoagulant when another population is comparatively more susceptible to the anticoagulant at a given dose (EPPO 1995, Buckle *et al.* 2007). EPPO (1995) defined resistance as a heritable trait in resistant rodents that will allow them to survive a dose of anticoagulant that would kill 99% of the susceptible individuals. The definition took into consideration phenotypic expression of genetic mutations but, not practical operational information. Greaves (1994) defined resistance as "a major loss of efficiency in practical conditions where the anticoagulant has been applied correctly, the loss in efficiency being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant". This definition seems to be more valid for field conditions and such resistance including loss of efficiency in practical conditions is called practical resistance (Buckle 2006, Buckle *et al.* 2010).

For example, low grade resistance to brodifacoum was found in UK (Gill *et al.* 1992) when Norway rats survived the dose of 5ppm in a laboratory test. However, there was no evidence of any practical effect of this low grade resistance on the efficiency of rodent control programmes in UK. This was due to the fact that the concentration of brodifacoum used in bait in field applications was 10 times the dose tested for in the laboratory test. To be able to provide information about operational importance in the forests in New Zealand, practical resistance needs to be evaluated. A "resistance factor" needs to be calculated for ship rats for each site of interest. The Resistance Factor is the multiple of the calculated effective dose required by the resistant animal to produce the same critical blood-clotting response as susceptible animal produce to the effective dose (Prescott *et al.* 2007, Endepols *et al.* 2015).

The resistance factor can also be calculated using the $LD_{50}$ dose instead of the $ED_{50}$ dose. Higher resistance factors will result in greater practical resistance and therefore, more adverse effects on rodent control using brodifacoum. A resistance factor above 5 for a particular anticoagulant may cause loss of efficiency of rodent control using that anticoagulant and may be demonstrated by need of longer treatment by anticoagulant in that area and/or application

of higher doses of anticoagulant (Greaves *et al.* 1982). Resistance factor above 10 is likely to have a significantly detrimental effect on anticoagulant efficiency (Prescott *et al.* 2017).

The $LD_{50}$ value of brodifacoum for ship rats in New Zealand is 0.46 mg/kg (O'Connor and Booth 2001). The dose administered to ship rats to test for resistance during this experiment was 2.9 mg/kg for males and 3.8 for females. If resistance factor is calculated for ship rats that were regarded as resistant in this study using the published 0.46 mg/kg ($LD_{50}$) dose, a resistance factor higher than 6.3 for males and 8.2 for females was obtained. The resistant rats need a dose higher than the administered effective dose to produce an INR ≥ 3.6. Therefore, the actual resistance factors for these rats will be higher than the calculated resistance factors.

In a recent experiment on Lord Howe Island in Australia (Wheeler *et al.* 2018), all the wild ship rats tested for brodifacoum resistance failed to survive a dose of 0.8 mg/kg of brodifacoum despite a long history of anticoagulant use dating back to the 1960s. Brodifacoum consumption on the island exceeds 400 kg per annum since 2000. The effective dose used for the present study was 3-5 times the dose used for testing in Lord Howe Island. Therefore, compared to ship rat population on Lord Howe Island, the individuals that survived the high effective dose of brodifacoum in the present study have highly reduced susceptibility and may actually have practical resistance.

*Impact on current rodent pest management in New Zealand*

There are two ready-to-use commonly available brodifacoum baits in New Zealand i.e. PESTOFF® and Talon® (Fisher 2005). PESTOFF® rodent bait contains 0.002g/kg of brodifacoum while Talon® contains 0.005 g/kg of brodifacoum. As per the pest control regime followed by the Greater Wellington Regional Council, bait stations are set up approximately 100-150m apart from each other and refilled with brodifacoum 4-6 times a year at each site (Uys 2017). In Turitea reserve, each bait station is filled with 400g of brodifacoum four times a year. There may be an additional intermediate refill of bait stations if necessary (Unpublished data).

To consume a lethal dose, a male ship rat resistant to effective dose of brodifacoum needs to consume more than 2.9 mg/kg of brodifacoum. The average weight of male ship rats calculated from the present study is 157 g. Taking into consideration effective dose of brodifacoum for male ship rats, an average weighed male rat needs to consume approximately 0.46mg of concentrated brodifacoum to consume a lethal dose. PESTOFF® rodent bait contains 0.002g/kg of brodifacoum while Talon® contains 0.005 g/kg of brodifacoum. Hence, a ship rat resistant to effective dose of brodifacoum needs to feed on more than 230 g of PESTOFF®

rodent bait or more than 92g of Talon®. Similarly a female of average weight (141.5 g) that is resistant to the effective dose of brodifacoum needs to consume 0.54 g of active concentration brodifacoum. Therefore, to consume a lethal dose of brodifacoum, the female needs to ingest more than 270g of PESTOFF® rodent bait or more than 108g of Talon® bait.

An average rat consumes 25g of food a day in multiple bouts (Krizova *et al.* 1996). Therefore, a rat needs to visit the bait station multiple times to consume a lethal dose of brodifacoum. Brodifacoum was introduced as a single feed anticoagulant. Due to reduced susceptibility to brodifacoum in ship rats, ship rats may no longer be able to consume a lethal dose of brodifacoum in one feed in the case of both PESTOFF® or Talon® baits.

PESTOFF® Hi-Strength possum baits containing 0.05g/kg of active brodifacoum are used in New Zealand at several locations to control possum populations. As possums and rodents may share bait-stations, Hi-strength baits may become available to rodents in bait stations. Ship rats resistant to the effective dose of brodifacoum need to consume more than 9.2g of Hi-strength bait in case of the males and 10.8g of bait in case of the females. Hence, there are good chances of ship rats consuming a lethal dose in one-feed from PESTOFF® Hi-Strength possum bait. However, an increase in concentration of brodifacoum in rodent baits to control brodifacoum resistant ship rats may only encourage further development of resistance to brodifacoum if sub-lethal doses are consumed.

There is another important issue with emergence of resistance to brodifacoum in ship rats in New Zealand. Brodifacoum is among the most toxic of the anticoagulants used against rodents (Erickson and Urban 2004). If a population becomes resistant to a more toxic anticoagulant, none of the less toxic anticoagulants can be used to control it (Pelz and Prescott 2015). Since there is no anticoagulant more toxic than brodifacoum at present, none of the other anticoagulant poisons remains an option to control brodifacoum resistant populations of rodents.

*Cause of resistance*

There are three pathways of anticoagulant resistance known in rats. The pathway of anticoagulant resistance in ship rats is known to be multifactorial (Ishizuka *et al.* 2008).

The first pathway of resistance is dietary-based. This resistance is caused by enhanced Vitamin K availability in their food source. Several strains of rats have been reported to synthesise Vitamin K at enhanced rate from pro-vitamin menadione (Vitamin K3) (Thijssen 1995). However, this pathway is considered to be of subsidiary importance only (Buckle and Prescott 2012) and the acclimatisation period of 15 days in our laboratory should have utilised any additional Vitamin K consumed by the rats in the forest. Also, the pelleted food provided

to the rats contained only the minimum amount of Vitamin K required by rats. Therefore, a high intake of Vitamin K can be ruled out as a cause for low susceptibility to brodifacoum in tested ship rats.

The second pathway of resistance in rats is pharmacodynamic resistance whereby conformation change in the Vitamin K epoxide reductase (VKOR) enzyme leads to lower susceptibility for anticoagulants in rats (Thijssen *et al.* 1989). This is a well-known mechanism of resistance in Norway rats and house mice (Meisenheimer *et al.* 1993, Rost *et al.* 2009, Lasseur *et al.* 2006). Recent research on this pathway of resistance enabled researchers to find Vitamin K epoxide reductase complex sub-unit 1 gene to be associated with functioning of VKOR enzyme. It was found that the C132-X-X-C135 motif in the VKORC1 gene comprises the site of redox activity that catalyses the Vitamin K epoxide reduction (Rost *et al.* 2005, Goodstadt and Ponting 2004), and mutations in this gene were found to be responsible for warfarin resistance. However, genetic resistance may or may not be accompanied by structural change in the VKOR enzyme (Rost *et al.* 2009). The first evidence of heritable genetic resistance was established in Japan in 2012 with multiple genetic mutations (Ala41Thr, Ala41Val, Arg61Trp, Leu76Pro, Tyr139Phe) identified in VKORC1 gene in ship rats (Tanaka *et al.* 2012). Recently, another SNP (Tyr25Phe) was reported in Spain to be associated with resistance to second-generation anticoagulants, bromadiolone and difenacoum (Goulois *et al.* 2016). The Tyr25Phe mutation has also been reported in ship rats in New Zealand (Cowan *et al.* 2017).

The third pathway of resistance in rats is pharmacokinetic-based resistance whereby an animal has increased ability to metabolise and excrete anticoagulant compounds from their body (Ishizuka *et al.* 2008, Buckle and Prescott 2012). Ship rats with evidence of metabolic resistance were first described in Tokyo by Sugano *et al.* (2001). Later Ishizuka *et al.* (2006) investigated VKORC1 gene in resistant ship rats in Tokyo but failed to detect VKORC1 mutations. On further investigation, it was found that warfarin metabolic activities were significantly higher (nearly twice) in resistant rats as compared to susceptible rats after administration of oral dose of warfarin. It was therefore evident from these results that cytochrome P450 dependent metabolism of warfarin is an important pathway of anticoagulant resistance in ship rats (Ishizuka *et al.* 2008, Takeda *et al.* 2016).

### *Conclusion*

Resistance to anticoagulants, as indicated by BCR tests, was apparently present in ship rats from the Wellington Region and Turitea Reserve in Palmerston North. The approximately linear relationship between INR and number of years of brodifacoum use provides tentative

evidence that areas with longer brodifacoum use have more resistance as compared to areas with low brodifacoum use. This should be taken as a warning to not rely completely on this poison for future rodent control programmes and it is recommended to use alternative poisons. Also, although ship rats are probably the most common rodent species in New Zealand forests, BCR tests need to be run for all species of rodents in New Zealand to establish their status of susceptibility towards brodifacoum. House mice are currently becoming an increasing important pest problem in areas like Bushy Park and Zealandia in Wellington where other predators have been successfully eradicated from the area. Brodifacoum is failing to eradicate house mice populations completely from such fenced predator free areas (Chapter 4).

Also, it is important to find if the phenotypic resistance obtained from BCR test is due to presence of genetic mutations. Tail samples have been collected and preserved from all the captured rats to analyse presence and frequency of genetic mutations associated with brodifacoum resistance.

# CHAPTER 4

## Comparison of blood-clotting responses to brodifacoum between house mice (*Mus musculus*) populations captured from areas of low, medium and high brodifacoum use

## *4.1 Introduction*

Many successful rodent eradication programmes have been achieved in New Zealand and around the world using anticoagulant poisons, particularly brodifacoum (Towns *et al.* 2013, Russell and Broome 2016). Total rodent eradication from discrete areas has been achieved in many cases (Bell 2002, Merton *et al.* 2002, Elliott *et al.* 2015), but house mice (*Mus musculus*) are often the only rodents that survive attempted mammal control operations on offshore islands (Micol and Jouventin 2002, Mckay *et al.* 2007, Watts *et al.* 2017) and in fenced mainland island sanctuaries (Innes *et al.* 2012). In New Zealand, larger predators like Norway rats (*Rattus Norvegicus),* ship rats (*R. rattus),* stoats (*Mustela ermine*a), ferrets (*Mustela putorius*), weasels (*Mustela nivalis*), brush-tailed possums (*Trichosurus vulpecula*) and cats (*Felis catus*) are known to limit the population of house mice in an area (Ruscoe and Murphy 2005, Bridgman *et al.* 2013). The absence of larger predators and competitors leads to higher population abundances of house mice (Angel and Cooper 2006, Russell 2012). Mice have therefore become a hurdle to achieve total pest eradication in such scenarios (Goldwater *et al.* 2012), with a failure rate of 19-38% for house mice eradication (Howald *et al.* 2007, MacKay *et al.* 2007).

Wheeler *et al.* (2018) proposed that failure to eradicate house mouse populations from discrete areas with 100% success rate may be due to two main factors. Firstly, smaller home ranges in mice as compared to rats result in a lower chance of the mice encountering bait stations (McKay *et al.* 2011). Secondly, mice are considered to have a natural tolerance towards anticoagulants as compared to rats (Buckle and Prescott 2012). In addition, they are known to thrive in the absence of rats due to reduction of predation and competition for food and space (Ruscoe *et al.* 2011).

However, a recent study was done to study movement of house mice after invasion on offshore predator-free Saddle Island (Mackay *et al.* 2019). A total of 16 mice comprising 8 mice of each sex were introduced to the island and were found to have 10 times the range size as compared to mice found on the island before eradication. The larger range size was associated with searching for mates and suggested high chances of breeding in invasive mice even at low densities. This indicates increased risk of successful population establishment at even very low densities after mouse invasion in predator-free fenced sanctuaries as well as predator-free offshore islands.

House mice are known to cause unwanted harmful impacts on native species and ecosystems (Wilson *et al.* 2007, Wanless *et al.* 2007). However, it is usually difficult to quantify their impacts (StClair 2011). Recently a 5-year study was done to assess the impact of house

mice on native flora and fauna in New Zealand in the absence of other small mammals (Watts *et al.* 2017). Mice were found to have an adverse effect on populations of invertebrates like weta (families Anostostomatidae and Rhaphidophoridae) due to predation or competition for food as has been suggested by many prior studies (Ramsay 1978, Bull 1967, Chown and Smith 1993). Although house mice cause less obvious damage than larger predators found in New Zealand, they have been reported as a cause of extinction of insects such as carabid beetles and weta on Antipodes Island (Marris 2000). House mice have been known to eat eggs of small birds up to the size of a quail (Frogley 2013, O'Donnell *et al.* 2017). They may even attack large-sized seabird chicks and lizards such as Otago skinks *Oligosoma otagense* (Jones and Ryan 2010, Norbury *et al.* 2014). They have been considered a potential threat to kakapo chicks in New Zealand (Watts *et al.* 2017).

There is great reliance on the use of rodenticides to control house mouse populations in New Zealand. Anticoagulants, particularly brodifacoum, are used extensively by City Councils, Regional Councils and private landowners on the New Zealand mainland, as well as on offshore islands by the Department of Conservation for rodent control and eradication programmes. Various cereal-based and wax-based formulations containing anticoagulant poisons are registered in New Zealand. There are two types of brodifacoum baits in New Zealand that are available for rodent control, 0.002 g/kg (20ppm) and 0.005 g/kg (50 ppm) brodifacoum containing baits. 20ppm bait pellets were found to be more palatable and effective for mouse control than 50ppm (Fisher 2005). The $LD_{50}$ dose of brodifacoum for house mice is known to be 0.52 mg/kg (O'Connor and Booth 2001). Therefore, an average mouse of 25g needs to eat 0.65 g of 20ppm brodifacoum bait or 0.26g of 50 ppm brodifacoum bait to ingest a lethal dose of brodifacoum (Fisher 2005).

Heavy long-term reliance on anticoagulants for rodent control may lead to the development of resistance to anticoagulants (Pelz *et al.* 2005, Bailey and Eason 2000). Anticoagulant resistance has been reported in house mice to almost all first-generation anticoagulants and second-generation anticoagulants like bromadiolone and difenacoum around the world (Rost *et al.* 2009, Guidobono *et al.* 2010, Pelz *et al.* 2012, Endepols *et al.* 2012). Reduced susceptibility has been reported to brodifacoum in house mice in Denmark, United Kingdom, Canada and Australia (Siddiqui and Blaine 1982, Wheeler *et al.* 2018, Berny *et al.* 2018). In a review of the susceptibility of house mice towards different anticoagulants, house mice were reported to be more susceptible to second-generation anticoagulants, particularly brodifacoum, as compared to first-generation anticoagulant, and female mice were found to be less susceptible than male mice to anticoagulants (Fisher 2005). No reduced

susceptibility or resistance to brodifacoum in house mice has previously been investigated or reported in New Zealand.

The aim of this study was to assess presence of brodifacoum resistance in the Wellington Region and Turitea Reserve in Palmerston North. These areas were chosen due to the extensive use of brodifacoum by the Wellington Regional Council (GWRC) and Palmerston North City Council over the last 20 years. The extended use of anticoagulants in these areas may have resulted in selection for anticoagulant resistance in rodent populations.

Blood-clotting response (BCR) tests were chosen for assessing susceptibility of house mice against brodifacoum. A BCR test involves collection of blood samples and measuring coagulation times of captured wild animals both before and after administration of a known anticoagulant dose. Animals that show considerably increased blood-clotting times after administration of a dose of anticoagulant are considered susceptible, whereas animals that show little or no increase in blood-clotting times are considered to be resistant (Kerins *et al.* 1993). This test does not rely on mortality of animals as in case of the lethal feeding period (LFP) test (see Chapter 2), and hence is considered more humane, has fewer ethical constraints, and can be performed in 24 h so is time efficient. BCR tests are very sensitive and can detect even small differences in susceptibility of rodent species to specific anticoagulant rodenticides (Prescott and Buckle 2000). The International Normalised Ratio (INR) is used to analyse blood-clotting times in BCR tests (see Chapter 2). INR is the ratio of post-treatment blood-clotting time to pre-treatment blood-clotting time. A rodent is considered to be susceptible to a dose of anticoagulant, if its plasma percent coagulation activity (PCA) is less than 17% after administration of that dose after 24h period (Martin *et al.* 1979, MacNicoll and Gill 1993, Precott and Buckle 2000). In case of house mouse, 17% plasma PCA activity corresponds approximately to an INR of 5. Therefore, a house mouse is considered to be a responder (susceptible) to a given dose of anticoagulant when it has an INR ≥ 5 (Prescott *et al.* 2007).

For my research, I followed the BCR test protocol developed by Prescott *et al.* (2007). This protocol provides a standardised BCR test for Norway rats and house mice for all the anticoagulant rodenticides. This method enables a system where all the future data generated from different anticoagulant-based tests involving different rodent species can be compared if the protocol is followed precisely and same dose of the specific anticoagulant for a specific species of rodent is administered (Rymer 2017). This administered dose is called the effective dose ($ED_{50}$). An effective dose first needs to be calculated for each species before screening animals for resistance, as it is essential to determine what level of change in response will be

considered as significant for each species of rodent. An “effective dose” is a sub-lethal dose of anticoagulant that will not kill the animal but will produce a critical measurable change in blood-clotting time (Prescott *et al.* 2007). The effective dose ($ED_{50}$) of brodifacoum for house mice is sex-specific and is known to be 0.39 mg/kg for male mice and 0.35 mg/kg for female mice (Prescott *et al.* 2007).

During assessment of resistance in wild rodent population, animals are tested with a higher multiple of the effective dose called a ‘discriminating dose’ (Gill *et al.* 1994). The discriminating dose usually represents a high response percentile such as the $ED_{99}$ dose. For a particular anticoagulant and species of rodent, using $ED_{99}$ dose as the discriminating dose provides similar results to those expected with previous BCR tests that are based on the response of susceptible animals (EPPO 1999) and has been successfully used to quantify resistance in wild Norway rats before conducting full field trials (Gill *et al.* 1992, Endepols *et al.* 2007). For non-resistant house mice, the $ED_{99}$ value of brodifacoum has been reported as 0.52 mg/kg for males and 0.46 mg/kg for females (Prescott *et al.* 2007). As an initial screen for resistance, these previously published $ED_{99}$ doses will be used as the discriminating dose for the present study and failure to respond to this discriminating dose will be presumed to indicate presence of resistance to brodifacoum in house mice.

## *4.2 Material and methods*

*Study Sites*

Nine different sites in the Wellington region were selected and were categorised as having high, moderate or low brodifacoum use areas based on their recorded management histories (Table 4.1; Figure 4.1). All the sites have had continuous brodifacoum use with bait stations serviced 4-6 times a year and bait is available in bait stations around the year (Uys 2017). Another site, Turitea reserve from Palmerston North, was added later due to its history of long continuous use of brodifacoum. A detailed recorded pest control history for each site for past 25 years is provided in Appendix 2.

**Table 4.1 Study sites in Wellington and Palmerston North categorised into high, moderate and low brodifacoum use.**

| Brodifacoum use | Site | Brodifacoum Use History (Years) |
|---|---|---|
| High | Otari-Wilton's Bush | 23 |
| | Porirua Scenic Reserve | 20 |
| | Khandallah Park | 19 |
| Moderate | Belmont regional Park | 13 |
| | Turitea Reserve | 12 |
| | Wainui Mainland Island | 12 |
| | East Harbor Regional Park | 12 |
| | Bothamley Park | 9 |
| Low | Hayward Scenic Reserve | 2 |
| | Akatarawa Forest | 0 |

*Trapping*

Twenty steel live-catch mouse traps measuring 260mm x 160mm x 45mm (Manufactured by Pestgrad, New Zealand) were set up at each selected site successively for a period of three weeks in February-December 2017. The traps have one-way doors so once a mouse entered a trap, it could not exit. Traps were placed 150 m apart and baited with cheese, peanut butter and a grape. Traps were cleaned every alternate day to replace the cheese, peanut butter and fresh grape. Traps were checked every morning and captured animals were transported in their traps to the Ecology Department at Massey University, Palmerston North, within 24 h. Animals were provided with cheese, peanut butter, a slice of apple and a grape before transportation. On arrival at Massey University, each animal was transferred from its trap to an individual mouse cage.

*Maintenance of captive mice*

Only mature and healthy mice were used for the experiment. Any pregnant females and immature mice caught were not used in the experiment but euthanased by cervical dislocation while under general anaesthesia (isoflurane). In the laboratory, mice were acclimatised individually in 30 cm x 30 cm x 25 cm cages for 15 days before the commencement of the experiment. Every cage included a 30 cm (l) x 15 cm (w) x 25 cm (h) nest box. Cages were provided with sawdust for bedding and shredded newspaper for nesting material. Cages were marked with a unique code for each mouse along with information about its weight and sex. Cages were cleaned weekly and food and water were provided *ad libitum*. Mice were provided

with cereal feed pellets named Diet 86 manufactured at the feed mill at Massey University. Diet 86 contains all the nutritional components necessary for mice (National Research Council 1995). A slice of apple or carrot was provided each day for variety as well as an acorn or walnut every few days. The laboratory was maintained at 20±2°C on a 12-hour light/dark cycle.

Vitamin K is a particular important component of the captive diet due to its role in the blood-clotting system of mammals. Deficiency of Vitamin K can lead to prolonged blood-clotting time while excess of Vitamin K in the body from diet can act as an antidote to administrated brodifacoum dose (Prescott *et al.* 2007). The level of Vitamin K in mice captured from wild was unknown, and it was therefore important both that mice had adequate Vitamin K before the experiment and also that they were held for an acclimatisation period to reduce any initial variation. The diet provided to the mice contained a minimum amount of Vitamin K required by a rat i.e. 1 mg/kg per diet (Fu *et al.* 2007) to avoid Vitamin K deficiency. Any initial excesses of Vitamin K present in their bodies was expected to have been utilized during the 15-day acclimatisation period.

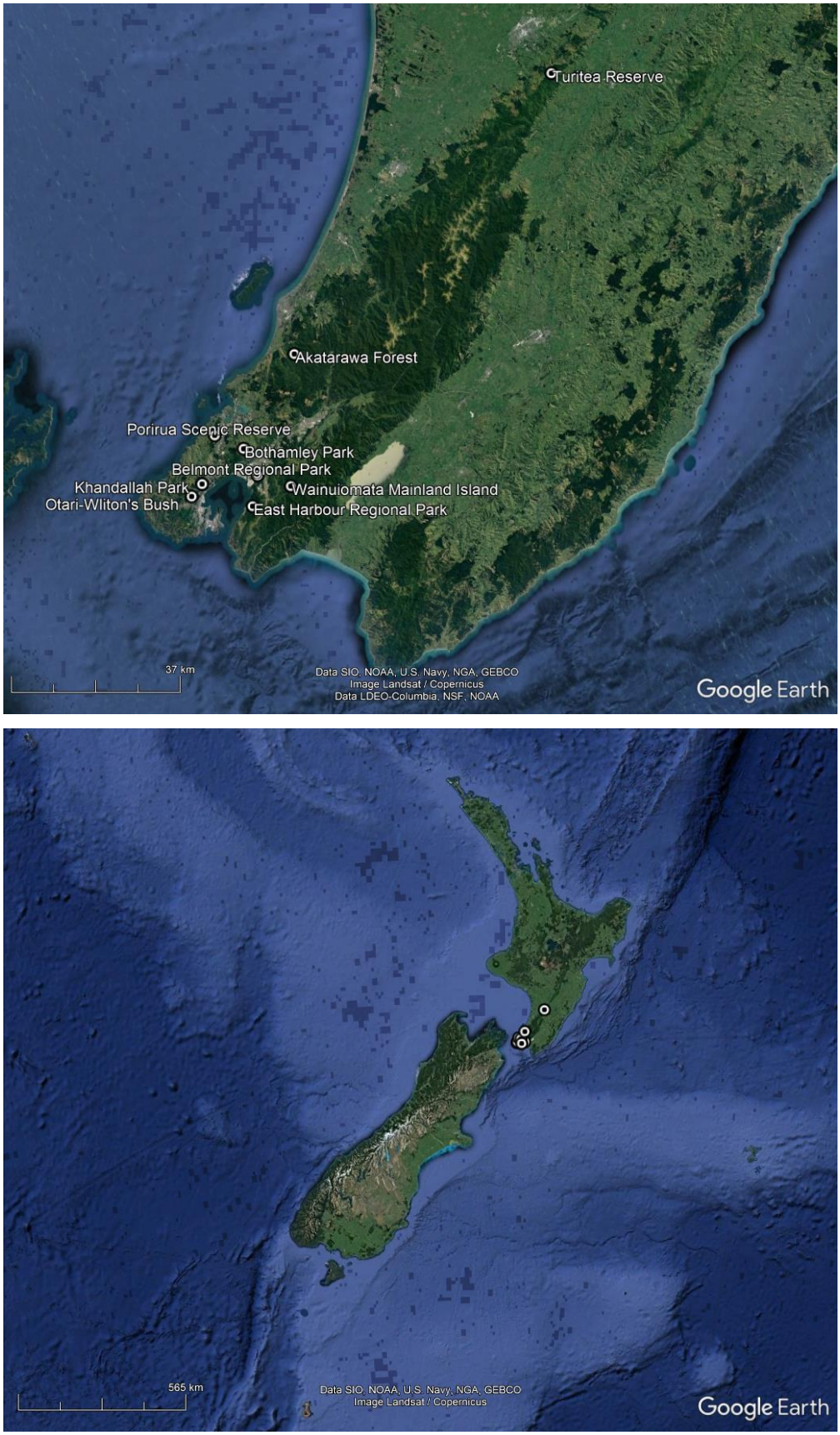



**Figure 4.1 (a) Study sites used for mice trapping in the Wellington and Palmerston North regions (b) along with their relative positions in New Zealand.**

*Experimental Design*

The aim of this experiment was to assess brodifacoum resistance in house mouse captured from populations from selected sites of known brodifacoum use history. Phenotypic anticoagulant resistance was assessed by their blood-clotting responses (prothrombin times) using a Coaguchek analyser.

A control group comprised of 6 mice was used to evaluate the baseline (pre-treatment) blood-clotting time for house mice. This was done because two blood samples, one before and one after the administration of brodifacoum dose could not be obtained from individual mice. Attempts to obtain blood via saphenous or jugular vein before dosing failed, so I was only able to obtain a single, terminal samples from each mouse.

A post-treatment blood sample was collected from each mouse captured from selected low, moderate and high brodifacoum use areas 24 h after the treatment. The treatment involved subcutaneously injecting the mouse with a discriminating dose of brodifacoum of 0.52 mg/kg for males and 0.46 mg/kg for females. These post-treatment blood-clotting times were converted into estimated INR (International Normalised Ratio) values using data on the baseline blood-clotting time evaluated from control group of mice. INR is the ratio of post-treatment blood-clotting time to the baseline blood-clotting time. A house mouse was considered a responder (susceptible) to brodifacoum dose if it had an INR value of ≥5 (Prescott *et al.* (2007). Failure to respond to the calculated discriminating dose was considered to be evidence of resistance.

*Laboratory methods*

Baseline blood-clotting time: Out of 19 mice captured from the high brodifacoum site (Otari-Wilton's bush), 6 mice (3 males and 3 females) were used to measure baseline blood-clotting times. The term baseline blood-clotting time has been used instead of pre-treatment blood-clotting times in this chapter due to the fact that house mice used to calculate baseline blood-clotting time were not treated with brodifacoum. After the acclimatisation period of 15 days, a baseline blood sample was taken from each mouse in the control group. Mice were placed under general anaesthesia using inhalational isoflurane gas in oxygen, delivered via an induction anaesthetic chamber initially and then via a facemask for blood sampling. Blood was collected via cardiac puncture using a 22-gauge needle. Up to 20 µl of blood was collected from each mouse, and blood-clotting time was measured immediately using the blood-clotting time analyser. The animals were euthanased by cervical dislocation while still under anaesthesia.

*Brodifacoum dose-preparation*

A stock solution of 0.1 mg/ml of brodifacoum in MPeg (1,2-Propandiol) was prepared by the toxicology laboratory, Landcare Research, Lincoln (Appendix 4). A discriminating dose ($ED_{99}$) of 0.52 mg/kg for males and 0.46 mg/kg

for females was prepared by diluting the stock solution with Polyethylene Glycol, molecular weight 200 (PEG200, Sigma Aldrich Company Ltd, Auckland, New Zealand) in a beaker to achieve a concentration of 0.065 mg/ml for males and 0.060 mg/ml for females. Each mouse was then given 0.08 ml of the solution per 10g bw to give the correct dose of brodifacoum per bw. The solution was shaken and heated to 60°C in a laboratory incubator overnight to aid thorough mixing. The beaker was covered with aluminum foil to avoid light throughout the procedure.

Treatment: All the captured mice (except for control group) were placed under general anaesthesia using inhalational isoflurane gas in oxygen, delivered via induction anaesthetic chamber initially and then via facemask, to administer the appropriate subcutaneous dose of brodifacoum dissolved in PEG200 by 22-gauge needle. The mice were returned to their cages while they were still under influence of anaesthesia and woke up in 2-3 minutes.

Post-treatment: 24 h after administering the dose of brodifacoum, up to 20 µl of blood was collected from each mouse. Mice were anaethestised as described above, then the post-treatment blood sample was collected via cardiac puncture using a 22-gauge needle. After blood collection, the mice were euthanased by cervical dislocation while still under anaesthesia. Blood-clotting time was measured immediately as for the baseline blood sample.

The analyser could only read blood-clotting time ≥ 9.6 s; therefore for some mice, we were only able to determine that this was the maximum blood-clotting time.

*Tissue conservation*

After euthanisation, all mice were dissected to remove a section of liver after perfusion through hepatic vein as per the method described by Thijssen (1987). Section of distal end of tail up to 2cm was preserved in ethyl alcohol for genetic analysis.

*Statistical Analysis*

I fitted a general linear mixed model to assess the relationship between years of exposure to brodifacoum and the INR for each house mouse. The sex of the mouse was included as a fixed effect (along with years of exposure) and the site included as a random effect. I could not

directly measure INR values because it was impossible to obtain both baseline- and post-treatment blood-clotting times from the same animal. However, I could obtain probability distributions for INR values by modelling the pre-treatment blood-clotting times as missing values using multiple imputation (Carpenter & Kenward 2013), assuming they followed the same distributions as the mice in the control group. There was only a maximum clotting time available for six mice (one in the control group and five of the treated mice), so I also modelled these as missing values with the maximum set as the upper limit. Variances were allowed to be different for pre- and post-treatment. I used OpenBUGS (Spiegelhalter *et al.* 2014) to model the data and the code is provided in Appendix 5. Parameter estimates are reported ± standard error.

## *4.3 Results*

### *Trapping*

A total of 33 live mice (18 males and 15 females) were captured from different selected sites use (Table 4.2). Although there were specialized mice catch traps, many of these mice were captured in rat traps used for the ship rat studies (Chapters 2-3). The rat traps ended up catching ship rats along with Norway rats and house mice as bycatch. Norway rats were euthanized, but house mice were used in the present study. The captured mice included 20, 10 and 3 house mice from high, moderate and low brodifacoum use sites respectively (Table 4.2). All 33 mice were mature and healthy, and none were pregnant. The average weight of males 16.35g (10.4-23.0) was more than the average weight of females 13.54g (9.6-16.7). No signs of lethargy, distress or hemorrhages were identified before the end of 24 h period after administration of the discriminating dose of brodifacoum.

**Table 4.2 Numbers of mice captured from each individual trapping site in comparison to the numbers of ship and Norway rats.**

| Site of study | No. ship rats | No. Norway rats | No. house mice |
|---|---|---|---|
| Akatarawa Forest | 38 | 0 | 2 |
| Otari Wilton's Bush | 4 | 1 | 19 |
| Porirua Scenic Reserve | 1 | 1 | 1 |
| Khandallah Park | 0 | 0 | 2* |
| Bothamley Park | 16 | 7 | 7 |
| Wainuiomata Mainland Island | 7 | 0 | 0 |
| Turitea Reserve | 14 | 0 | 2* |
| Belmont Regional Park | 7 | 1 | 0 |
| East Harbour Regional Park | 7 | 1 | 3 |

| | | | |
|---|---|---|---|
| Hayward Scenic Reserve | 1 | 1 | 1 |

*Mice found dead in traps so BCR test could not be performed. Tail samples preserved for genetic analysis.

*Baseline blood-clotting time*

The baseline blood-clotting times produced by control group were 9.6 s or less for males and 9.6-10.8s for females (Table 4.3). The average blood-clotting time was estimated to be 9.6s including modelling of the missing values. The baseline blood-clotting time was consistent for males and females.

**Table 4.3 Baseline blood-clotting times for the control group of house mice captured from Otari-Wilton's bush. The blood-clotting time analyser only provided a maximum clotting time if the value was < 9.6 s. In these cases the clotting time was modelled as a constrained missing value, hence there is a standard error (SE) around the INR.**

| Animal ID. | Sex | Body weight (g) | Baseline blood-clotting time (seconds) |
|---|---|---|---|
| H1 | M | 19.0 | <9.6 |
| H2 | M | 14.5 | 9.6 |
| H3 | M | 17.8 | 9.6 |
| H4 | F | 13.0 | 10.8 |
| H5 | F | 16.7 | 9.6 |
| H6 | F | 11.0 | 9.6 |

*BCR test*

A total of 27 mice were tested for resistance to brodifacoum using the BCR test. For high brodifacoum use areas, 12 mice (8 males and 4 females) were successfully tested for resistance. Post-treatment blood-clotting times could not be obtained for two additional mice captured from Otari Bush due to rejection of the blood sample by the blood-clotting analyser (Table 4.4). Seven out of 9 tested males and 3 out of 4 tested females were found to be resistant to the administered discriminating dose with an INR <5. Ten mice (5 males and 5 females) were captured from moderate brodifacoum use sites. Four out of five female mice tested were found to be resistant (INR < 5) while 3 out 5 male mice were found to be resistant. In two low brodifacoum use sites, only three mice were captured including two females from Akatarawa Forest and one male from Hayward Scenic Reserve. All the mice were found to be resistant to the discriminating dose of brodifacoum and produced INR < 5.

**Table 4.4 Blood-clotting responses of house mice captured from different sites to discriminating doses of brodifacoum (0.52 and 0.46 mg brodifacoum per kg of rat body weight for males and females respectively). INR is the post-treatment blood-clotting time (BCR) divided by the baseline blood-clotting time. No baseline blood-clotting times were available for these animals and hence, baseline blood-clotting times treated as missing values from the control group of house mice. Consequently, the INR values are reported as estimates accompanied by standard errors (SE).**

| Site | No of rats trapped | Sex | Post-treat. BCR (s) | INR est. | INR SE |
|---|---|---|---|---|---|
| Otarl-Wilton's Bush | 13 | M | <9.6 | 0.53 | 0.56 |
| | | M | 22.8 | 2.39 | 0.38 |
| | | M | 28.8 | 3.02 | 0.61 |
| | | M | 36 | 3.77 | 0.57 |
| | | M | 51.6 | 5.38 | 1.07 |
| | | M | 64.8 | 6.73 | 1.93 |
| | | M | NS | - | - |
| | | M | NS | - | - |
| | | F | 9.6 | 1.02 | 0.37 |
| | | F | 9.6 | 1.01 | 0.74 |
| | | F | 10.8 | 1.14 | 0.35 |
| | | F | 13.2 | 1.39 | 0.58 |
| | | F | 74.4 | 7.69 | 1.10 |
| Porirua Scenic Reserve | 1 | M | <9.6 | 0.53 | 0.38 |
| Bothamley park | 7 | M | <9.6 | 0.53 | 0.33 |
| | | M | <9.6 | 0.53 | 0.39 |
| | | M | 9.6 | 1.01 | 1.12 |
| | | M | 55.2 | 5.74 | 0.72 |
| | | F | 9.6 | 0.99 | 3.43 |
| | | F | 9.6 | 1.01 | 0.73 |
| | | F | 115.2 | 11.78 | 1.15 |
| East Harbour Regional Park | 3 | M | 88.8 | 9.14 | 0.89 |
| | | F | 10.8 | 1.14 | 0.89 |
| | | F | 10.8 | 1.21 | 9.17 |
| Akatarawa Forest | 2 | F | <9.6 | 0.52 | 0.41 |
| | | F | 9.6 | 1.02 | 1.09 |
| Hayward Scenic Reserve | 1 | M | 31.2 | 3.26 | 0.49 |

The INR values obtained from the captured house mice suggest there was no relationship between number of years of brodifacoum use and susceptibility to brodifacoum (Fig 4.2). Also, there was no significant difference in INR values produced by male and female mice in response to the discriminating dose.

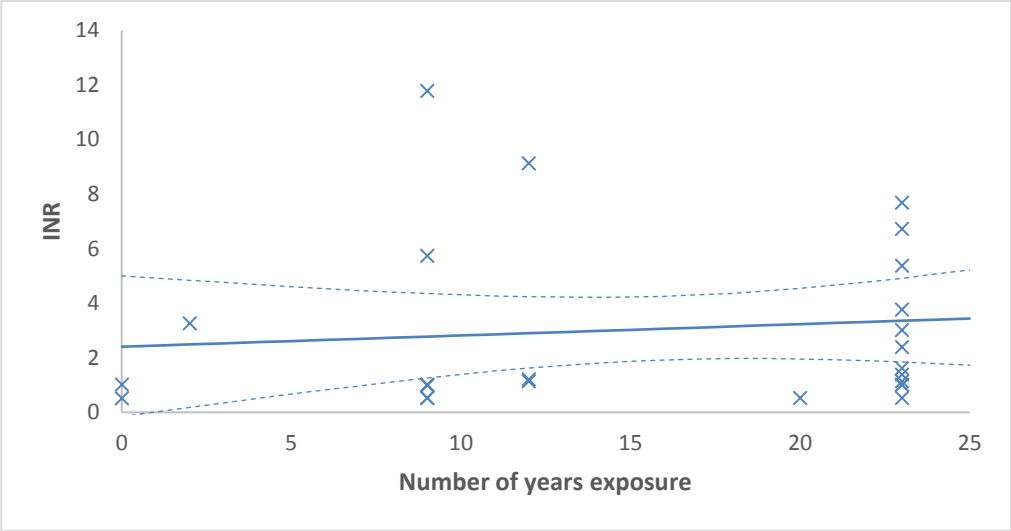



**Figure 4.2. Relationship between number of years of brodifacoum exposure at sites and the INR values of mice captured from those sites. Crosses show estimated INR values for individual mice, the solid line shows a linear function fitted to the data, and the dotted lines show the 95% credible intervals around that relationship.**

## *4.4 Discussion*

Live trapping of house mice for three weeks at each of the selected 10 brodifacoum use areas resulted in a sample size of only 33 live mice. Mice were captured from six of the 10 selected sites, with the highest number of mice (19) captured from Otari-Wlton's bush. The reason that no or very few numbers of mice were captured from other sites may have been the extensive predator control programmes aimed at control of possums, rabbits, mustelids and rodents in action at most of these by the City Council, local staff and the community groups. However, Otari-Wilton's bush also had a large-scale predator control programme being run by GWRC with help of local management staff and community group named RAMBO (Rats and Mustelid Blitzing Otari). The relatively high number of mice captured may be due to low rat numbers in the bush as suggested by a recent rodent monitoring report (Uys 2017). This may also have been due to movement of mice into the site from the surrounding residential neighbourhood.

An inverse relationship is known to exist between number of rats and mice in a given area (Brown *et al.* 1996, King *et al.* 1996). This may be due to interspecific competition that exists between rats and mice (Ruscoe *et al.* 2011). Drops in rat density have been associated

with rises in mouse density due to reduction of predation and competition for food and space from rats. Similarly, increases in rat density seem to supress mouse density in the forest (Watts *et al.* 2017, Bowie *et al.* 2018). Based on capture rates the pattern of high rat and low mouse density seemed to occur in Wainuiomata Mainland Island, Akatarawa forest, Belmont Regional Park and Turitea reserve (Table 4.2). In contrast, Norway rats, ship rats and house mice appeared to co-exist in relatively high numbers in East Harbour Regional Park and Bothamley Park (Table 4.4). This may be due to availability of different niches for the three species in same area. This also indicates the need for better management of rodent populations in these areas as compared to other sites.

The baseline blood-clotting times of the control group (mean 9.6 s, SE 0.45) was consistent with baseline blood-clotting times for ship rats (Chapters 2-3). A more precise estimate of mean baseline blood-clotting response in mice may be obtained by using equipment that can precisely measure blood-clotting time below 9.6 as well as using higher numbers of individual per sex in a control group. However, these numbers can be justified based on limitations of practical field situations. With only 33 live house mice captured, a larger control group couldn't be justified.

A total of 20 out of 26 successfully assessed house mice were found to be resistant to the administered dose of brodifacoum. Females in general are known to be less susceptible to anticoagulants as compared to males (Fisher 2005). In my study, four out of 15 males were found to be resistant in comparison to two out of 12 females. However, there was no significant difference in blood-clotting response to the discriminating dose of brodifacoum in male and female mice.

The results show widespread brodifacoum resistance in house mice irrespective of number of years of brodifacoum exposure. This may be due to multiple reasons but it difficult to explain the lack of any relationship between brodifacoum resistance in mice and years of brodifacoum use. This may be due to general high tolerance to brodifacoum in house mouse populations. Also, brodifacoum is available over the counter and is used widely by the general public. Immigration of mice from surrounding brodifacoum-using areas into the sites with low brodifacoum use may be another reason for the presence of brodifacoum resistance in house mice in areas with negligible brodifacoum use. Also, most of the pest control operations target possums; therefore, high concentration brodifacoum baits are used in the forest. Unintentional access to highly concentrated brodifacoum baits may also be encouraging resistance in house mice.

The discriminating dose used for BCR tests for house mice in the present study was evaluated by Prescott *et al.* (2007) and is known to provide initial identification of resistance in house mice populations. Such resistance is known as technical resistance (Pelz and Prescott 2015) and may or may not have any effect on practical control of house mice populations in the wild. If there is difficulty in achieving efficient rodent control by anticoagulants in wild populations, such resistance is called practical resistance (RRAG 2018).

To evaluate efficiency of brodifacoum for mice control under practical conditions in the wild, a resistance factor needs to be calculated for each site. The resistance factor is the multiple of the calculated effective dose required by the resistant animal to produce the same critical blood-clotting response as a susceptible animal produces to the effective dose (Prescott *et al.* 2007, Endepols *et al.* 2015). The resistance factor is calculated using higher multiples of the effective dose (Prescott *et al.* 2007, Buckle *et al.* 2007). For example, let's take the hypothetical situation that all the house mice from a particular area administered with the effective dose of brodifacoum are found to be resistant. However, when individuals from same population are administered a dose of five times the effective dose of brodifacoum, 50% of the individuals are reported as susceptible to the administered dose. This implies that the resistance factor for the hypothetical mice population is approximately 5 (Pelz and Prescott 2015).

The brodifacoum dose used to evaluate the resistance factor may depend on either the effective dose or the lethal dose. The effective dose corresponds to the dose at which 50% individuals respond with critical delayed blood-clotting response to the administered dose of anticoagulant whereas, the lethal dose corresponds to the dose which kills 50% of the population. Lethal feeding period trials may also be done on the same animals once blood-clotting response tests have been performed. This provides another parameter, mortality along with blood-clotting response. However, there are ethical constrains to the methodology.

A recent study was done on Lord Howe Island, Australia, to access susceptibility of ship rats and house mice before rodent eradication using brodifacoum. This study was based on a lethal feeding trial test. House mice were captured from settlement areas for the study. Only 10% of the mice died at 1.2mg/kg (2 times X the previously published $LD_{50}$ dose) whereas 100% mortality was achieved at a dose of 6 mg/kg which is about 10-15 times the published $LD_{50}$ dose (Wheeler *et al.* 2018). Hence, the resistance factor for brodifacoum resistant house mice on the island was > 2 but < 10-15. A resistance factor above 5 for a particular anticoagulant may cause loss of efficiency of rodent control operations using that anticoagulant and may be demonstrated by a need for longer treatment by anticoagulant in that area and/or application

of higher doses of anticoagulant (Greaves *et al.* 1982). Therefore, it was suggested to provide prolonged exposure of brodifacoum baits (20 ppm of brodifacoum) to house mice on Lord Howe Island for at least 7 days after eradication of rats to allow resistant mice to ingest a lethal dose of brodifacoum in multiple bouts in the absence of other food sources. A key concept in understanding this strategy is that resistance to anticoagulants does not confer immunity from higher doses. Thus, in some instances, a resistant population may still be eliminated by the anticoagulant provided it is ingested at sufficiently high doses. However, prolonged exposure to brodifacoum and/or increasing brodifacoum concentration may help to achieve 100% eradication of brodifacoum-resistant house mouse populations, but this may also impair the successful management of resistance in future if some resistant individuals survive the eradication programme. This is because continuous increased use of brodifacoum may promote selection of resistance in rodent populations (Pelz and Prescott 2015).

Since technical resistance has been identified, the pathway of resistance to brodifacoum in house mice in New Zealand needs to be established. Resistance in house mice is known to be multi-factorial as for ship rats (Chapter 3). The three main pathways hypothesised are pharmacodynamics-based resistance, pharmacokinetic-based resistance and dietary-based resistance.

In pharmacodynamics-based resistance, a change in conformation of Vitamin K epoxide reductase complex subunit 1 (VKORC1) protein due to mutation in the VKORC1 gene leads to lower susceptibility for anticoagulants in mice (Thijssen *et al.* 1989). Many different amino acid substitutions, for example, W59G, R12W, R58G, R61L causing mutations in the VKORC1 gene in resistant strains of mice have been described, leading to reduced Vitamin K epoxide reductase enzyme activity by up to 49% (Lasseur *et al.* 2006, Rost *et al.* 2009). Another type of resistance linked with genetic-based resistance is “spretus group” resistance. It was developed because of a group of DNA sequence changes due to hybridisation of house mice and Algerian mice (*Mus spretus*) (Song *et al.* 2011).

In pharmacokinetic-based resistance, an anticoagulant rodenticide may be ineffective for controlling a house mouse population due to the increased ability of some animals to metabolise and excrete anticoagulant compounds from their bodies (Buckle and Prescott 2012). Sutcliffe *et al.* (1990) found that the metabolic profile of warfarin 4’-, 6-, 7- and 8-OH was very different in susceptible and resistant mice when treated with various Cytochrome P450 inducers. This showed that cytochrome P450 played an important role in resistance of house mice.

Dietary-based resistance refers to resistance due to increased Vitamin K availability. Synthesis of Vitamin K from the pro-vitamin compound Vitamin $K_3$ (Menadione sodium bisulphate) has been evident in many different strains of rats (MacNicoll and Gill 1993). However, studies done on both susceptible and resistant mice have shown that some animals have not been able to synthesise the compound in the same way. Vitamin $K_3$ presence in many animal feeds may be the reason for levels of resistance in certain strains of rodents. Due to these reasons, dietary-based resistance is not regarded to be a significant mechanism of resistance (Buckle and Prescott 2012). Also, the acclimatisation period of 15 days should have enabled metabolisation of any extra Vitamin K in the bodies of house mice. The diet fed to the captive mice during this period contained the minimum required amount of Vitamin K for house mice i.e. 1 mg/kg of the diet (Fu *et al.* 2007). Therefore, high intake of Vitamin K can be ruled out as a cause for low susceptibility to brodifacoum in tested ship rats.

In conclusion, although technical resistance has been established for house mice, the degree of this resistance is still unknown. This may or may not have any practical implications on house mice control programmes. There is a need to calculate resistance factors and/or lethal feeding trial tests for house mouse populations that appear to be resistant to brodifacoum, to have a clear picture of the degree of resistance. This will help to plan effective management for control of the house mice populations concerned. Also, it is important to determine whether the phenotypic resistance obtained from BCR tests is due to the presence of genetic mutations. Tail samples have been collected and preserved from all the captured mice to analyse presence and frequency of genetic mutations associated with brodifacoum resistance (Chapter 6). It is also recommended to assess the other known pathways of resistance in house mice to develop a better understanding of cause of resistance in house mice in New Zealand.

# CHAPTER 5

## Distribution and frequency of VKORC1 nucleotide mutations and their implications for resistance against anticoagulant rodenticides in New Zealand *Rattus rattus*

## *5.1 Introduction*

Anticoagulant resistance is defined as a heritable factor that allows an animal to survive a dose of an anticoagulant that would kill 99% of susceptible individuals of the population. This trait can evolve naturally in rodent populations and becomes increasingly prevalent when subjected to strong selection by prolonged and intensive exposure to anticoagulant compounds. Increased prevalence of resistance genetics results from elimination of the more susceptible individuals, whereas resistant individuals survive and pass on the trait to their offspring (Bailey *et al.* 2005).

Anticoagulant resistance in rodents was discovered soon after first-generation anticoagulant rodenticides (FGARs) were introduced. A failure of warfarin to control Norway rat (*Rattus norvegicus*) populations was reported first in Scotland (Boyle 1960) and then Denmark (Lund 1964). Subsequently, tolerance of warfarin as well as other first-generation anticoagulants including coumatetralyl and diphacinone was reported throughout Europe (e.g. Lund 1966, Bentley 1968, Greaves and Renninson 1973, Buckle 2013). The rapid and widespread failure of these first-generation anticoagulants as rodenticides led to development of second-generation anticoagulants (SGARs) that were marketed in the 1970s (Hadler and Shadbolt 1975). Bromadiolone and difenacoum were the first to be marketed and were initially found to be effective against FGAR-resistant rats (Halder te al 1975, Marsh 1977). However, resistance was soon reported to these compounds (Redfern and Gill 1978, Greaves and Cullen-Ayers 1988, Quy *et al.* 1995), and three additional SGARs were introduced to the market; brodifacoum, flocoumafen and difethialone. These were sometimes referred to as “resistance breakers” (Buckle *et al.* 2013) as they were found to be very effective against rodent populations that showed resistant to first- and second-generation compounds.

Anticoagulant rodenticides act as antagonists of the enzyme Vitamin K1-epoxide reductase and so hinder the recycling of Vitamin K necessary for blood clotting (Lowenthal and Macfarlane 1964, Parmar *et al.* 1987, Huckle *et al.* 1988). Hindering the blood-clotting process by anticoagulants can lead to fatal haemorrhages and internal bleeding (Thijssen *et al.* 1986), although warfarin is also in use for management of certain cardiac conditions in humans where precise dosage can be managed and monitored to optimise blood thinning (Keller *et al.* 1999).

The genetic basis of anticoagulant resistance in rodents was established for the first time in the 1960s from studies using captive breeding of wild-caught warfarin-resistant Norway rats and laboratory rat strains (Greaves and Ayers 1967). Research on warfarin resistance in house mice (*Mus musculus*) indicated a single locus that was mapped by linkage

studies to chromosome 7 in *Mus musculus* and found to have an analogous position in *Rattus norvegicus* (Wallace and MacSwiney 1976). Kohn and Pelz (1999) used recombination analysis to infer that, in Norway rat, a mutation at a single locus was sufficient to explain inheritance of anticoagulant resistance and modern studies of genome sequences have helped clarify the details of locus position on chromosome 1 in *R. norvegicus* (Kohn and Pelz 2000, Lasseur *et al.* 2005). The putative gene known as Rw was identified as coding for subunit 1 of the Vitamin K epoxide reductase enzyme complex 1 (VKORC1). The VKORC1 gene is 6126 base pairs long with 3 exons coding for 163 amino acids (Tie *et al.* 2005), and it has been identified that non-synonymous single nucleotide mutations in the coding exons of VKORC1 are the source of anticoagulant resistance in the Norway rat and house mouse (Pelz *et al.* 2012).

An important consideration of genetic resistance in regard to rodent management is incidence of resistance. Incidence of resistance is the proportion of individuals within a population that carry the mutation-conferring resistance. The greater the number of individuals resistant to the anticoagulant, the more difficult is control of a rodent population in the wild. The incidence of resistance and the incidence of resistance alleles in a population may not be perfectly correlated and depends on the level of heterozygosity. Although anticoagulant resistance in Norway rats was initially inferred to be an autosomal dominant trait (Pelz *et al.* 2005), more recent evidence indicates co-dominant resistance such that heterozygous individuals show a moderate level of resistance to respective anticoagulant (Grandemange *et al.* 2010, Berny *et al.* 2018). Populations with a higher frequency of homozygotes for a mutation-conferring resistance are more difficult to control using the anticoagulant concerned (Clarke and Prescott 2012, Buckle and Prescott 2012). Critically, as it is the application of the anticoagulant that selects for increasing prevalence of alleles conferring resistance, management practice is important. If anticoagulant resistance in rodents carries a metabolic cost, it is expected that natural selection could favour loss of resistance in populations not continuously exposed to the anticoagulant. In such cases recessive resistance alleles may be retained in heterozygous individuals.

In recent years, anticoagulant resistance in rats has focused on European Norway rats *Rattus norvegicus* in which there appears to be a close correlation between certain VKORC1 alleles and anticoagulant resistance. Pelz *et al.* (2005) screened Norway rats from European countries and described nine different amino acid replacements (arising from non-synonymous nucleotide mutations) with the VKORC1 exons. These are described in terms of amino acid positions 1-163 translated from the three concatenated VKORC1 exons numbered 1 to 3 (Tie *et al.* 2005, Pelz *et al.* 2012). Five of these (Leu120Gln, Leu128Gln, Tyr139Cys, Tyr139Phe and

Tyr139Ser) were indicated as having significant influence on susceptibility to anticoagulants. Leu120Gln is known to cause significant practical impacts to the use of bromadiolone and difenacoum to control Norway rats, and Leu120Gln is also associated with technical resistance to brodifacoum (Redfern and Gill 1978, Gill *et al.* 1994, Quy *et al.* 1995). Tyr139 mutations have been known to cause serious practical resistance to all FGARs along with resistance to SGARs like bromadiolone. Since being reported in 2005, these and other mutations have been reported in Norway rats across Europe (Lodal 2001, Grandemange *et al.* 2010, Prescott *et al.* 2010, Buckle *et al.* 2013, Mooney *et al.* 2018). Resistance to anticoagulants in Norway rats has also been reported in many other countries outside Europe, including Canada, USA, Australia, Korea and China (Siddiqui and Blaine 1982, Jackson and Ashton 1986, Saunders 1978).

Identification of specific single-nucleotide polymorphisms associated with amino acid replacements and evidence of their influence on expressed phenotypic traits in rats that lead to anticoagulant resistance helps in establishing genotypic expression of the independent mutation (Pelz *et al.* 2007, Pelz *et al.* 2012). With a clear link established between nucleotide substitutions and resistance, it would be relatively easy to screen wild rat/mouse populations for presence of influential amino acid mutations.

Ship rats (*Rattus rattus*) are considered to be generally less susceptible to anticoagulants as compared to Norway rats (Buckle 1994). Indeed in the mid-1970s, large-scale tolerance of anticoagulant poisons in British ship rats was noted and the possibility of a polygenic architecture to this resistance was inferred from unstable patterns of inheritance that were observed under experimental conditions (Greaves *et al.* 1976). This contrasts with the situation in Norway rats. Most of the studies concerning anticoagulant resistance are focused on Norway rats because they are the more significant pest in many countries (Berny *et al.* 2018). Resistance of ship rats to warfarin has been reported in Denmark, France, Germany, UK, USA, Australia and Japan (Saunders 1978, Jackson and Ashton 1981, Myllymaki 1995, Ishizuka *et al.* 2007), and resistance to both bromadiolone and difenacoum has also been reported in French ship rats (Desidiri *et al.* 1978, Lund 1984).

Anticoagulant resistance in ship rats has a more complex genetic basis than in *R. norvegicus*. For Norway rats, VKORC1 gene has been the focus of studies and is strongly associated with emergence of resistance to anticoagulants in the rat species. Anticoagulant resistance is known to be multi-factorial in ship rats. Although the VKORC1 gene has been sequenced and studied in ship rats, there is limited understanding of an association of the VKORC1 gene with anticoagulant resistance in ship rats (Table 5.1) (Ishizuka *et al.* 2007, Diaz *et*

*al.* 2010, Garg *et al.* 2017). The first evidence of heritable genetic resistance to anticoagulants in ship rats that was linked to the VKORC1 gene was established in ship rats in Japan, with multiple amino acid mutations inferred from DNA nucleotide sequencing (Ala41Thr, Ala41Val, Arg61Trp, Leu76Pro and Tyr139Phe) (Tanaka *et al.* 2012). A complaint of reduced efficiency of bromadiolone-based rodent control in Zaragoza (Spain) led to identification of the Tyr25Phe (exon 1) mutation as the cause of resistance to all first-generation anticoagulants (e.g. warfarin) and some second-generation anticoagulants (bromadiolone and difenacoum) in ship rats (Goulois *et al.* 2016). Resistance to SGARs was established in ship rats in India using lethal feeding period tests and blood-clotting response (BCR) tests (Garg and Singla 2015, Garg *et al.* 2017). Although BCR tests revealed presence of resistance to bromadiolone in ship rats, no genetic mutations associated with anticoagulant resistance were identified (Singla *et al.* 2017). Resistance to more potent anticoagulants namely, brodifacoum and flocoumafen, has not yet been reported in ship rats.

Few studies are available documenting the susceptibility of rodents to anticoagulants in New Zealand. Anticoagulant resistance was suspected as early as 1978 in parts of Auckland and on Raoul Island but no investigation took place (Wodzicki 1978). In 2002, ship rats were eradicated successfully from Raoul Island using brodifacoum by aerial application (Cowan *et al.* 2017). A preliminary study of Norway rats captured from different parts of New Zealand using blood-clotting response tests with a sub-lethal dose (~$LD_{15}$ value) of brodifacoum suggested no resistance to brodifacoum (Bailey *et al.* 2005). However, considering the small sample size (54) of animals tested, it was suggested that resistant individuals might have been present at low density in the Norway rat population. In New Zealand, it is the ship rat rather than Norway rat that is most abundant and widespread in native forests (Atkinson and Moller 1990, Cunningham and Moors 1996), so screening ship rats for resistance is needed.

In a recent (2010-12) study using rat tail samples collected across New Zealand, the distribution and frequency of nucleotide point mutations in rat VKORC1 exons was reported (Cowan *et al.* 2017). Multiple single nucleotide polymorphisms (SNPs) were encountered in the ship rats surveyed far more frequently that in Norway rats and kiore (*Rattus exulans*). Among 482 ship rats, 6 synonymous and 3 non-synonymous DNA variants were found, and of these, one of the non-synonymous mutations (Tyr25Phe) was previously reported to be associated with practical anticoagulant resistance in ship rats in Spain (Goulois *et al.* 2016).

**Table 5.1 Known amino acid replacement mutations in exons of the VKORC1 gene thought to confer anticoagulant resistance in ship rats. Amino acid replacements are numbered in order through the three VKORC1 exons, with the typical amino acid shown to the left of the number and replacement to the right. For example, in the first mutation shown Tyrosine (Tyr) is replaced by Phenylalanine (Phe).**

| Mutation | Abbreviations | Country reported from | Impact on Pest Control | Reference |
|---|---|---|---|---|
| Tyr25Phe | Y25F | Spain, New Zealand | Resistant | Goulois *et al.* 2016, Cowan *et al.* 2017 |
| Ala14Val | A14V | New Zealand | Unknown | Cowan *et al.* 2017 |
| Ala26Val | A14V | | | |
| Ala41Thr | A41T | Japan | Resistant | Tanaka *et al.* 2012 |
| Ala41Val | A41V | | Resistant | |
| Leu76Pro | L76P | | Resistant | |
| Arg61Trp | R61W | | Resistant | |
| Tyr139Phe | Y139F | | Resistant | |
| Arg35Pro | R35P | | Susceptible | |
| Arg58Trp | R58W | | Susceptible | |
| Ala21Thr | A21T | | Unknown | |
| His21Gly | H21G | | Unknown | |
| Arg40Gly | R40G | Japan, France and Spain | Susceptible\Resistant | Tanaka *et al.* 2012\ Pernik *et al.* 2015 |
| Ala26Pro | A26P | France and Spain | Resistant | Pernik *et al.* 2015 |
| His68Asn | H68N | | Unknown | |
| Arg40Gly | R40G | | Resistant | |
| Ser57Phe | S57F | | Unknown | |
| Trp59Cys | W59C | | Unknown | |
| Trp59Arg | W59R | | Unknown | |
| Lys152Thr | K152T | | Resistant | |

The two other non-synonymous mutations (Ala26Val and Ala14Val) were novel and need to be evaluated (Cowan *et al.* 2017). The New Zealand study did not correlate these observation with any specific anticoagulant used and did not present any phenotypic evidence of resistance in the sampled rats. There remains therefore, a gap in understanding of the significance of amino acid mutations found at the VKORC1 locus in relation to anticoagulant resistance and specific anticoagulant compounds; information is essential for practical management of rodents in New Zealand.

The development of anticoagulant resistance in rats is apparently not compound-specific as rodent populations that are resistant to one anticoagulant may have resistance to other anticoagulants that they have never encountered. This "cross resistance" (Pelz and Prescott 2015) may be due to the similar mode of action of all anticoagulant compounds. Notably, a rodent that is resistant to a highly toxic anticoagulant is also resistant to all less toxic anticoagulants, and in New Zealand this is significant because brodifacoum is one of the most toxic and also widely used anticoagulants. Our hypothesis is that long-term, continuous and widespread use of brodifacoum in New Zealand by several city councils, landowners and private individuals is likely to lead to emergence of resistance to in ship rats. If so, future coordinated rodent management, such as for example envisaged by the PredatorFree 2050 New Zealand campaign, may suffer due to high reliance on brodifacoum.

In Chapters 2-3 I reported BCR tests that suggested that phenotypic anticoagulant resistance had developed in ship rats in the Wellington Region and Palmerston North. In this chapter, I report genotypic analysis undertaken to test for nucleotide substitutions and inferred amino acid replacements associated with anticoagulant resistance in ship rats.

## *5.2 Material and methods*

*Study site and sampling*

Rats were trapped at several locations in the vicinity of Wellington and Palmerston North, New Zealand, using single live-catch rat traps (Table 5.2). These trapping areas were selected based on the history of brodifacoum use. Detailed information of brodifacoum use history at each site is provided in Appendix 2. A total of 103 rats were trapped, and of these 12 were identified as Norway rats based on their morphology (Cunningham and Moors 1996) and so were excluded from the study. Ship rats were maintained in captivity and subject to BCR tests (Chapters 2-3) before being euthanized by cervical dislocation while under general anaesthesia. Tail tips of about 2 cm length were collected and immediately preserved in absolute alcohol for genetic analysis. Another five tail samples were collected and preserved from DOC 200 kill traps near Hunterville, New Zealand.

*DNA extraction*

Genomic DNA was extracted from the preserved tail segments using a Genomic DNA Purification Kit (dnature Diagnostics and Research Ltd, Gisborne, New Zealand) following the manufacturer's instructions. It was used to amplify the mitochondrial DNA Control Region for species determination and the VKORC1 gene to detect mutations associated with anticoagulant resistance.

**Table 5.2 Ship rats collected from selected sites in Wellington and Palmerston North, New Zealand.**

| Site | Brodifacoum use history (Years) | No. ship rats |
|---|---|---|
| Otari Wilton's Bush | 23 | 4 |
| Porirua Scenic Reserve | 20 | 1 |
| Khandallah Park | 19 | 0 |
| Belmont Regional Park | 13 | 7 |
| Wainuiomata Mainland Island | 12 | 7 |
| Turitea Reserve | 12 | 14 |
| Bothamley Park | 9 | 16 |
| East Harbour Regional Park | 12 | 5 |
| Hayward Scenic Reserve | 2 | 1 |
| Hunterville | Unknown | 5* |

*Only tail samples, no BCR test

*DNA sequencing for species identification*

To ensure accurate identification of species of captured rats, genetic confirmation of species was undertaken using screening of mitochondrial DNA haplotypes. Two primer pairs were employed, EGL4L/RJ3R (Robins *et al.* 2008) and L15774/H16498 (Shields and Kocher 1991) (Table 5.2), to target the partial control region (D-loop) and Cytochrome b oxidase of the mtDNA genome. PCR amplification of these mitochondrial regions used 20µl reactions containing: 2µl DreamTaq™ Green buffer (10x) including 20Mm magnesium chloride, 0.2 µl of 5U/µl 10X DreamTaq™ DNA polymerase, 2µL of 2mM dNTP, 0.4 µl of 25mM ABgene™ Magnesium chloride (Thermo Fisher Scientific, Waltham, USA), 0.8µl of each 1000 µM primer, 2µL of DNA template at approximately 10ng/ml. Polymerase chain reaction (PCR) thermal cycling conditions used were as follows: 95˚C for 2 min 15 s, 35 cycles of 94˚C for 30 s, 60˚C for 30 s, 72˚C for 1 min, followed by an extension period of 72˚C for 10 min and an idling temperature of 10˚C for 9 min 40 s. The PCR product was preserved at 4°C temporarily after running gel electrolysis to check quality of PCR product. These were later sent to Macrogen, Korea, for sequencing.

*VKORC1 gene variation – Sanger sequencing*

The three exons of VKORC1 gene were amplified using three set of primers: RrVKORC1-F, RrVKORC1-R; RrVKORC2-F, RrVKORC2-R; and RrVKORC3-F, RrVKORC3-R which have been previously applied in studies of rats (Diaz *et al.* 2010) (Table 5.3). PCR amplifications were

carried out in 20 µl reactions using thermal cycling at 95˚C for 5 min, 30 cycles of 95˚C for 1 min, 56˚C for 1 min and 72˚C for 2 min; and an extension period of 72˚C for 10 min. PCR products obtained were run through gel electrolysis to verify product quality and stored at 4$^{o}$C until sent to Macrogen, Korea, for sequencing.

Resulting sequences were edited and aligned in Geneious v10 (Kease *et al.* 2012). Sequences of mtDNA haplotypes were compared with published data available on Genbank: *Rattus rattus* (Accession #KJ603317- Varudhkar and Ramakrishnan 2015, Accession #HQ588111- Russell *et al.* 2011), *Rattus norvegicus* (Accession #DQ673916- Schlick *et al.* 2006) and *Rattus exulans* (Accession # EU273711- Robins *et al.* 2008).

The SNPs were identified in VKORC1 gene sequences and compared to published VKORC1 gene sequences in *Rattus norvegicus* (Accession #AY423047), *R. rattus frugivorus* (Accession #HM181985) and *R. rattus alexandrinus* (Accession #HM181979). Numbering of nucleotide and amino acid positions was in reference to the published VKORC1 sequence for the Norway rat (Accession #AY423047) to simplify comparisons with published data. The data were also compared to known SNPs in the VKORC1 gene reported from Spain (Goulois *et al.* 2015) and New Zealand (Cowan *et al.* 2017).

*VKORC1 gene variation – Amplicon sequencing*

To clarify allele variants where ship rats' VKORC1 sequences were heterozygous, a next-generation amplicon sequencing approach was implemented on the Miseq platform (Ravi *et al.* 2018). This generated 250 bp paired end reads (Fadrosh *et al.* 2014) for a set of overlapping fragments of 450–500 bp. To achieve coverage of the VKORC1 gene including exons and introns, a set of 7 overlapping pairs of primers was designed for use in multiplex PCR of the *Rattus rattus* VKORC1 locus using the Primer3 tool (Mukhopadhyay and Choudhary 2017) implemented in Geneious version 10 (Geneious 10.0 Manual) (Figure 5.1). Forward and reverse overhang adapter sequences were added to all primers (i.e. Forward primer: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3' and reverse primer: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG 3.

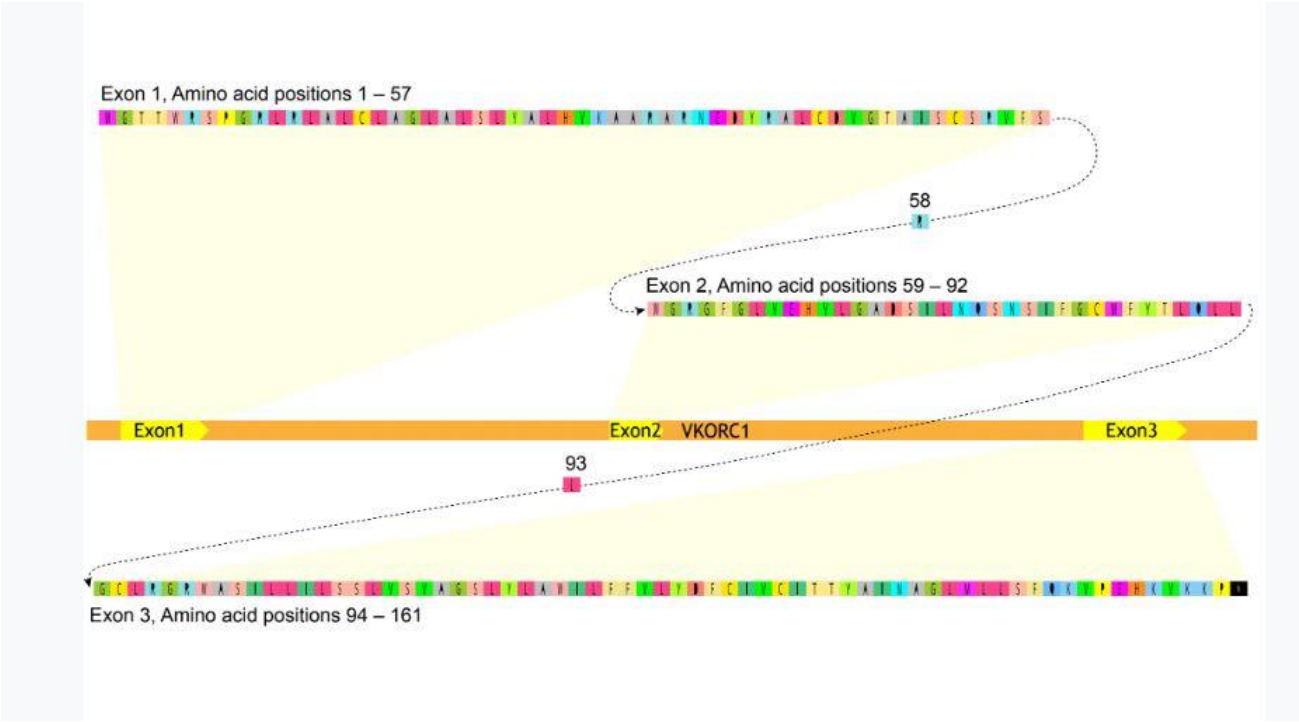
To achieve optimal PCR conditions, these primers were processed in two multiplex PCR reactions of 20 µl for each rat. Each reaction contained 2µl DreamTaq$^{TM}$ Green buffer (10x), 0.2 µl of DreamTaq$^{TM}$ DNA polymerase, 2µL of 2Mm dNTP, 0.4 µl of ABgene$^{TM}$ Magnesium chloride (Thermo Fisher Scientific, Waltham, USA), 1.6 µl of primer mix, 2µl of betaine and 2µL of DNA

template. One multiplex contained primers SuStRat 18F-487R, SuStRat 796F-1217R, SuStRat 1308F-1711R and SuStRat 1864F-2283R, and the other SuStRat 459F-998R, SuStRat 1122F-1535R and SuStRat 1516F-1934R. Alternate primers were used in a primer mixture to avoid formation of any additional unwanted products by adjacent primers. PCR conditions were set at 95$^{o}$C for 15 min followed by 35 cycles of 94˚C for 30s, a gradient temperature of 57-63˚C for 90 s and 72˚C for 90 s; and a final extension period of 10 min at 72$^{o}$C. An Agarose gel was run for each rat sample obtained from each master mix to quantify the quality of the PCR product. For any PCR product that indicated poor quality, PCR was performed again until the required quality was achieved. The PCR products obtained from both master mixes were combined after gel electrolysis.

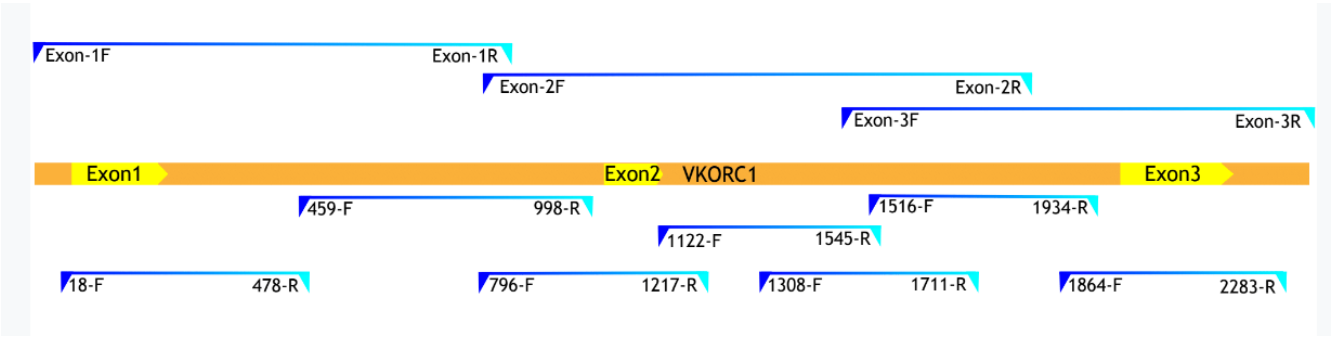
Combined PCR products for each rat sample were purified using AMPure XP beads (Detailed protocol provided in Appendix 6) and the cleaned product was submitted for amplicon sequencing using a Miseq platform (Massey Genome Services, Massey University, Palmerston North) to generate sequencing data for VKORC1 gene for 96 samples. The resulting pair end sequences for each rat sample were trimmed of primers and adapters. The detailed description for each type of sequences obtained along with the margin of error was provided by Massey Genome Services (See Appendix 7). These were paired and mapped to a reference sequence of *R. rattus alexandrinus* (Accession #HM181979) using Geneious v10. Consensus sequence obtained for each rat at threshold 65% was used to identify any single nucleotide polymorphisms in the VKORC1 gene. Data obtained for Amplicon sequencing was compared to published VKORC1 gene sequences the same way as data from the Sanger sequencing above.

**Table 5.3 Primers used for species identification of ship rats as well as Sanger and Amplicon sequencing of the VKORC1 gene in ship rats.**

| Name of forward primer | Sequence | Name of reverse primer | Sequence | Reference |
|---|---|---|---|---|
| EGL4L | CCACCATCAACACCCAAAG | RJ3R | CATGCCTTGACGGCTATGTTG | Robins *et al.* 2008 |
| L15774 | GTAAAACGACGGCCAGTACAT GAATTGGAGGACAACCAGT | H16498 | CCTGAACTAGGAACCAGATG | Shields and Kocher 1991 |
| RrVKORC1-F | TAGCTGTCACGCCTAAGAA | RrVKORC1-R | GCAAATAAGTGCCTGCTGCC | Diaz *et al.* 2010 |
| RrVKORC2-F | ACTTTCGGGAGCTGATTCTC | RrVKORC2-R | CAGACTGATTTTATGTAATG | |
| RrVKORC3-F | CAGGGTTTCTCTGTGTAAC | RrVKORC3-R | CAGACTTGACCAACATAGAA | |
| SuStRat18F | CCTGTGTCTGGGCTGTACTG | SuStRat487R | ATTCGCTATACTGCCCTGCC | This study |
| SuStRat495F | GGCAGGGCAGTATAGCGAAT | SuStRat998R | TAAGTGCAGTCAGCCTGGTG | |
| SuStRat796F | ATCCAGTCGACAGGTTTGGC | SuStRat1217R | GCTGTCAACTGTCTGGGTCA | |
| SuStRat1122F | CTGTTGCTAGGTGAGTGGCT | SuStRat1535R | GGCAGAGGCAGGCAAATTTC | |
| SuStRat1308F | AGGGCCCTCAAAATTTCCCA | SuStRat1711R | GGTTGTGTACAGTCCCAGCA | |
| SuStRat1516F | GAAATTTGCCTGCCTCTGCC | SuStRat1934R | GTAACGCCTCCCCAATGACA | |
| SuStRat1864F | AGGCCCATCAGATCCTGTCT | SuStRat2283R | ATGAGGGGGATAGGGCCTTT | |




a.




b.

**Figure 5.1 The VKORC1 gene in *Rattus rattus* indicating a. position of three exons b. primers used for Sanger sequencing and primers designed for amplicon sequencing.**

## *5.3 Results*

*Species Identification*

A segment of mtDNA Control Region of 96 rats was screened to identify species of the rodent. Of 96 rat samples examined, three were identified as Norway rats as they were haplotypes identical to a published sequence (Accession #EF186346) for this species reported in the Society Islands in French Polynesia (Robins *et al.* 2008). The remaining 93 rat samples were assigned to *Rattus rattus* (Table 5.4) and comprised three haplotypes. One haplotype was found in 90 (96.8%) of sampled individuals and has been previously reported (e.g. Accession #KR559035, Big South Cape Island, New Zealand) (Robins *et al.* 2016). The two other haplotypes came from ship rats captured in the Akatarawa forest and were found to be new.

**Table 5.4 Haplotype diversity in ship rat populations collected from different sites in Wellington and Palmerston North.**

| Sites | Haplotype 1 *Rattus rattus* (KR559035) | Haplotype 2 (New) | Haplotype 3 (New) |
|---|---|---|---|
| Akatarawa Forest | 33 | 2 | 1 |
| Otari Wilton's Bush | 2 | | |
| Porirua Scenic Reserve | 1 | | |
| East harbour regional park | 5 | | |
| Hayward scenic reserve | 1 | | |
| Wainuiomata mainland island | 7 | | |
| Bothamley park | 16 | | |
| Turitea reserve | 14 | | |
| Belmont regional park | 7 | | |
| Hunterville | 4 | | |
| TOTAL | 90 | 2 | 1 |

*VKORC1 Sanger sequencing*

Unambiguous sequences were obtained from 84 ship rats and these included evidences of several single-nucleotide polymorphisms compared to reference VKORC1 sequences. Many of these were non-synonymous and so can be inferred as resulting in amino acid replacements (Table 5.5).

When compared to Norway rat sequences from the NCBI database, 97.9% of the ship rats differed from Norway rats by amino acid substitution at nucleotide position 268 (exon 2) leading to mutation Ile90Leu that has been reported earlier (Cowan *et al.* 2017, Garg *et al.* 2017). Three rats including two from Otari Bush and another from Huntersville were found to be Norway rats and excluded from the study. Synonymous amino acid mutations were recorded at positions Ala14, Tyr25 and Ala26 where mutations have been reported earlier in New Zealand. However, the resulting amino acid substitution differed with Ala14Gly, Tyr25Ser

and synonymous mutation leading to no change in amino acid at Ala26Ala in present study compared to Ala14Val, Tyr25Phe and Ala26Val (Cowan *et al.* 2017).

However, it was notable that most of the VKORC1 sequences had minor ambiguities consistent with heterozygous individuals. Only 15 rats in the sample had unambiguous 'homozygous' sequences. In addition, many of the sequences appeared to contain numerous SNPs suggesting a higher than expected level of genetic variation (Table 5.5). To help resolve uncertainty about the precision of these results, an alternative amplicon sequencing and mapping approach was used to explore this apparent variation. Using the MiSeq NGS platform it was possible to generate short-read sequences spanning ~500 bp representing single sequence variants.

**Table 5.5 Single nucleotide polymorphisms in the VKORC1 gene leading to change in synonymous and non-synonymous mutations in 84 *Rattus rattus* individuals captured from different sites of known brodifacoum history. The data were obtained from Sanger sequencing and has been provided with reference to nucleotide substitution site numbering in *Rattus norvegicus* wild-type sequence (Accession #AY423047).**

| EXON | Postion in comparison to *Rattus norvegicus* GenBank - AY423047 | Codon (AY423047) | Codon Mutation | Amino acid change | No. of rats with mutation | Homozygous for presence | Heterozygous for presence |
|---|---|---|---|---|---|---|---|
| 1 | 10 | ACC | TCC | **Thr4Ser** | 19 | 0 | 19 |
| | 36 | CGG | CGA | Arg12Arg | 84 | 84 | 0 |
| | 41 | GCA | GGA | **Ala14Gly** | 17 | 0 | 17 |
| | 53 | GCT | GAT | **Ala18Asp** | 35 | 0 | 35 |
| | 56 | GGC | GCC | **Gly19Ala** | 15 | 0 | 15 |
| | 59 | CTA | CAA | **Leu20Gln** | 13 | 0 | 13 |
| | 74 | TAC | TCC | **Tyr25Ser** | 26 | 0 | 26 |
| | 78 | GCA | GCG | Ala26Ala | 8 | 0 | 8 |
| | 78 | GCA | GCC | Ala26Ala | 1 | 0 | 1 |
| | 83 | CAC | CCC | **His28Pro** | 17 | 0 | 17 |
| | 90 | AAG | AAG | Lys30Lys | 0 | 0 | 0 |
| | 123 | GCG | GCA | Ala41Ala | 16 | 3 | 13 |
| | 138 | GGC | GGT | Gly46Gly | 18 | 0 | 18 |
| | 139 | ACG | GCG | **Thr47Ala** | 16 | 0 | 16 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 150 | AGC | AGA | **Ser52Arg** | 2 | 0 | 2 |
| | 165 | TTC | TTA | **Phe57Leu** | 3 | 0 | 3 |
| | 168 | TCC | TCA | **Ser58Ser** | 1 | 0 | 1 |
| 2 | 181 | CGG | AGG | Arg61Arg | 7 | 0 | 7 |
| | 186 | GGC | GGA | Gly62Gly | 9 | 0 | 9 |
| | 193 | CTG | TTG | Leu65Leu | 1 | 0 | 1 |
| | 196 | GTG | ATG | **Val67Met** | 6 | 0 | 6 |
| | 199 | GAG | CAG | **Glu67Gln** | 12 | 0 | 12 |
| | 204 | CAT | CAC | His68His | 9 | 0 | 9 |
| | 206 | GTG | GAG | **Val70Glu** | 5 | 0 | 5 |
| | 211 | GGA | AGT | **Gly72Ser** | 34 | 0 | 34 |
| | 214 | GCT | CCT | **Ala72Pro** | 11 | 0 | 11 |
| | 217 | GAC | AAC | **Asp73Asn** | 34 | 0 | 34 |
| | 220 | AGC | GGC | **Ser74Gly** | 34 | 0 | 34 |
| | 227 | CTC | CAC | **Leu76His** | 27 | 0 | 27 |
| | 242 | AGC | ACC | **Ser81Thr** | 36 | 0 | 36 |
| | 246 | ATA | ATC | Ile82Ile | 5 | 0 | 5 |
| | 268 | ATA | TTA | **Ile90Leu** | 84 | 84 | 0 |
| | 275 | CTG | CGG | **Leu92Arg** | 3 | 0 | 3 |
| | 280 | TTA | CTA | Leu94Leu | 84 | 84 | 0 |
| | 282 | CTA | CTC | Leu94Leu | 1 | 0 | 1 |
| 3 | 317 | CTG | CWG | **Leu106Gln** | 2 | 0 | 2 |
| | 333 | CTG | CTR | **Leu111Leu** | 1 | 0 | 1 |
| | 411 | ACC | ACT | Thr137Thr | 84 | 84 | 0 |
| | 429 | GCG | GCA | Ala143Ala | 84 | 84 | 0 |

*Amplicon Sequencing*

More than 500 trimmed and cleaned sequence reads were obtained from most rat samples using the MiSeq platform (Massey Genome Service) (Figure 5.2). The quality of results was good with low margin of error (See Appendix 7). Short reads were mapped to reference VKORC1 sequence and following individual curation from each sample, consensus sequences were aligned. Nucleotide variation across the whole gene as well as within the exons was studied and SNPs were identified by comparison to published data and amino acid translation.

Three quarters (76%) of the ship rat individuals were found to be homozygous with 22 being identified as heterozygous. Three rats were found to be Norway rats with non-synonymous substitution at Ile90Leu and were excluded from further consideration, the same as for Sanger sequencing. With respect to exons, 2 nucleotide variants of VKORC1 gene were identified on exon 1 and 3 on exon 2 and 2 on exon 3 when compared to VKORC1 gene in Norway rat #AY423047 (Table 5.6, Figure 5.3). Among the non-synonymous nucleotide

substitutions that were revealed, none corresponded to amino acid replacements previously reported as conferring anticoagulant resistance.

**Table 5.6 Single nucleotide polymorphisms in the VKORC1 gene in 93 *Rattus rattus* individuals captured from sites of known brodifacoum history near Wellington and Palmerston North, New Zealand. The data were obtained from amplicon sequencing and has been provided with reference to nucleotide substitution site numbering in *Rattus norvegicus* wild-type sequence (Accession # AY423047).**

| Exon | Postion in comparison to *Rattus norvegicus* GenBank - AY423047 | Codon (AY423047) | Codon Mutation | Amino acid change | No. of rats with mutation (out of 96) | Homozygous | Heterozygous |
|---|---|---|---|---|---|---|---|
| 1 | 36 | CGG | CGA | Arg12Arg | 93 | 93 | 0 |
| | 123 | GCG | GCA | Ala41Ala | 19 | 4 | 15 |
| 2 | 246 | ATA | ATC | Ile82Ile | 1 | 0 | 1 |
| | 268 | ATA | TTA | Ile90Leu | 93 | 93 | 0 |
| | 280 | TTA | CTA | Leu94Leu | 93 | 93 | 0 |
| | 411 | ACC | ACT | Thr137Thr | 93 | 93 | 0 |
| | 429 | GCG | GCA | Ala143Ala | 93 | 93 | 0 |

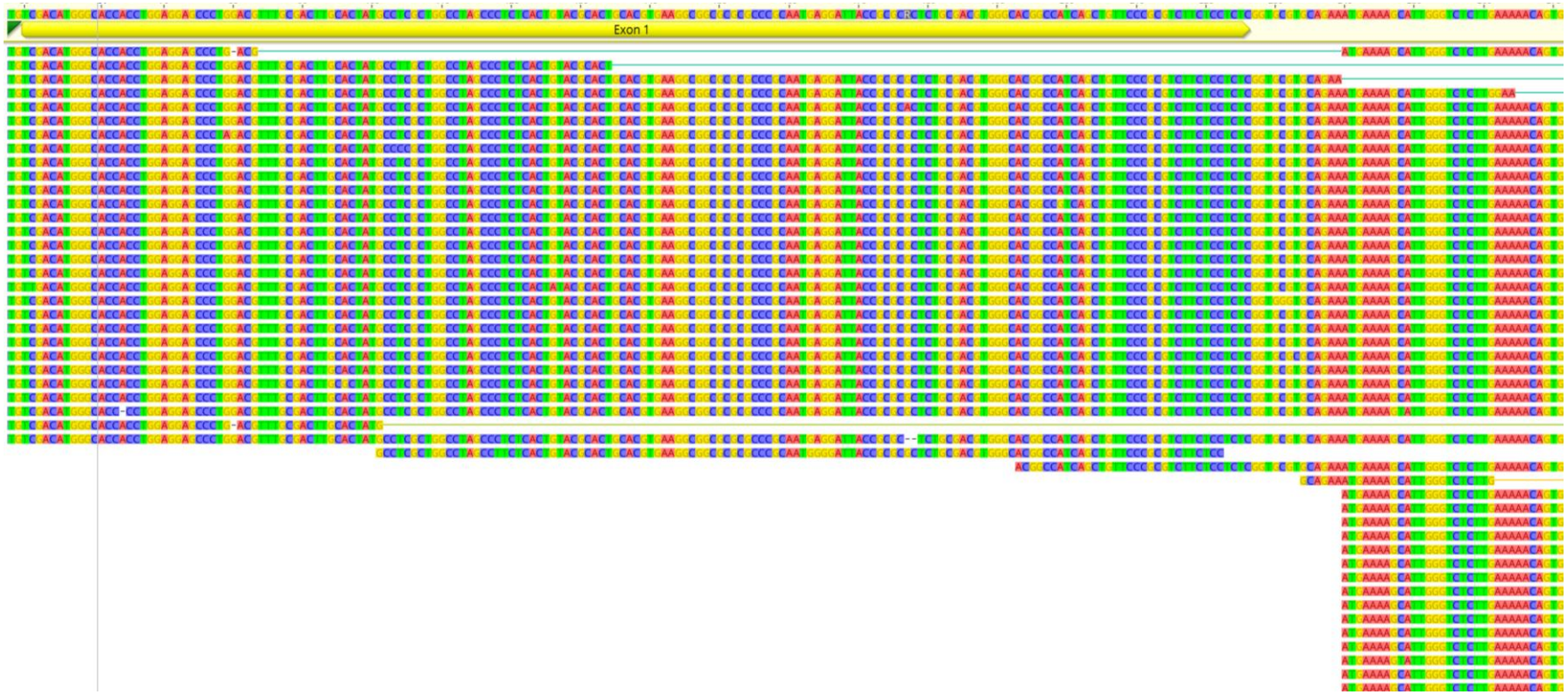



**Figure 5.2 Example of multiple reads obtained for exon 1 of VKORC1 gene for a ship rat (Rr008) captured in Akatarawa forest. The data were generated using Amplicon sequencing of overlapping PCR products obtained from species=specific primers. Unique SNPs in reads are inferred as sequence errors excluded from analysis on the basis of consensus from numerous reads.**

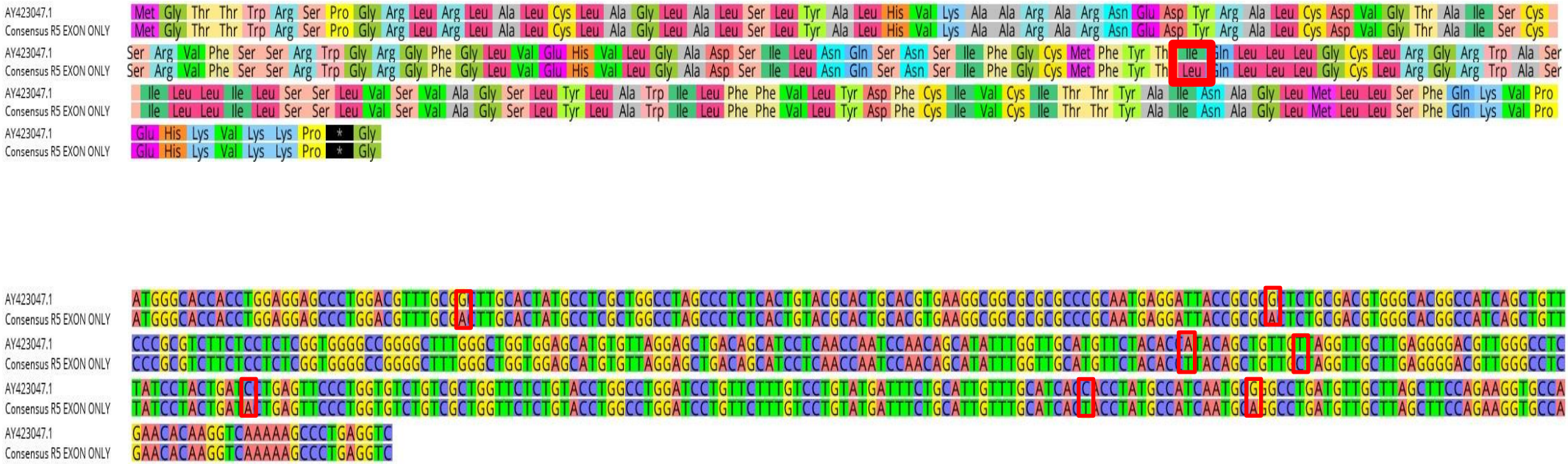



**Figure 5.3 Concatenated exons of VKORC1 gene in one of the ship rats (Rr005) compared to published Norway rat VKORC1 sequence (AY423047) highlighting a. amino acid substitution b. single nucleotide polymorphism**

## *5.4 Discussion*

*Sanger vs Amplicon sequencing*

In the present study, VKORC1 gene sequences of reasonable quality were obtained for 84 ship rats by Sanger sequencing and these provided good overlapping between three PCR products to obtain clean sequences of the entire VKORC1 gene. Apart from 15 individuals the ship rats appeared to be heterozygous and there were consistent ambiguities in heterozygous individuals along with very high levels of genetic variation, leading to suspicion of errors in sequencing (Figure 5.4). Such dilemmas may lead to false positives/negatives as well as inaccurate interpretation of results.

On the other hand, data obtained from Amplicon sequencing provided multiple reads for segments amplified by different pair of primers with optimal overlapping of the VKORC1 gene including introns and exons. All the pairs of primers used for amplicon sequencing were tested individually to obtain good quality products. However, one of the primers (SumaSteRat 1122F, SumaSteRat 1535R) did not amplify in most of the rats, leading to loss of some data for the intron between exon 1 and 2. However, data collected from introns is unlikely to be important for assessement of VKORC1 amino acid variation with respect to anticoagulant resistance. This issue may be easily resolved by repeating the PCR followed by sequencing for required rat samples with the concerned pair of primers, though it may be time-consuming.

Amplicon sequencing was able to identify positive SNPs in exons and well as introns successfully. Unique SNPs obtained in reads were inferred as sequence errors and were excluded from the analysis on the basis of consensus set at 65% threshold from numerous reads. Therefore, the interpretation of sequences was more accurate.

Most existing studies involving the VKORC1 gene in rodents to assess anticoagulant resistance use Sanger sequencing along with evaluation of inferred amino acid substitutions found in the study involving study of VKOR enzyme activity (Tanaka *et al.* 2012, Goulois *et al.* 2016). In some cases, DNA cloning from PCR products has been used to confirm the presence of two different VKORC1 alleles and so infer heterozygotes (Rost *et al.* 2009*,* Goulois *et al.* 2016). Without DNA cloning, Sanger sequencing of PCR amplified VKORC1 form heterozygous individuals will yield ambiguous calls at SNP sites. The fact that most of the studies reporting amino acid substitutions conferring anticoagulant resistance have not made their data publically available via NCBI GenBank or a similar database (Cowan *et al.* 2017, Goulois *et al.* 2016, Tanaka *et al.* 2012) means that it is not possible to analyse or compare results obtained from other studies, and raises the question of why the sequences were not made available.

One possibility is that others have found the quality of Sanger sequences using the popular VKORC1 primers similar unsatisfactory as the present study. An additional problem with many reports is that sequence results are without any verification of results by supporting phenotypic data (Cowan *et al.* 2017), so there is a possibility of getting inaccurate results as in the present study.

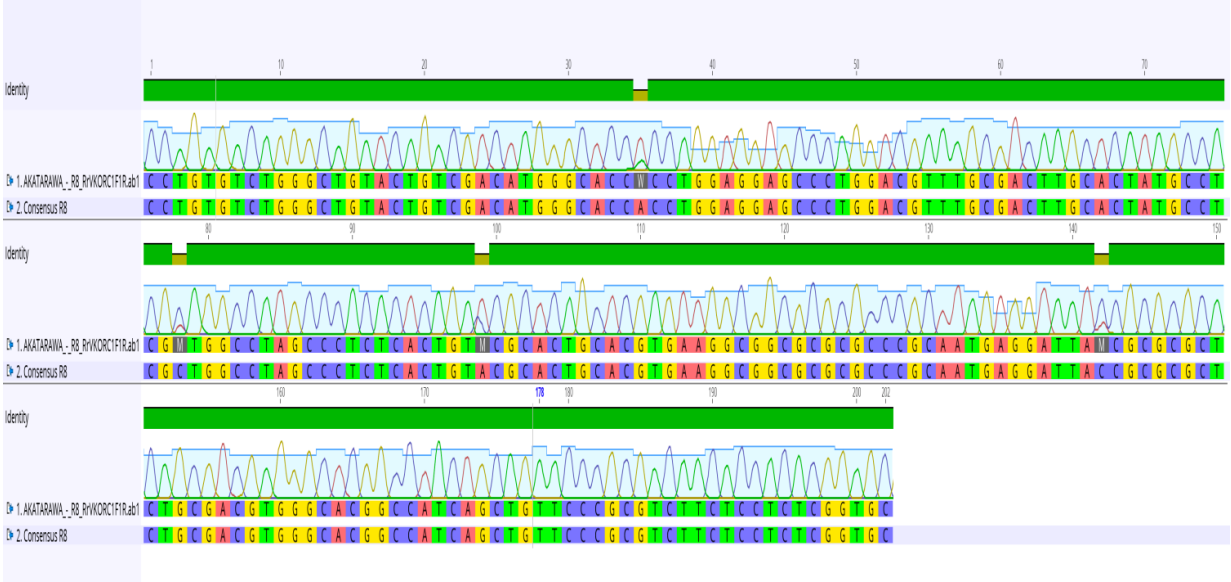


**Figure 5.4 Exon 1 of the VKORC1 gene in one of the ship rats captured from Akatarawa forest (Genetic ID-R8) amplified by Sanger sequencing (Akatarawa_-_R8_RrVKORC1F1R.AB1) as well as Amplicon sequencing (Consensus R8). R8 was found to be heterozygous at position 35, 78, 99 and 142 by Sanger sequencing in the above figure as opposed to the homozygous expression depicted by Amplicon sequencing at same positions.**

*Phenotypic vs genotypic expression*

Ship rats were captured from different areas of known brodifacom use and blood-clotting response (BCR) tests were performed to gather phenotypic expression of susceptibility to brodifacoum (Chapters 2-3). During the present study, the VKORC1 gene was studied in ship rats to access presence and distribution of any SNPs associated with anticoagulant resistance. No BCR data were available for rat samples from Huntersville.

Ship rats collected from Akaratarawa forest, an area where brodifacoum is not known to have been used, provided data from a ranging study performed to calculate the effective dose of brodifacoum. The results implied reduced susceptibility to brodifacoum indicating some level of anticoagulant resistance (see Chapter 2). Many of the ship rats (41% males and 72% females) collected from areas where it is known brodifacoum has been widely used, were

found to be resistant to the administered calculated effective dose of brodifacoum (Chapter 3). Despite this high prevalence of phenotypic expression of resistance shown in these rats, no amino acid replacements at the VKORC1 locus that might confer anticoagulant resistance were revealed in the genetic analysis.

A similar finding has been reported for ship rats in India where rats found to be resistant to bromadiolone (second-generation anticoagulant) through blood-clotting tests followed by lethal feeding trials, but the VKORC1 gene showed no inferred amino acid replacements known to confer resistance to anticoagulants (Garg *et al.* 2017). Ishizuka *et al.* (2006) also investigated the VKORC1 gene in warfarin-resistant ship rats in Tokyo and did not detect any SNP substitutions associated with anticoagulant resistance. On further investigation of these rats it was found that warfarin metabolic activities were significantly higher (nearly twice) in resistant rats compared to susceptible rats after administration of an oral dose of warfarin. It was evident from these results that Cytochrome P450 dependent metabolism of warfarin is a pathway of resistance in these ship rats (Ishizuka *et al.* 2008). More recently analysis of ship rats in Tokyo has confirmed that warfarin resistance does not depend solely on VKORC1 gene mutations but can also involve high anticoagulant metabolism due to high levels of CYP450 isoforms (Takeda *et al.* 2016).

Based on the results obtained in the present study, Amplicon sequencing appears to be a more reliable tool to study the VKORC1 gene than Sanger sequencing. Although no known resistance-conferring amino acid substitutions were identified in ship rats collected from the Wellington region and Palmerston North, I recommend investigating other pathways of resistance given that BCR tests indicate resistance to brodifacoum. It is revealing that two closely related rodent species appear to have quite distinct mechanisms of resistance. Perfused liver samples were collected from some of the ship rats, including both susceptible as well as resistant ship rats as indicated by the BCR tests reported in Chapter 3. These samples have been stored at -80$^{o}$C and can be used to study in-vitro analysis of the VKOR enzyme activity as well as CYP450 enzyme activity. Reduced VKOR activity and increased CYP450 activity are both potential other known pathways of resistance in ship rats (Lasseur *et al.* 2005, Ishizuka *et al.* 2007). Similarly, equivalent data can now be collected for Norway rats in New Zealand to confirm that the local population expresses the same mechanism of resistance as conspecifics in Europe, as it is possible that separate populations evolve novel resistance strategies.

# CHAPTER 6

## Genetic analysis for determining distribution and frequency of VKORC1 mutations conferring resistance against brodifacoum in *Mus musculus*

## *6.1 Introduction*

Anticoagulants are the most widely used rodenticides to manage wild rats (*R. Norvegicus, R. rattus*) and mice (*Mus musculus*). In New Zealand, anticoagulants are used in both rodent control and eradication programmes on offshore islands and mainland islands (Choquenot *et al.* 1990, Veitch *et al.* 2011). Although, anticoagulants have proven to be very effective to date, there are major concerns for long-term use of anticoagulants like brodifacoum, which are persistent in the environment as well as causing non-target poisoning (Taylor and Thomas 1993). Another major issue for the continued use of anticoagulant toxins is the emergence of resistance. Resistance has been formally defined as a heritable genetic trait that enables a resistant individual to survive a dose of an anticoagulant poison that would kill 99% of a susceptible population (Bailey *et al.* 2005).

The basis of genetic resistance has been well established in rats and house mice in Europe and other parts of the world. The development of anticoagulant resistance has been associated with single nucleotide polymorphism (SNP) in the Vitamin K Epoxide Reductase Enzyme Complex 1 (VKORC1) gene in rodents, as in other mammals including humans. Early investigation of warfarin resistance in house mice indicated that the VKORC1 gene was present on chromosome 7 (Wallace and MacSwiney 1976). House mice are also known to have some degree of natural tolerance to anticoagulants (Buckle and Prescott 2012) resulting in anticoagulant poisons being less effective against house mice in general as compared to Norway or ship rats (Prescott *et al.* 2017).

The first reports of warfarin-resistant house mice came in the 1960s from Europe shortly after failure of warfarin to control mouse infestations was recorded in several different locations in the UK (Dodsworth 1961). Since then, resistance of house mice to first-generation anticoagulant rodenticides (FGARs) has been reported from numerous countries worldwide (Rost *et al.* 2009). Following failure of first-generation anticoagulants, more potent second-generation anticoagulant rodenticides (SGARs) were introduced. In a survey conducted by the European Plant Protection Organisation in 1992, it was found that warfarin-resistant mice had varied degrees of resistance to even second-generation anticoagulants (Myllymaki 1995). Resistance was soon reported to SGARs like bromadiolone and difenacoum from many different countries (Lund 1984, Misenheimer *et al.* 1993, Guidobono *et al.* 2010). After failure of bromadiolone and difacinone, further development led to more potent SGARs (brodifacoum and flocoumafen) being introduced that proved to be effective against anticoagulant-resistant house mice (Rowe and Bradfield 1976, Rowe *et al.* 1985). In Denmark and the UK, some degree of resistance has been reported to even brodifacoum in house mice (Buckle *et al.* 1994, Rowe

and Bradfield 1976). Reduced effectiveness of brodifacoum for mouse control has also been reported in Canada, UK and Denmark (Siddiqi and Blaine 1982, Berny *et al.* 2018).

Numerous studies have documented single nucleotide polymorphisms (SNPs) on the VKORC1 locus leading to amino acid substitutions that are linked to anticoagulant resistance in house mice. In the UK, allele L128S (where the numbering refers to the amino acid position in the protein by reference to GenBank Accession #AY423047) has been identified as conferring resistance to the FGARs warfarin, coumatetralyl and chlorophacinone demonstrated by analysing VKOR enzyme activity (Rowe *et al.* 1981). House mice carrying the Tyr139Cys mutation are fully resistant against FGARs and less potent SGARs like bromadiolone (Prescott *et al.* 2017). These two mutations in the VKORC1 gene along with several associate amino acid changes (Arg12Trp/Ala26Ser/Ala48Thr/Arg61Leu) have been inferred as being responsible for conferring resistance in mice (Kohn and Endepols 2009, Rost *et al.* 2009, Pelz *et al.* 2012). In a recent study of mice across Germany, Switzerland and Azores, 90% of the house mice screened for VKORC1 gene variants were found to be carrying one or more of the above mentioned three mutations (Pelz *et al.* 2012).

**Table 6.1 Known amino acid replacement mutations in exons of the VKORC1 gene thought to confer anticoagulant resistance in house mice. Amino acid replacements are numbered in order through the three VKORC1 exons, with the typical amino acid shown to the left of the number and replacement to the right.**

| Mutation | Country reported from | Reference |
|---|---|---|
| Arg12Trp<br>Ala26Ser<br>Glu37Gly<br>Ala48Thr<br>Arg58Gly<br>Arg61Leu<br>Tyr139Ser | France, Germany, Switzerland, Azores | Rost *et al.* 2009, Pelz *et al.* 2012, Goulois *et al.* 2017 |
| Leu128Ser<br>Tyr139Cys | Ireland, France, Germany, Switzerland, Azores | Rost *et al.* 2009, Pelz *et al.* 2012, Gouois *et al.* 2017, Mooney *et al.* 2018 |
| Trp59Leu<br>Trp59Ser<br>Leu124Gln | Germany | Pelz *et al.* 2012 |
| Ala26Thr<br>Trp59Gly<br>Leu124Met | France | Goulois *et al.* 2017 |

At present, brodifacoum is the most potent and commonly used anticoagulant in New Zealand. Its continuous long-term use over decades in several areas has raised concern. The susceptibility of house mice to anticoagulants in New Zealand varies potency of anticoagulant indicating that wild mice were more susceptible to second-generation anticoagulants than first-generation anticoagulants as is the case for other rodents (Fisher 2005). However, further field assessments for testing the efficiency of anticoagulants in house mice were recommended. In a more recent preliminary study, 30 house mice from Hawke's Bay and Gisborne were genetically analysed for Exon 1 of the VKORC1 mutations that have been reported to be associated with anticoagulant resistance, but none were found (Bradley *et al.* 2017). However, the study did not sequence all three exons of the VKORC1 gene so could potentially have missed relevant mutations.

No other study has been done in New Zealand to assess phenotypic or genotypic expression of anticoagulant resistance in house mice. Here I used house mice collected from the Wellington and Palmerston North areas where brodifacoum is used extensively for rodent control. Phenotypic anticoagulant resistance in individual house mice was established using blood-clotting response (BCR) tests (see Chapter 4), and genetic analysis was undertaken to access the presence and frequency of nucleotide substitutions and inferred amino acid replacements associated with anticoagulant resistance in house mice.

## *Material and methods*

### *Study site and sampling*

House mice were trapped from 10 locations in the vicinity of Wellington and Palmerston North, New Zealand, using single live-catch rat traps (Table 6.2). These trapping areas were selected based on the history of brodifacoum use. Detailed information of brodifacoum use history at each site is provided in Figure 6.2. A total of 33 mice were maintained in captivity and subject to BCR tests (Chapter 4) before being euthanized by cervical dislocation while under general anaesthesia. Tail tips of about 2 cm length were collected and immediately preserved in absolute alcohol for genetic analysis. Additional tail samples were collected from mice caught in kill traps at the same sites and at two additional sites: a site near Hunterville and Bushy Park, a fenced sanctuary near Whanganui.

**Table 6.2 House mice collected from selected sites in the lower North Island, New Zealand.**

| Site | Brodifacoum use history (Years) | No. house mice |
|---|---|---|
| Otari Wilton's Bush | 23 | 20 + 2* |
| Porirua Scenic Reserve | 20 | 1 |
| Khandallah Park | 19 | 2* |
| Belmont Regional Park | 13 | 0 |
| Wainuiomata Mainland Island | 12 | 0 |
| Turitea Reserve | 12 | 2* |
| Bothamley Park | 9 | 7 |
| East Harbour Regional Park | 12 | 3 + 1* |
| Hayward Scenic Reserve | 2 | 1 |
| Akatarwara Forest | 0 | 2 |
| Hunterville | Unknown | 3* |
| Bushy Park | | 16* |

*Only tail samples, no BCR test

*DNA extraction*

Genomic DNA was extracted from the preserved tail segment using a Genomic DNA Purification Kit (dnature Diagnostics and Research Ltd, Gisborne, New Zealand) following the manufacturer's instructions and used to amplify mitochondrial DNA Control Region for species determination and the VKORC1 gene to detect mutations associated with anticoagulant resistance.

*DNA sequencing for species identification*

As juvenile of rats sometimes may be difficult to distinguish from adult house mice, it is possible that some juvenile rats were included in the sample of house mice. To ensure accurate identification of captured house mice, genetic confirmation of species was undertaken using screening of mitochondrial DNA haplotypes. The polymerase chain reaction (PCR) primers L15735 and H00072 (Prager *et al.* 1993, Bradley *et al.* 2017) were employed to target partial Control region (D-loop) of the mtDNA genome. PCR amplification of these mitochondrial regions used 20 µl reactions containing: 2µl DreamTaq™ Green buffer (10x) including 20Mm magnesium chloride, 0.2 µl of 5U/µl 10X DreamTaq™ DNA polymerase, 2 µL of 2mM dNTP, 0.4 µl of 25 mM ABgene™ Magnesium chloride (Thermo Fisher Scientific, Waltham, USA), 0.5 µl of each 1000 µM primer, and 2 µL of DNA template at approximately 10 ng/ml. Polymerase chain reaction (PCR) thermal cycling conditions used were as follows: 95˚C for 2 min 15 s, 38 cycles of 95˚C for 15s, 64˚C for 15s, 72˚C for 1 min, followed by an extension

period of 72˚C for 4 min and an idling temperature of 10˚C for 5 min. The PCR product was preserved at 4$^{o}$C after running gel electrolysis to check quality of the PCR product. PCR products did not require any cleaning and were sent to Macrogen, Korea, for sequencing.

Resulting sequences were edited and aligned in Geneious v10 (Kease *et al.* 2012). Sequences of mtDNA haplotypes were compared with published data available on Genbank: *M. musculus domesticus* Accession #U47495; *M. muculus musculus* #U47504 and *M. musculus castaneus* #U47534 (Prager *et al.* 1996).

*Sanger sequencing*

Amplification of the three exons of VKORC1 gene used the primer pairs: Mus VKORC1-ex1F, Mus VKORC1-ex1R; Mus VKORC1-ex2F, Mus VKORC1-ex2R and Mus VKORC1-ex3F, Mus VKORC1-ex3R previously applied in studies of house mice (Espinosa 2013, Bradley *et al.* 2017) (Table 6.2) PCR conditions used were as follows: 95$^{o}$C for 2 min followed by 28 cycles of 95˚C for 20s, 55˚C for 20s and 72˚C for 25s; and a final extension period of 5 min at 72$^{o}$C PCR products obtained were run through gel electrolysis to verify product quality. PCR products obtained for three exons of the VKORC1 gene amplified using these three set of primers were of poor quality as revealed by weak product bands and smears on electrophoresis gels. Only two PCR products were considered suitable for sequencing but resulted in short poor quality sequences.

Due to poor quality of products obtained from first set of primers, a second set of primers: sVKOR-S1, sVKOR-AS1 and sVKORC1-S2, sVKORC1-AS2 (Goulois *et al.* 2017) was used. PCR amplifications were carried out in 20µl reactions; 2µl DreamTaq$^{TM}$ Green buffer (10x), 0.2 µl of DreamTaq$^{TM}$ DNA polymerase, 2µL of 2Mm dNTP, 2 µl of primer mix and 2µL of DNA template. PCR conditions were set as described by Goulois *et al.* 2017; 94$^{o}$C for 3 min followed by 40 cycles of 94˚C for 20s, 64˚C for 20s and 68˚C for 50s; and a final extension period of 10 min at 68$^{o}$C. PCR products obtained were run through gel electrophoresis to verify product quality and size and stored at 4$^{o}$C until sent for sequencing to Massey Genome Services, Massey University, Manawatu. Resulting sequences were edited and aligned in Geneious v10 (Kease *et al.* 2012). Obtained sequences were compared to published VKORC1 gene sequences in *Mus musculus domesticus* Accession: #GQ905715, and *Mus spretus* #GQ905711. The PCR product obtained using the Goulois *et al.* 2015 primers (sVKOR-S1, sVKOR-AS1 and sVKORC1-S2, sVKORC1-AS2) were of better quality but resulting sequences were ambiguous, a problem also encountered by Bradley *et al.* (2017). Attempts were made to obtain clean sequences by adding 0.4 µl of ABgene$^{TM}$ Magnesium chloride (Thermo Fisher Scientific, Waltham, USA) to

PCR mix. However, clean sequences could not be obtained. Sanger sequencing was therefore excluded from the study.

*Amplicon sequencing*

In order to obtain DNA sequences of consistent quality a novel next-generation amplicon sequencing approach was implemented on the Miseq platform (Ravi *et al.* 2018). This generated 250 bp paired end reads (Fadrosh *et al.* 2014) for a set of overlapping fragments of 450–500 bp generated by PCR. To achieve coverage of the VKORC1 gene including exons and introns, a set of 8 novel overlapping pairs of primers was designed for use in multiplex reactions using the Primer3 tool (Mukhopadhyay and Choudhary 2017) implemented in Geneious version10 (Geneious 10.0 Manual) (Table 6.3, Figure 6.1). Forward and reverse overhang adapter sequences was added to all primers (i.e. Forward primer: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3' and reverse primer: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG 3. To achieve optimal PCR conditions, these primers were processed in two multiplex PCR reactions of 20 µl for each house mouse. Each reaction contained 2µl DreamTaq™ Green buffer (10x), 0.2 µl of DreamTaq™ DNA polymerase, 2µL of 2Mm dNTP, 0.4 µl of ABgene™ Magnesium chloride (Thermo Fisher Scientific, Waltham, USA), 1.6 µl of primer mix, 2µl of betaine and 2µL of DNA template. One multiplex contained primer pairs SuStMus42F-473R, Song2F-2R, SuStMus1425F-1844R and SuStMus 1922F-2343R, and the other SuStMus319F-758R, SuStMus1052F-1581R, SuStMus1562F-2017R and Espinosa 3F-3R. Alternate primers were used in a primer mixture to avoid preferential formation of short products by adjacent primers. PCR conditions were set at 95$^{o}$C for 15 min followed by 35 cycles of 94˚C for 30s, a gradient temperature of 57–63˚C for 90s and 72˚C for 90s; and a final extension period of 10 min at 72$^{o}$C. Agarose gel elecrophorsis was used to check quality of PCR product. For any PCR product that indicated poor quality, PCR was performed again until required quality was achieved. PCR products obtained using the two primer sets were combined for each mouse sample and purified using AMPure XP beads (Detailed protocol provided in Appendix 6) prior to submission for amplicon sequencing using the Miseq platform (Illumina, San Diego).

Resulting pair end sequence reads for each house mouse sample were automatically trimmed of primers and adapters and a detailed description of the data with error margin was provided by Massey Genome Services (see Appendix 7). Sequence reads were paired and mapped to a reference sequence of *Mus musculus* (Accession #GQ905714; Song *et al.* 2011) using Geneious v10 with default settings. For each mouse sample a consensus sequence was generated at threshold of 65% and used for comparison and identification of single nucleotide

polymorphisms in VKORC1 gene. Single nucleotide polymorphisms (SNPs) identified were compared to published VKORC1 gene sequences in *Mus musculus domesticus* Accession: #GQ905715, and *Mus spretus* #GQ905711.

**Table 6.3 Primers used for species identification of house mice as well as Sanger and Amplicon sequencing of the VKORC1 gene in house mice.**

| Name of forward primer | Sequence | Name of reverse primer | Sequence | Reference |
|---|---|---|---|---|
| L15735 | CCAATGCCCCTCTTCTCGCT | H00072 | TATAAGGCCAGGACCAAACCT | Prager *et al.* 1993 |
| Mus VKORC1-ex1F | GACCAATCTTCCGGTAGGAG | Mus VKORC1-ex1R | CGACCCCAGACTCCAAAAT | Epinosa *et al.* 2015 |
| Mus VKORC1-ex2F | TGGAGCTTCTTGCTAATCACT | Mus VKORC1-ex2R | GGTGTCAATTGTCTGGGTCA | Epinosa *et al.* 2015 |
| Mus VKORC1-ex3F | GAAGCACCTGCTGTCTGTCA | Mus VKORC1-ex3R | GCCTTCTAGGAACCCACACA | Epinosa *et al.* 2015 |
| sVKOR-S1 | GATTCTTCCCTCCTGTCC | sVKOR-AS1 | AGACCCTGTCTCAAAACCTA | Goulois *et al.* 2017 |
| sVKORC1-S2 | GAAAGCAGAACACTTAGCAGG | sVKORC1-AS2 | AACCAACAGCAGAATGCAGCC | Goulois *et al.* 2017 |
| SuSteMus-42F | ATCCTGTTTACCAGCCCAGC | SuSteMus-473R | CCTTTCTGTGGACACTGCGA | Present study |
| SuSteMus-319F | ATGAAAATTACCGCGCGCTC | SuSteMus-758R | GCTACAGTTCCCTCACCACC | Present study |
| Song2F | GATGATACAAAGCGGGAGGA | Song-2R | TAAGTGGGGTAGGCCAAGTG | Song *et al.* 2011 |
| SuSteMus-1052F | CCAGGACATGCCAGGTCAAT | SuSteMus-1581R | GGTGAGAGAGGGAGAGGGAG | Present study |
| SuSteMus-1425F | AGCAGAACACTTAGCAGGAGG | SuSteMus-1844R | AGCACTCTGATGGCAGACAC | Present study |
| SuSteMus-1562F | CTCCCTCTCCCTCTCTCACC | SuSteMus-2017R | GTGTACAGTCCCAGCACTCC | Present study |
| SuSteMus-1922F | ACCCTAGTTGTCCCGGAGTT | SuSteMus-2343R | GGGAACTCAGCACCAGTAGG | Present study |
| Espinosa-3F | GAAGCACCTGCTGTCTGTCA | Espinosa-3R | GCCTTCTAGGAACCCACACA | Espinosa 2013 |

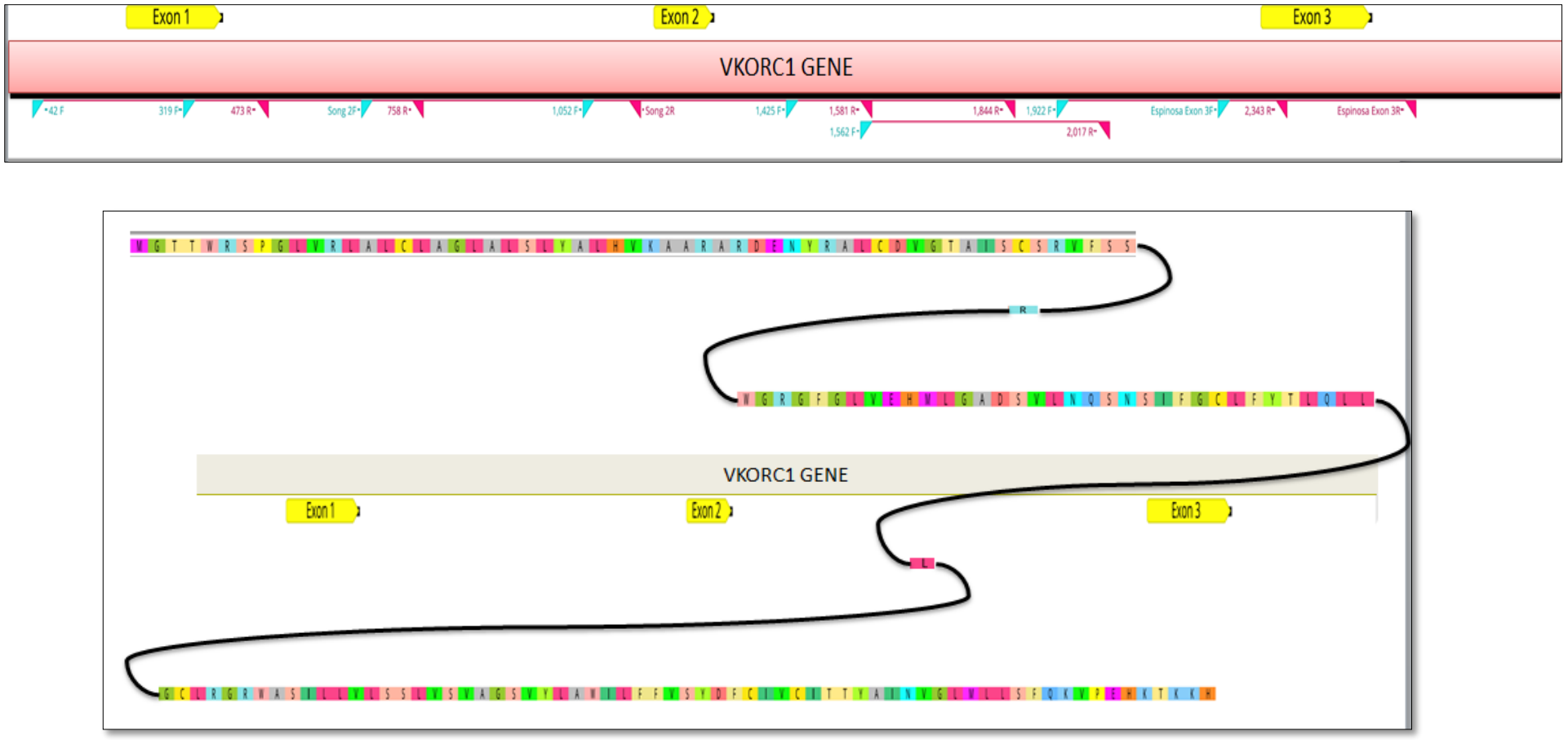



**Figure 6.1 The VKORC1 gene in *Mus musculus domesticus* wild-type sequence (#GQ905714) indicating (above) primers designed for amplicon sequencing and (below) position of exon 1, 2 and 3 on VKORC1 gene.**

## *6.3 Results*

*Species Identification*

All the tail samples were confirmed to be *Mus musculus,* and comprised five haplotypes associated with three sub-species; *M. musculus musculus*, *M. musculus domesticus* and M*. musculus castneus* (Table 6.4). Four of these haplotypes have already been reported earlier and preserved with GenBank #U47504, U47430, U47495 and AF74514. One of the haplotypes found near Huntersville belonging to the sub-species *M. musculus domesticus* was found to be new.

**Table 6.4 Haplotype diversity in house mouse populations collected from different sites in the lower North Island along with sample size (N) from each site.**

| **Site** | **N** | **H 1** ***M. m. musculus*** **(U47504)** | **H2** ***M. m. domesticus*** **(U47430)** | **H 3** ***M. m. domesticus*** **(U47495)** | **H 4** ***M. m. casteneus*** **(AF74514)** | **H 5** ***M. m. domesticus*** **(New)** |
|---|---|---|---|---|---|---|
| Akatarawa | 2 | | | 2 | | |
| Otari Wilton's Bush | 22 | 5 | 1 | 4 | 12 | |
| Porirua Scenic Reserve | 1 | | | | 1 | |
| Khandallah Park | 2 | | | 2 | | |
| East Harbour Regional Park | 4 | | | 4 | | |
| Hayward Scenic Reserve | 1 | | | 1 | | |
| Bothamley Park | 7 | | | 1 | 6 | |
| Turitea Reserve | 2 | | | 2 | | |
| Hunterville | 3 | | 2 | | | 1 |
| Bushy Park | 16 | | 13 | 3 | | |
| Total | 60 | 5 | 16 | 19 | 19 | 1 |

*Amplicon Sequencing*

More than 500 trimmed and cleaned sequence reads were obtained from each of 57 house mouse samples using the MiSeq platform. For three samples, including two from Otari Bush and one from Bothamley Park, no sequence reads were obtained. Examination of the data revealed that two of the primer pairs (1052F-1581R; Song2F, 2R) failed to amplify in most of the individuals. The fragment of the VKORC1 gene that was covered by these primers included exon 2, thus no results were obtained for sequencing for exon 2 in most house mice. As a result, exon 2 was excluded from the study. The quality of results for the remaining mice was good with a low margin of error (See Appendix 7).

There was optimal overlapping of the VKORC1 gene in most house mice including introns and exons for the region covered by successful PCR, demonstrating the efficacy of this approach.

For each mouse short reads were mapped to published VKORC1 sequences in *Mus musculus* (GeneBank Accession #GQ905714) and following individual curation from each sample, consensus sequences were aligned. Consensus sequence obtained for each house mice at threshold 65% was used to identify any single nucleotide polymorphisms across the whole VKORC1 gene as well as exons. Consensus sequences for each house mouse were compared to published VKORC1 gene sequences for *Mus musculus domesticus* (Accession: #GQ905715, and *Mus spretus* #GQ905711) using Geneious v10. Among the 60 specimens, no SNPs were reported on exon 1 while one SNP leading to an amino acid substitution was revealed on exon 3. At Exon 3, a change in nucleotide at position 2223 from Adenine to Guanine led to amino acid substitution Tyrosine / Cysteine, i.e. Tyr139Cys (Figure 6.2, 6.3). This SNP was observed in seven house mice including four from Otari Bush and one each from Bothamley Park, East Harbour Regional Park and Bushy Park. All of these individuals were heterozygous for the mutation.

Tyr139Cys is also a commonly found mutation in Norway rats (Pelz *et al.* 2005, Prescott *et al.* 2017). Another SNP, Tyr139Phe has been reported at same position on VKORC1 gene in ship rats (Tanaka *et al.* 2012). As expected, *Rattus norvegicus* (GeneBank Accession #AY423047) and *Rattus rattus* (Rr005) captured from Akatarawa Forest (Chapter 5), and house mouse sequences for the VKORC1 gene were significantly different (Figure 6.4). Similar differences have been shown by prior studies (Goulois et al 2016, Rost et al 2009).


**Figure 6.2 One of the house mice (Mm013) captured from Otari Bush was heterozygous for Y139C mutation at position on exon 3 of VKORC1 gene with reference to published *Mus musculus domesticus* (#HM027479).**

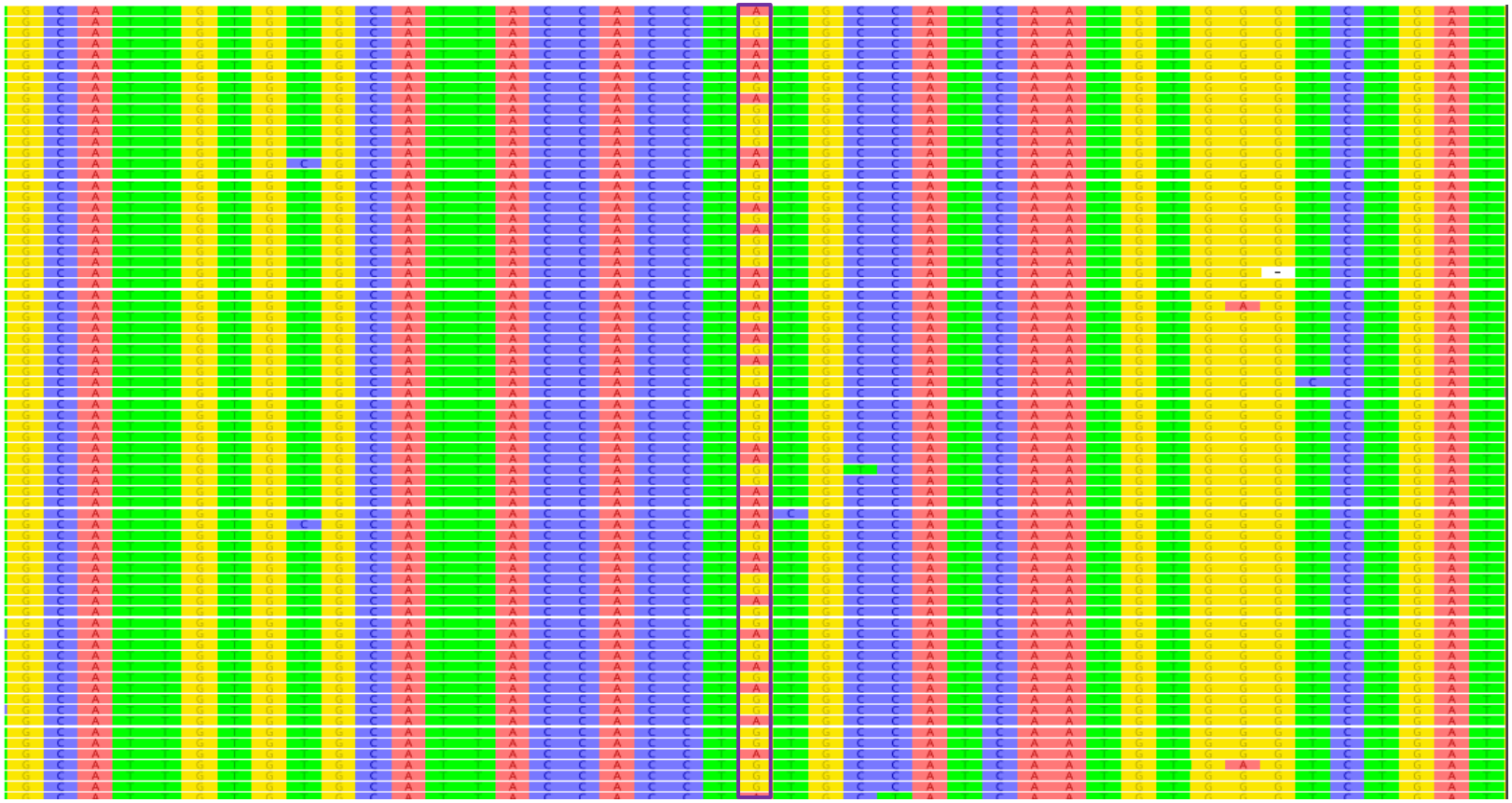


**Figure 6.3 Multiple reads for a part of exon 3 of VKORC1 gene in one house mouse (Mm010) captured in Otari bush was heterozygous at position 2223 (indicated). This nonsynomous nucleotide substitution from Adenine to Guanine resulted in amino acid replacement Tyr139Cys. Any individual was considered heterozygous based on consensus obtained from multiple reads at threshld of 65%. Other rare point mutations are inferred to be read sequencing errors.**

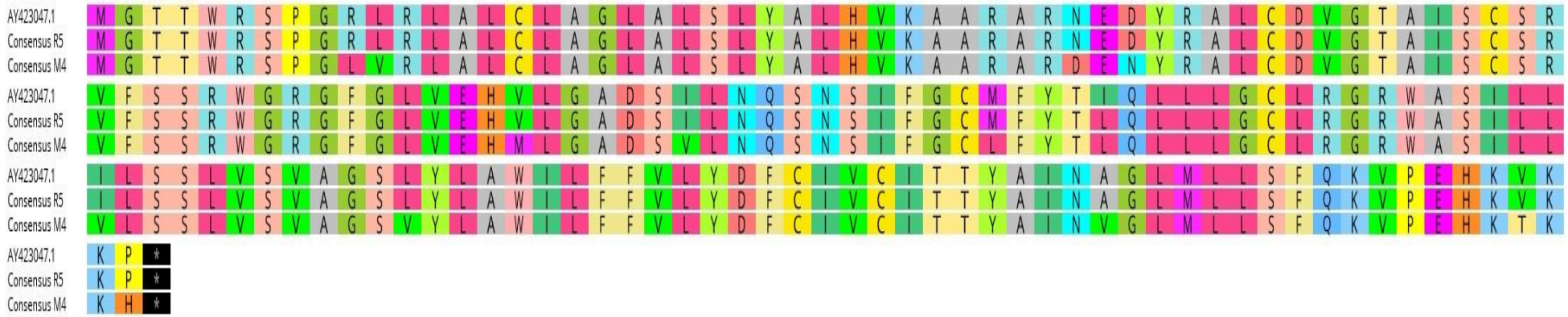



**Figure 6.4 Concatenated exons in the VKORC1 gene in one of the house mice (M4) captured from Otari Bush compared to published Norway rat (#AY423047) and ship rat (R5) VKORC1 gene sequences.**

## *6.4 Discussion*

A nested amplicon sequencing approach was used to study the VKORC1 gene in 60 house mice from southern North Island, New Zealand. Pairs of PCR primers were designed and applied to amplify the entire VKORC1 gene in a series of overlapping sections. Sequencing on the MiSeq platform yielded more than 500 reads per sample with sufficient overall overlap among the sequences amplified with different pairs of primers. However, one pair of primers amplifying the fragment of VKORC1 gene including exon 2 did not produce any PCR product and thus any sequences, leading to a gap in the data. However, this absence most likely reflects a technical error in primer design and use and can be rectified to enhance the utility of this approach.

The results revealed for the first time the presence among New Zealand mice of the Tyr139Cys amino acid substitution in exon 3 of the VKORC1 gene. Tyr139Cys is one of the most commonly found mutations in house mice identified as conferring resistance to anticoagulant poisons in European mice (Goulois *et al.* 2017, Prescott *et al.* 2017). This mutation is known to confer resistance to all FGARs and SGAR, including bromadiolone (Pelz *et al.* 2012). The Tyr139Cys mutation has been studied thoroughly in the European mice, and resistance factors to various anticoagulants have been calculated for Tyr139Cys-carrying house mice using blood-clotting response tests (Prescott *et al.* 2017) (Table 6.5). A resistance factor is the multiple of the calculated effective dose required by the resistant animal to produce the same critical blood-clotting response as a susceptible animal produces to the effective dose (Prescott *et al.* 2007, Endepols *et al.* 2015). Higher resistance factors are correlated with greater practical resistance to anticoagulant compounds and therefore lowered effectiveness of brodifacoum on rodent control. Resistance factors above 5 for a particular anticoagulant may cause loss of efficiency of rodent control using that anticoagulant and may be demonstrated by need of longer treatment by anticoagulant in that area and/or application of higher concentrations of doses of anticoagulant (Greaves *et al.* 1982). Resistance factors above 10 are likely to have a significantly detrimental effect on anticoagulant efficiency (Prescott *et al.* 2017). However, these resistance factors calculated in the UK were for different pest control regimes from those used in New Zealand. Therefore, resistance factors need to be calculated for Tyr139Cys-carrying mice in New Zealand for a more accurate assessment of practical control of these resistant populations. A resistant factor below five is unlikely to cause any practical resistance affecting the management of house mice in the field.

**Table 6.5 Resistance factors to first- and second-generation anticoagulants (FGARs, SGARs) evaluated for male and female *Mus musculus* carrying the Tyr139Cys mutation in the VKORC1 gene.**

| Generation | Anticoagulant | Resistance factor | | Reference |
|---|---|---|---|---|
| | | Males | Females | |
| FGARS | Warfarin | 275 | 534 | RRAC Resistance Guide |
| | Chlorphacinone | 628 | 628 | RRAC Resistance Guide |
| | Coumatetralyl | 32 | 21 | RRAC Resistance Guide |
| | Difacinone | 539 | 539 | RRAC Resistance Guide |
| SGARS | Bromadiolone | 17 | 20 | Prescott *et al.* 2017 |
| | Difenacoum | 1.2 | 2.7 | Prescott *et al.* 2017 |
| | Brodifacoum | 1.7 | 1.9 | Prescott *et al.* 2017 |
| | Flocoumafen | 0.9 | 1.2 | Prescott *et al.* 2017 |
| | Difethialone | 1.5 | 1.5 | Prescott *et al.* 2017 |

Apart from bromadiolone, all other second-generation anticoagulants including brodifacoum are known to be effective against Tyr139Cys-carrying house mice. Although difenacoum has a low resistance factor, many Tyr139Cys-carrying house mice survived field trials and difenacoum is no longer considered to be fully effective in populations carrying this mutation. Mouse populations with a high proportion of individuals homozygous for the Tyr139Cys mutation are generally more difficult to control using difenacoum as compared to populations with a high proportion of heterozygous individuals (Pelz *et al.* 2012). All the individuals carrying the Tyr139Cys mutation in the present study were heterozygous for this allele, and likely to have less tolerance to anticoagulants than homozygotes. However, the population sample size used for the present study was small and only 11.6% of the tested individuals had the concerned mutation. A larger sample size would provide a more accurate indication of the incidence of the Tyr139Cys mutation in mouse populations and enable Hardy-Weinberg testing of allele frequency.

*Phenotypic v/s genotypic data*

Phenotypic expression of susceptibility to brodifacoum was studied in 27 of the individuals used in the present study (Chapter 4). Twenty of the 27 house mice were found experimentally to be resistant to brodifacoum irrespective of documented past brodifacoum use at the locations they were sampled from. Of the seven house mice carrying the Tyr139Cys mutation,

BCR data are available for six mice including four from Otari Bush, one from Bothamley Park and one from East Harbour Regional Park (Table 6.6). There are no phenotypic data available for the house mice from Bushy Park. There was no readily apparent relationship between phenotype and genotype at first glance based on the amplicon sequencing results. Most (20 out of 27) house mice tested for resistance to brodifacoum using blood-clotting response tests were resistant (see Chapter 4) while there were only seven individuals in which Tyr139Cys mutation was found and in heterozygous state. Also, two of the seven individuals that have the mutation at one allele were found to be susceptible in blood-clotting response test. There was therefore no correlation between the genetic and phenotype information. However, the lack of information for exon 2 could mean additional VKORC1 mutations that provide anticoagulant resistance are present in New Zealand mice.

**Table 6.6 Blood-clotting response data for mice found to be heterozygous for the Tyr139Cys mutation in exon 3 of VKORC1 gene. Data were collected after administration of a discriminating dose of 0.46 mg/kg and 0.52 mg/kg of brodifacoum in males and female house mice respectively (see Chapter 4). The INR (International Normalised Ratio) values show the proportional increase in blood-clotting time, with a value > 5 indicating resistance.**

| Mice ID | Site | Male/Female | INR |
|---|---|---|---|
| M1 | Otari Bush | M | 1.9 |
| M2 | Otari Bush | M | 4.3 |
| M3 | Otari Bush | M | 1.2 |
| M4 | Otari Bush | F | 6.2 |
| M5 | Bothamley Park | M | 0.8 |
| M6 | East Harbour Regional Park | M | 7.4 |
| M7 | Bushy Park | Only tail sample collected | |

*Mechanism of resistance*

Anticoagulant resistance in house mice is known to be multi-factorial. VKORC1 gene mutations leading to changes in configuration and/or activity of the VKOR enzyme seems to be one of the pathways. Although the presence of the Tyr139Cys mutation demonstrates the role of this mechanism of resistance in house mice, the consequences of the Tyr139Cys mutation on the functional properties of the VKORC1 gene still need to be assessed. The catalytic ability of the VKOR enzyme against different anticoagulants needs to be studied in New Zealand house mice (Rost et al. 2009, Goulois et al. 2017). From the present study, liver samples from all house

mouse samples were preserved after perfusion and stored at -80$^{o}$C (Chapter 4), so VKOR activity can be studied for these mice using a protocol such as that described by Thijssen (1987).

Another pathway to anticoagulant resistant known in mice is pharmaco-kinetic based resistance. An anticoagulant rodenticide can be ineffective for controlling a house mouse population due to the increased ability of some animals to metabolise and excrete anticoagulant compounds from their bodies (Buckle and Prescott 2012). Sutcliffe et al. (1990) found that the metabolic profile of warfarin 4'-, 6-, 7- and 8-OH was different in susceptible and resistant mice when treated with various cytochrome P450 (CYP450) inducers. This showed that CYP450 played an important role in resistance of house mice. The metabolic pathway can be studied by assessing the activity of CYP450 enzymes in liver tissue. The preserved perfused liver samples (See Chapter 4) from the present study can also be used to analyse CYP450 enzymes as described by Ishizuka et al. (2007).

Since a mutation conferring anticoagulant resistance, Tyr139Cys, has been identified from a small sample of mice from southern North Island of New Zealand, I recommend wider screening for Tyr139Cys including exon 2 and screening of house mice for other potential mutations associated with anticoagulant resistance.

# CHAPTER 7

## General Discussion

## *7.1 Discussion*

A second-generation anticoagulant, brodifacoum, has been used extensively to control rodent populations by private landowners, private pest control contractors and city councils on mainland islands in New Zealand for a few decades. Rodents may evolve resistance to anticoagulants after continuous use of the same or related rodenticides for long periods of time, meaning control will become ineffective unless application rates are substantially increased, creating even greater risks for non-target species.

The overall aim of my PhD was to assess the presence of brodifacoum resistance in ship rat (*Rattus rattus*) and house mouse (*Mus musculus*) populations in the lower North Island of New Zealand. I first conducted blood-clotting response (BCR) tests to evaluate the effective dose of brodifacoum for ship rats (*Rattus rattus*) (Chapter 2). Generally, an effective dose is calculated using susceptible laboratory rats but due to a lack of laboratory ship rats in New Zealand, the effective dose was calculated using wild ship rats captured from Akatarawa forest, an area with no known prior anticoagulant use. This was intended to provide a baseline for comparison with rats from other areas with a history of brodifacoum exposure. However, the effective dose was estimated to be 2.9 mg/kg for males and 3.8 mg/kg for females, which is nearly 5 and 8 times the published $LD_{50}$ value in New Zealand for males and females respectively. This relatively high effective dose indicates highly reduced susceptibility to brodifacoum in the ship rat population in Akatarawa forest. Although no brodifacoum use history is known for Akatarawa forest, brodifacoum is used extensively by the Greater Wellington Regional Council (GWRC) and private landowners in areas adjoining Akatarawa forest. This reduced susceptibility in ship rats may be due to invasion of ship rats exposed to brodifcaoum from areas that adjoin Akatarawa forest.

The calculated effective dose of brodifacoum was then used to assess relative resistance to brodficoum in ship rats captured from areas of high, moderate and low brodifacoum use history using BCR tests (Chapter 3). A discriminating dose, i.e. a multiple of the effective dose, is generally used to test for resistance in wild ship rats. However, the evaluated effective dose was very high compared to effective doses of brodifacoum known for other species of rodent. Also, it was 5-8 times higher than the published $LD_{50}$ doses of brodifacoum for ship rats in New Zealand. Therefore, instead of using a discriminating dose, the $ED_{50}$ was used to test for brodifacoum resistance in captured ship rat individuals and failure to respond to the evaluated $ED_{50}$ was regarded as evidence of resistance. 41% of the tested male rats and 72% of the tested female rats were found to be resistant to the relatively very high $ED_{50}$ of brodifacoum. There was a significant decrease in International Normalised Ratio (INR) value, meaning less

responsiveness to the brodifacoum dose, with increasing number of years of brodifacoum use in an area. Similar blood-clotting response tests were also performed in house mice using the published discriminating dose of brodifacoum; 0.52 mg/kg for males and 0.46 mg/kg for females (Chapter 4). A total of 20 out of 27 assessed house mice were found to be resistant to the administered dose of brodifacoum (INR < 5), with no significant difference in responsiveness in male and female mice. The results showed widespread brodifacoum resistance in house mice irrespective of the number of years of brodifacoum exposure.

To study the pathway of resistance in ship rats and house mice, genetic analysis of the VKORC1 gene was done to assess presence and frequency of any mutations associated with anticoagulant resistance (Chapters 5-6). Sanger sequencing was used initially. For ship rats, the VKORC1 gene sequences obtained had minor ambiguities consistent with heterozygous individuals along with a higher than expected level of genetic variation. For house mice, no clean sequences could be obtained using Sanger sequencing. Therefore, a new next generation sequencing technique, amplicon sequencing, was adopted. No known anticoagulant-resistance-conferring mutations were found in the VKORC1 gene sequences obtained from amplicon sequencing in ship rats. For house mice, a known non-synonymous mutation Tyr139Cys, was found. The Tyr139Cys mutation is known to cause complete resistance to all first-generation anticoagulants as well as less toxic second-generation anticoagulants like bromadiolone. Brodifacoum is known to be still effective against Tyr139Cys-carrying house mouse populations. No relationship was found to exist between phenotypic data (blood-clotting response test) and genotypic data (VKORC1 gene sequences) for individual house mice, and as noted above I found no evidence of VKORC1 mutations in ship rats.

## *7.2 Recommendations for future research*

The effective dose evaluated for the present study was very high, indicating reduced susceptibility in ship rats captured from Akatarawa forest. I recommend evaluating effective dose using ship rats captured from isolated areas with no brodifacoum use, such as offshore islands, so that an accurate effective dose can be estimated and compared to the current effective dose. Ship rats collected from such islands with no past anticoagulant history may show an increased susceptibility to anticoagulants that can be compared to data collected from ship rat populations on the mainland.

Resistance to anticoagulants seems to be widespread in ship rats and house mice from the Wellington Region and Turitea Reserve in Palmerston North as indicated by BCR tests. This resistance suggested by BCR tests is known as technical resistance and may or may not have

any practical importance at present. Therefore, further study involving lethal feeding period (LFP) trials and/or calculation of resistance factors for ship rats and house mice for each site of interest needs to be done. LFP trials measure mortality as a parameter that can be easily related to the practical outcome of the brodifacoum baiting in the field; however, it is questionable in terms of humaneness. On the other hand, the resistance factor is the multiple of the calculated effective dose required by the resistant animal to produce the same critical blood-clotting response as a susceptible animal produces to the effective dose and can be evaluated for any ship rat populations found to be resistant to an effective dose or lethal dose. Higher resistance factors will result in greater practical resistance and therefore, more adverse effects on rodent control using brodifacoum.

Anticoagulant resistance in ship rats and house mice is known to be multi-factorial meaning there may be more than one active pathways of resistance. Based on my present results, technical resistance to brodifacoum was found to be widespread ship rat populations in the Wellington Region and Turitea Reserve in Palmerston North. However, no mutations conferring anticoagulant resistance were found in the VKORC1 gene. On the other hand, Tyr13Cys, a mutation conferring anticoagulant resistance, was found in tested house mice, but no relationship was observed between the phenotypic data and the genotypic data. It seems that there may be other pathways causing resistance to brodifacoum in both ship rats and house mice. I recommend assessing the other known pathways of resistance in ship rats and house mice to develop a better understanding of the cause of resistance to brodifacoum in New Zealand.

## *7.3 Management implications*

Brodifacoum was registered in New Zealand for the first time in 1982 for rodent control but no lethal feeding laboratory trials or field trials were done to record susceptibility of ship rat populations to brodifacoum. The same is true for Norway rats (*R. norvegicus*), kiore (*R. exulans*) and house mouse (*Mus musculus*) populations. This led to a lack of any baseline data to compare to future studies after introduction of anticoagulant poisons in native forest and residential areas. At present it is nearly impossible to find an area with no prior anticoagulant use on mainland New Zealand that may not have possibly been invaded by brodifacoum-exposed ship rats from surrounding areas. Brodifacoum is available over the counter so is easily accessible to the general public to be used on farmlands as well as in residential areas without any documentation of history of use or extent of use. It is very important to record susceptibility of any rodent poison to all rodents before it is used for the first time in an area. This not only provides data for future reference but, also provides important information for

planning effective rodent control programme in the respective area. Also, there is a need to monitor anticoagulant resistance at regular intervals in rats and mice in New Zealand especially, in areas with long-term continual use to plan effective anticoagulant-based rodent control programmes.

Brodifacoum is a highly potent and persistent second-generation anticoagulant that has been used widely in New Zealand on mainland islands. Less persistent anticoagulants like diphacinone are often recommended to be used instead of brodifacoum, especially in areas of long-term brodifacoum use, to reduce risk of non-target poisoning. However, long-term continuous brodifacoum use can lead to emergence of resistance to brodifacoum in exposed rodent populations as suggested by the results obtained in the present study. Rodent populations resistant to one anticoagulant are known to develop resistance to other anticoagulants even if they have never been exposed to the other anticoagulants. Also, it is a known fact that a rodent population resistant to a more potent anticoagulant can not be effectively controlled by a less potent anticoagulant. Therefore, recommendations to use a less potent anticoagulant in the areas of known brodifacoum use may not be very effective for practical control of rodents. A lack of known anticoagulant exposure in a rodent population may also seem like a good reason to use less potent anticoagulants instead of brodifacoum. However, there may be some areas like Akatarawa forest where there has been no known anticoagulant use but the rodent population still appears to have been exposed to brodifacoum from surrounding areas. Therefore, I recommend screening the susceptibility of the target rodent population to the potential anticoagulant before using it in the fields. This may be done using LFP trials or using BCR tests, although BCR testing methodology will need to be established for each anticoagulant to be tested.

Overcoming anticoagulant resistance is critical to the success of rodent control operations, and there is a need to formulate compounds that have efficient rodenticidal potential, are humane, non-persistent, do not bio-accumulate, are safe against non-target species, cost effective and do not develop resistance in rodents. Presently New Zealand relies greatly on anticoagulant poisons. Non-anticoagulants like acute poisons were widely used as vertebrate pesticides before the introduction of anticoagulants in the 1950s. After first-generation anticoagulants like warfarin were introduced, the use of acute poisons reduced considerably, especially for rodent control. After the fall of first-generation anticoagulants due to reports of resistance from different parts of the world, more potent second-generation anticoagulants replaced them. However, non-target poisoning of these highly persistent

anticoagulant poisons like brodifacoum has resulted in renewed interest in less persistent vertebrate pesticides (Eason *et al.* 2015).

To achieve better sustainable pest control programmes in New Zealand, initiatives are being taken to retrieve and retain older alternatives as well as developing novel rodenticides (Eason *et al.* 2015). A three-pronged approach has been identified to overcome problems related to anticoagulant use. This approach includes improving the performance of older non-anticoagulant rodenticides such as acute poisons like zinc phosphide, optimising the performance of first-generation anticoagulants, and identifying other alternatives to anticoagulant rodenticides with similar modes of action (Eason *et al.* 2012). In Europe, cholecalciferol has been added to bait containing first-generation anticoagulants to overcome anticoagulant resistance in rats and mice (Eason *et al.* 2008, Eason *et al.* 2011). Such synergism increases the rodenticidal activity of compounds and reduces the potential for anticoagulant resistance to develop (Zatsepin *et al.* 2006). Cholecalciferol was added to a first-generation anticoagulant, coumatetralyl in Europe to control resistant rat and mice populations. The mixture was attempted in New Zealand (Eason and Ogilvie 2009); however, coumatetralyl was reported to be less palatable and more persistent. As a result, another combination of first-generation anticoagulants, diphacinone and cholecalciferol (D+C), were tested for possum and rodent control in New Zealand (Eason *et al.* 2015). Diphacinone is a highly potent anticoagulant but has low persistence as compared to coumatetralyl. The D+C paste formulation containing 0.005% diphacinone and 0.06% cholecalciferol was found to be effective against possums, ship rats and mice, reducing the target populations by 94%, 94% and 80% respectively in a field trial in Opepe forest (Eason *et al.* 2019).

The chief acute poisons used for vertebrate pests at present in New Zealand are 1080, cholecalciferol and cyanide. Some vertebrate pesticides have been recently registered, namely zinc phosphide and sodium nitrite (Eason *et al.* 2013, Eason *et al.* 2014). A micro-encapsulated paste formulation of zinc phosphide has been developed to be used for possum control. Another micro-encapsulated solid cereal-bait formulation is being developed for used as a rodenticide in the near future (Shapiro *et al.* 2015).

Norbromide is the only known rat-specific acute rodenticide in New Zealand and is considered to be one of the best options for future rodenticides due to its target specificity. However, any efforts to establish it as a viable rodenticide have been disappointing in the past due to its low palatability in rats. Recently, fatty acid ester prodrugs added to the rodenticide

formulation appeared to provide a promising development of norbormide oxin with increased palatability along with efficiency to control rats (Choi *et al.* 2016).

Some other non-lethal rodent control options are also being explored by researchers worldwide. Gonadotrophin-releasing hormone, porcine zona pellucida, Trojan females and many other genetic approaches have recently been suggested for rodent control. The Trojan female technique, which involves identification of naturally occurring mutations that lead to male sterility, has great potential in future pest control. Such mutations have been identified in maternal mitochondrial DNA in house mice. Such techniques when fully developed and used alongside other control methods in integrated pest management may be quite effective (Thresher *et al.* 2014).

We do not have a miraculous single novel rodenticide that can be used as a solution to all rodent control problems in New Zealand or worldwide at present. However, the best can be attained by strategically planning pest control programmes using the best of what we have. Integrated pest management refers to using different methods of pest control systematically to achieve an economically justified sustainable pest-control strategy with minimum risk to environment and humans. Essential components of integrated pest management are surveys for appropriate estimation of the extent and intensity of pest infestation; physical control like kill traps; chemical control like acute or chronic poisons; environmental management like identification and removal of harbourage used by pests; and monitoring to check the effectiveness of control measures used (Endepols *et al.* 2015).

Another important tool that may be used to control resistant rodent populations is rotation of poisons. This means alternating between acute poisons and anticoagulant poisons. Each poison has its benefits and limitations but using them in rotation may help to minimise the limitations, providing more effective rodent control. For example, zinc phosphide and brodifacoum may be used alternatively after regular intervals. Zinc phosphide is an acute poison that is known to be effective against anticoagulant resistance in rodents, but its continuous long-term use may cause bait shyness. On the other hand, brodifacoum would be effective against zinc-phosphide bait-shy populations and can effectively control susceptible rodent populations after effective removal of resistant individuals by Zinc phosphide. Although this idea seems plausible, it still needs to be tested. A major drawback for rotation of poisons may be the cost-effectiveness, as it requires formation of two different baits to be used alternatively along with specially designed bait stations for each.

## *7.4 Conclusion*

At present, only technical resistance to brodifacoum has been reported in ship rats and house mice, and brodifacoum may still be used effectively to control these rodent populations. However, continual use of brodifacoum may encourage further resistance. The only way to overcome resistance is to increase the concentration of brodifacoum in baits, leading to higher risk of non-target poisoning. Effective long-term control of anticoagulant-resistant populations can only be achieved by use of alternative non-anticoagulant rodenticides and strategic use of chemical and non-chemical methods. Also, regular monitoring of rodent populations, using blood-clotting response tests and/or lethal period feeding trials, can be used to measure changing levels of resistance and therefore whether changes in management are necessary. There is also a need to reassess the strategies of predator control in New Zealand keeping in mind anticoagulant resistance. This is essential for the success of Predator Free 2050 campaign in New Zealand.

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# Appendices

## *1. OpenBUGS software (version 3.2.3 rev 1012) code used for statistical analysis of blood-clotting response data in ship rats to model unknown pre-treatment values*

```
#Starting values for MCMC chains
list(mu=0,tau.pre=10,tau.post=10,alpha=0,beta=0)

Model {

##Model priors
    mu.pre ~ dnorm(0,0.001)                    # mean pre-treatement blood-clotting time
        tau.pre ~ dgamma(0.001, 0.001)         # precision in pre-treatment blood-clotting time
        tau.post ~ dgamma(0.001, 0.001)        # precision post-treatment blood-clotting time
        alpha~dnorm(0,0.001)                   #model intercept
beta~dnorm(0,0.001)                            #effect of  years since exposure

#Likelihood
for(i in 1:n.rats) {
        #Pre-treatment blood-clotting time is sample from a normal distribution
        #(constrained to the maximum reading)
        x.pre[i]~dnorm(mu.pre,tau.pre) I(,max.pre[i])
        #INR is modelled linearly to the number of years (y) since exposure (with intercept alpha)
        inr[i] <- alpha+beta*y[site[i]]

        #Expected post-treatment clotting time is determined by pre-treatment and INR
         x.post.mu[i] <- x.pre[i]*inr[i]
        #Post-treatment blood-clotting time is sample from a normal distribution
         x.post[i]~dnorm(mu.post[i],tau.post)
}

#x.pre.new~dnorm(mu,tau)
```

## *2. Recorded rodenticide use history for last 25 years for sites of study to access anticoagulant sustainability in Wellington and Palmerston North as provided by Greater Wellington Regional Council and Palmerston North City Council.*

| USE OF BRODIFACOUM | SELECTED SITES OF STUDY | BRODIFACOUM HISTORY |
|---|---|---|
| **HIGH BRODIFACOUM USE AREAS** | Otari-Wilton's Bush | • Brodifacoum used for last 23 years since 1993<br>• Bait stations installed in 1997 at 150 m intervals and are serviced every 3 months till now |
| | Porirua Scenic Reserve | • Brodifacoum used for nearly 20 years now<br>• Brodifacoum used for first time in 1996-97 after initial knockdown by cholecalciferol<br>• Brodifacoum used till March 1998 with bait stations serviced every month<br>• No control till 2001.<br>• Brodifacoum use resumed in March 2001 after initial knockdown by cholecalciferol<br>• Brodifacoum serviced every 3 months now since then<br>• Pindone has been used occasionally but not for many years. |
| | Khandallah Park | • Started June 1998, Initial control with Potassium and Sodium Cyanide and cholecalciferol, followed by brodifacoum and pindone in bait stations at possum spacing at regular intervals (4 times a year) since started till now |
| **MODERATE BRODIFACOUM USE AREAS** | Wainui Mainland Island | • Pelifeed bait stations were installed on 150 m*100 m intervals in 2004 and activated in 2005.<br>• Baitstations are serviced with brodifacoum every 5 weeks. Dephacinone has also been used several occasions.<br>• Aerial 1080 operations done in 1999,2005 and 2012.<br>• In response to mast year leading to sharp increase in rat numbers, 1080 cereal pellet bait and cholecalciferol were used in 2014<br>• As per 3-4 August 2017 monitoring report, rats -36% and mice 12% |
| | East harbour regional Park | • Pest control carried out in parts by contractors and volunteers from 1997-2001<br>• Control extended to whole site in 2001 and 2003-04 using leghold and kill traps along with encapsulated cyanide and cholecalciferol<br>• Pelifeed baitstations installed in 2005-06. Additional lines set up in 209-10. Use only brodifacoum (Report |

| | | |
|---|---|---|
| | | GWRC Aug 2017) |
| | Bothamley park | • Brodifacoum used initially in May 2008<br>• Pindone Possum pellets used for 2-4-year period.<br>• Brodifacoum use was resumed and the operation size doubled in size in 2012 with a total of 45 bait stations |
| | Belmont regional Park | • Initial knockdown by cyanide and cholecalciferol in 2004 followed by brodifacoum in bait stations over 340 ha area.<br>• Another 150 ha added in control regime on western side in 2009.<br>• Bromadiolone was used as rodenticide in 2011 in eastern sides but brodifacoum use recommenced throughout site since 2014. |
| | Turitea reserve | • Cyanide used in 1995,1997 and 1999<br>• Bait-stations installed and filed with cholecalciferol in 1999-2000<br>• Initial knockdown by cyanide in 2004 followed by establishment of bait-stations for brodifacoum use<br>• Baitstations are refilled every 4 months |
| **LOW BRODIFACOUM USE AREAS** | Hayward Scenic Reserve | • A small brodifacoum history more than 10 years ago<br>• Pelifeed bait station network installed in 2015<br>• Brodifacoum used since then every 3 months now<br>• A large bait station network bordering the site to south-east to prevent incurtions.<br>• DOC 200 traps installed in 2015-2016 (GWRC REPORT 2015-16). |
| **NO BRODIFACOUM USE AREA** | Akatarawa forest | • Sodium fluoroacetate (1080) used in selected parts of forest in 1995-97<br>• An initial knockdown using cholecalciferol and brodifacoum followed by brodifacoum used in bait stations every 3 months in south eastern parts of forest around Cannon point walkway, Birchville dam and Totara Park<br>• 1080 used in 2007 followed by another drop in 2013 |

## *3. OpenBUGS software (version 3.2.3 rev 1012) code used for statistical analysis of blood-clotting response data in ship rats including sex effects and random site effects.*

```
#Starting values for MCMC chains
list(mu.pre=1, tau.pre=3, tau.post=3, alpha=3, beta.y=0, sd.site=0.1, beta.sex=0, beta.y.sex=0)

Model {
        ##Model priors
        mu.pre ~ dnorm(0,0.001)                 # mean pre-treatement blood-clotting time
        tau.pre ~ dgamma(0.001, 0.001)          # precision in pre-treatment blood-clotting time
        tau.post ~ dgamma(0.001, 0.001)         # precision post-treatment blood-clotting time
        sd.pre <- pow(tau.pre,-0.5)             #standard deviation pre-treatment blood-clotting time

        sd.post <- pow(tau.pre,-0.5)            #standard deviation post-treatment blood-clotting
time
        alpha ~ dnorm(0,0.001)                  #model intercept
        beta.y ~ dnorm(0,0.001)                 #effect of years since exposure
        sd.site ~ dunif(0,2)                    #random effect of site (in standard deviation)
        tau.site <- pow(sd.site,-2)             #precision of random effect of site
        beta.sex ~ dnorm(0,0.001)               #effect of sex
        beta.y.sex ~ dnorm(0,0.001)             #effect of interaction between sex and years since
exposure


        #Likelihood
        #Random effects for each site
        for (i in 1:n.sites) {
                re.site[i] ~ dnorm(0,tau.site)
        }


        for (i in 1:n.individuals) {
                #Pre-treatment blood-clotting time is sample from a normal distribution
                #(constrained to the maximum reading)
                x.pre[i] ~ dnorm(mu.pre,tau.pre) I(,max.pre[i])

                #INR is modelled as a function of site's years since exposure, gender of individual, an
                #interaction between these terms, plus a random effect of site
                INR.mu[i] <-
alpha+beta.y*y[site[i]]+re.site[site[i]]+beta.sex*sex[i]+beta.y.sex*y[site[i]]*sex[i]

                #Predicted post-treatment clotting time is determined by pre-treatment and predicted
INR
                mu.post[i] <- x.pre[i]*INR.mu[i]

                #Post-treatment blood-clotting time is sample from a normal distribution
                x.post[i] ~ dnorm(mu.post[i],tau.post) I(0,max.post[i])

                #Actual INR
                INR[i] <- x.post[i]/x.pre[i]
        }
```

***4. Certificate of preparation of stock solution of brodifacoum dissolved in MPeg (1,2 Propandiol) by Toxicology Laboratory, Lincoln.***

Manaaki Whenua
Landcare Research

**TOXICOLOGY LABORATORY**
Lincoln Region

P.O. Box 69
Lincoln
Ph: +61 3 325 6700
Fax: +61 3 325 2418

## CERTIFICATE OF PREPARATION

| **Product Identification** | | **Cert. No.: P16/18** |
|---|---|---|
| Description: | Toxicological assessment of Brodifacoum | |
| Active ingredient: | Brodifacoum., 2-[3-(4'bromo[1-1'-biphenyl]-4-yl)-1,2,3,4-tetra-hydro-1-napthalenyl]-4-hydroxy-2H-1-benzopyran-2-one | |
| Animal: | Rat | |
| Required dosage: | 0.8mg milligrams per Kilogram body weight (0.8mg-Kg) | |
| Dose volume: | 2000µL per 250gram Rat (8mL per Kilogram of Rat) | |
| Active concentration: | 0.1mg/mL (also known as 0.01% w/v) | |

**Preparation**

| | |
|---|---|
| General precautions: | Wear gloves when handling; avoid contact with skin and eyes. |
| Active ingredient: | Brodifacoum (Lot #SZBC214XV) |
| Weight of Active (mg): | 8.05 |
| Active strength *(if known)*: | 99.4%w/w |
| Final volume, mL: | 80 |

Remarks on Formulation: Eight milligrams (8.05mg) of Brodifacoum was added to a 100mL glass bottle. An 80mL aliquot of MPeg (1,2-Propandiol) was added to make a final weight of 83.2g (specific gravity of 1.04g per mL). This was vortexed for 5-minute and sonicated for a further 5-minute.

**Documented value of concentration:** 0.100mg-mL (or 0.01%w/v)

*(95% confidence level)*

Signature for Preparation
Date: 27 July 2016

Check Signature
for: Pest Control and Wildlife Toxicology
Lincoln Region
27 July 2016

T:\Lincoln\Projects P-T\Toxlab\Solution_prep\2016\CertP16.18.doc 27/07/16

## *5. OpenBUGS software (version 3.2.3 rev 1012) code used for statistical analysis of blood-clotting response data in house mice including sex effects and random site effects.*

```
    list(mu.pre=1, tau.pre=3, tau.post=3, alpha=3, beta.y=0, sd.site=0.1, beta.sex=0, beta.y.sex=0)

Model
{

# priors
mu.pre ~ dnorm(0,0.001)             # mean baseline blood-clotting time
tau.pre ~ dgamma(0.001, 0.001)      # precision in baseline blood-clotting time
tau.post ~ dgamma(0.001, 0.001)     # precision post-treatment blood-clotting time
sd.pre <- pow(tau.pre,-0.5)         #standard deviation baseline blood-clotting time

sd.post <- pow(tau.pre,-0.5)        #standard deviation post-treatment blood-clotting time

alpha ~ dnorm(0,0.001)              #model intercept
beta.y ~ dnorm(0,0.001)             #effect of years since exposure
sd.site ~ dunif(0,2)                #random effect of site (in standard deviation)
tau.site <- pow(sd.site,-2)         #precision of random effect of site
beta.sex ~ dnorm(0,0.001)           #effect of sex
beta.y.sex ~ dnorm(0,0.001)         #effect of interaction between sex and years since exposure


        #Likelihood

        #Random effects for each site
        for (i in 1:n.sites) {
                re.site[i] ~ dnorm(0,tau.site)
        }


        for (i in 1:n.individuals) {
                #Pre-treatment blood-clotting time is sample from a normal distribution
                #(constrained to the maximum reading)
                x.pre[i] ~ dnorm(mu.pre,tau.pre) I(,max.pre[i])

                #INR is modelled as a function of site's years since exposure, gender of individual, an
                #interaction between these terms, plus a random effect of site
                INR.mu[i] <-
alpha+beta.y*y[site[i]]+re.site[site[i]]+beta.sex*sex[i]+beta.y.sex*y[site[i]]*sex[i]

                #Predicted  post-treatment clotting time is determined by pre-treatment and
predicted INR
                mu.post[i] <- x.pre[i]*INR.mu[i]

                #Post-treatment blood-clotting time is sample from a normal distribution
                x.post[i] ~ dnorm(mu.post[i],tau.post) I(0,max.post[i])

                #Actual INR
                INR[i] <- x.post[i]/x.pre[i]
        }
```

## *6. Protocol used for Clean-up of PCR product before sending off for Amplicon sequencing as provided by Massey Genome Services, Massey University, Manawatu.*

Consumables:

| Items | Quantity required | Storage |
|---|---|---|
| 10 Mm Tris Ph 8.5 | 52.5 µl per sample | -15 to -25$^{o}$C |
| AmPure XP beads | 20 µl per sample | 2 to 8$^{o}$C |
| Freshly prepared 80% ethanol | 400 µl per sample | |
| 96-well PCR plate | 1 plate | |
| Microseal 'B' film (optional) | | |
| 96-well MIDI plate (optional) | 1 plate | |

Procedure:

1. Bring the AMPure XP beads to room temperature.
2. Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.
3. Using a multichannel pipette set to 25 µl, transfer the entire Amplicon PCR product from the PCR plate to the MIDI plate. Change tips between samples. NOTE Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.
4. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough depending on the number of samples processing.
5. Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate. Change tips between columns.
6. Gently pipette entire volume up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
7. Incubate at room temperature without shaking for 5 minutes.
8. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
9. With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.

10. With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.

b. Incubate the plate on the magnetic stand for 30 seconds. Carefully remove and discard the supernatant.

11. With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:

    a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    b. Incubate the plate on the magnetic stand for 30 seconds.
    c. Carefully remove and discard the supernatant.
    d. Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

12. With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

13. Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.

14. Gently pipette mix up and down 10 times, changing tips after each column (or seal plate and shake at 1800 rpm for 2 minutes). Make sure that beads are fully re-suspended.

15. Incubate at room temperature for 2 minutes.

16. Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

17. Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

18. If you do not immediately proceed to Index PCR, seal plate with Microseal “B” adhesive seal and store it at -15° to -25°C for up to a week.

## *7. Report generated by by Massey Genome Services, Massey University, Manawatu on Steve Trewick's MGS00189 samples on 11th April 2019*

1. PROJECT DESCRIPTION

   Type of Samples: PCR Amplicon

   Number of Samples: 167 samples

   Type of Sequencing: Custom Amplicon Sequencing

   Library Preparation Method: Custom Amplicon Library Preparation - Second PCR Step

   Libraries Indexed and pooled: Yes

   Number of Runs: 1

   Illumina MiSeq™ Kit: Illumina MiSeq Nano 500 cycle Kit_V1

   Read Length: Nano 2X 250 base PE

   Data Output Provided: Sequence reads provided in fastq format

   Special Client Requirements: None

2. LIBRARY PREPARATION DESCRIPTION

   Libraries were prepared using the following method:

   Custom Amplicon Library Preparation - Second PCR Step

   Date of Library Preparation: 2/04/2019

3. QC REPORT ON LIBRARY PREPARATIONS

   Method of Library QC check

   The library(s) were QC checked using the following method(s):

   - PerkinElmer GX Touch HT Instrument using LabChip DNA High Sensitivity Assay
   - Life Technologies Qubit Fluorometer_Version 3.0 using:

     - Quant-iT dsDNA HS Assay for quantification

4. RUN SUMMARY REPORT

| Level | Yield Total (G) | Projected Total Yield (G) | Aligned (%) | Error Rate (%) | Intensity Cycle 1 | % >= Q30 |
|---|---|---|---|---|---|---|
| Read 1 | 0.19 | 0.19 | 54.23 | 1.76 | 219 | 97.65 |
| Read 2 | 0.01 | 0.01 | 0 | NaN | 507 | 38.23 |
| Read 3 | 0.01 | 0.01 | 0 | NaN | 156 | 93.65 |
| Read 4 | 0.19 | 0.19 | 53.72 | 1.74 | 151 | 94.91 |
| Non-Indexed Total | 0.38 | 0.38 | 53.97 | 1.75 | 185 | 96.28 |
| Total | 0.39 | 0.39 | 53.97 | 1.75 | 258 | 95.45 |

**Read 1**

| Lane | Tiles | Density (K/mm2) | Clusters PF (%) | Legacy Phasing/Prephasing rate | Phasing slope/offset | Prephasing slope/offset | Reads (M) | Reads PF (M) | % >= Q30 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 562 ± 3 | 96.85 ± 0.20 | 0.043 / 0.057 | NaN / NaN | NaN / NaN | 0.78 | 0.76 | 97.65 |

| Lane | Yield(G) | Cycles Err Rated | Aligned (%) | Error Rate (%) | Error Rate 35 cycle (%) | Error Rate 75 cycle (%) | Error Rate 100 cycle (%) | Intensity Cycle 1 |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.19 | 250 | 54.23 ± 0.08 | 1.76 ± 0.01 | 0.10 ± 0.00 | 0.13 ± 0.00 | 0.18 ± 0.00 | 219 ± 0 |

**Read 2**

| Lane | Tiles | Density (K/mm2) | Clusters PF (%) | Legacy Phasing/Prephasing rate | Phasing slope/offset | Prephasing slope/offset | Reads (M) | Reads PF (M) | % >= Q30 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 562 ± 3 | 96.85 ± 0.20 | 0.000 / 0.000 | NaN / NaN | NaN / NaN | 0.78 | 0.76 | 38.23 |

| Lane | Yield(G) | Cycles Err Rated | Aligned (%) | Error Rate (%) | Error Rate 35 cycle (%) | Error Rate 75 cycle (%) | Error Rate 100 cycle (%) | Intensity Cycle 1 |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.01 | 0 | NaN ± NaN | NaN ± NaN | NaN ± NaN | NaN ± NaN | NaN ± NaN | 507 ± 4 |

**Read 3**

| Lane | Tiles | Density (K/mm2) | Clusters PF (%) | Legacy Phasing/Prephasing rate | Phasing slope/offset | Prephasing slope/offset | Reads (M) | Reads PF (M) | % >= Q30 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 562 ± 3 | 96.85 ± 0.20 | 0.000 / 0.000 | NaN / NaN | NaN / NaN | 0.78 | 0.76 | 93.65 |
| **Lane** | **Yield(G)** | **Cycles Err Rated** | **Aligned (%)** | **Error Rate (%)** | **Error Rate 35 cycle (%)** | **Error Rate 75 cycle (%)** | **Error Rate 100 cycle (%)** | **Intensity Cycle 1** | |
| 1 | 0.01 | 0 | NaN ± NaN | NaN ± NaN | NaN ± NaN | NaN ± NaN | NaN ± NaN | 156 ± 1 | |

**Read 4**

| Lane | Tiles | Density (K/mm2) | Clusters PF (%) | Legacy Phasing/Prephasing rate | Phasing slope/offset | Prephasing slope/offset | Reads (M) | Reads PF (M) | % >= Q30 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 562 ± 3 | 96.85 ± 0.20 | 0.114 / 0.028 | NaN / NaN | NaN / NaN | 0.78 | 0.76 | 94.91 |
| **Lane** | **Yield(G)** | **Cycles Err Rated** | **Aligned (%)** | **Error Rate (%)** | **Error Rate 35 cycle (%)** | **Error Rate 75 cycle (%)** | **Error Rate 100 cycle (%)** | **Intensity Cycle 1** | |
| 1 | 0.19 | 250 | 53.72 ± 0.15 | 1.74 ± 0.01 | 0.10 ± 0.01 | 0.16 ± 0.00 | 0.21 ± 0.00 | 151 ± 2 | |

## 5. DATA QC AND PREPROCESSING

With the latest version of the Illumina software, the sequences now come off the machine as the sample name, with suffixes as I shall explain below, and in a compressed format. I now run the sequence data through a standard pipeline process to check the data from a quality control point of view. Consequently, the output we are providing contains the raw sequences in a folder, as well as a folder of partially processed sequences, a folder with processed and quality-trimmed sequences and finally the output from 3 different QC programs.

### *a. Sequence file naming convention*

For example, one of your sequence files is called: M1_S97_L001_R1_001.fastq. This is named as per the following naming convention:

{sample name}_{sample number}_L001_{read number}_001.fastq

Some of the other parts of the name are due to a similar naming convention used on the Illumina HiSeq machine. The 'Undetermined' file contains those sequences that do not map to one of the input indices that was used, and has a sample number of 0, hence the file name is Undetermined_S0_L001_R1_001.fastq.

*b. Contents of the uploaded folder*

At the root is the folder name above, and all subsequent data is found in 4 folders, 'fQsequences', 'processed', 'processed_trimmed' and 'QCdata'. In addition, there are two other files supplied, a log file that results from the processing, plus a Linux/Mac executable file that shows the md5 calculations to check the data has been copied properly from source to destination. In order to execute it, open a terminal; navigate to the run's root folder and type: ./checksum.sh

*c. fQsequences*

These are the "raw" sequence files that result from the MiSeq. As before, the 'Undetermined' file contains those sequences that do not map to one of the input indices that were used, or to the PhiX reference sample that is loaded on every run.

*d. Processed*

For each of the sequences in fQsequences folder, they are then mapped against the PhiX genome using Bowtie2, any hitting PhiX sequences are removed from the resultant SAM file, and the fastq file is reconstructed using the SamToFastq.jar program from the Picard suite (http://picard.sourceforge.net/). This is then used as an input to an adapter removal procedure (through the "fastq-mcf" program from the ea-utils suite of tools (http://code.google.com/p/ea-utils/; version 1.1.2-621)), and the resultant file has the prefix 'processed_'. As you can see, these files are smaller than the input sequences from the fQsequences folder, though not by much.

*e. Processed_trimmed:*

For each of the sequences in the 'processed' folder, they are now also trimmed to their longest contiguous segment for which quality scores are less than a quality cutoff, set at 0.01, using the dynamictrim application from the SolexaQA++ software (http://solexaqa.sourceforge.net/). The resulting output from this process a file that has the suffix '.trimmed' at the end of its name. In order to maintain the order of the reads within the file, if the sequence has been trimmed to a length of 0 bases, a single base is left in the file in its place. Depending on the application, it might be necessary to remove these short reads from these files, which can be done with the lengthsort application from the SolexaQA++ software (http://solexaqa.sourceforge.net/).

6. QCDATA

For a QC analysis, I am now running three processes, SolexaQA++, fastQC and fastQscreen on the original sequences in the fQsequences folder:

*SolexaQA++:*

Website: http://solexaqa.sourceforge.net/

As each sequence generates 7 files for each read direction, there are 14 files per sample (including the 'Undetermined' sequences). For a given read a default number of 10000 sequences are taken per tile, and analysed with the programme. Four kinds of outputs are generated, either as a PDF or a png file, along with the appropriate text file that made it. Data is reported in terms of error probabilities, rather than the NGS equivalent of Phred scores that some other tools use.

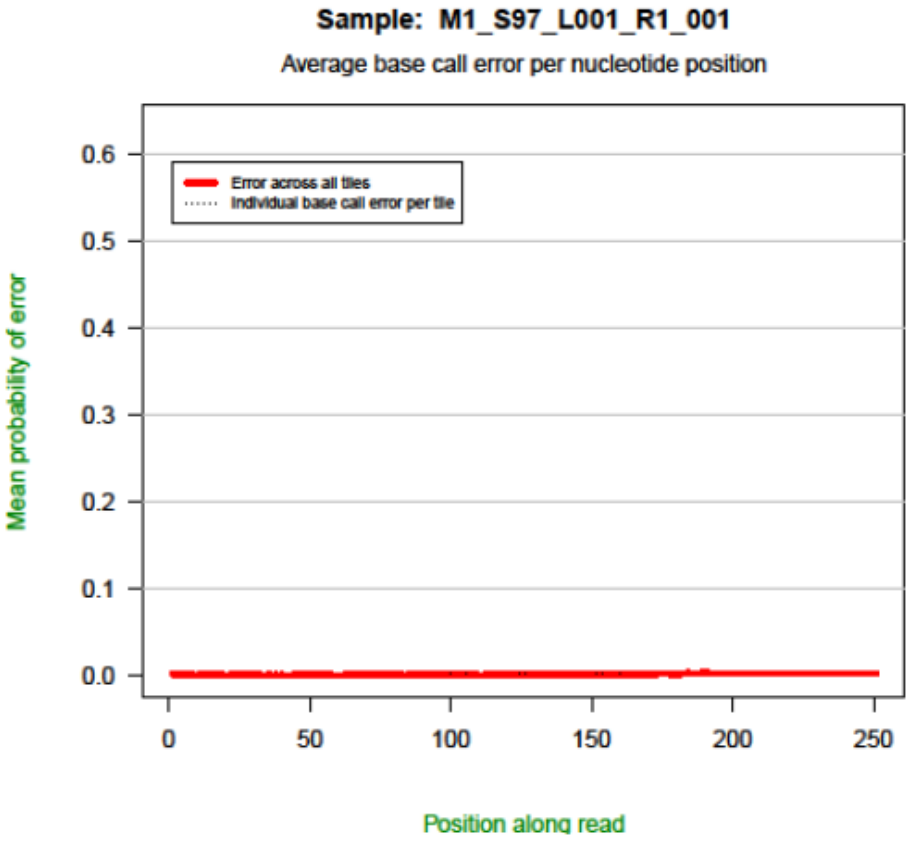
**Note for this project:** due to the large amount of samples in your run, the SolexaQA++ folder has been organized into 4 subfolders (3 folders containing 50 samples each + 1 folder containing 18 samples). Each subfolder contains the graphs described below.

*Heatmap:*

This shows the quality on a cycle by cycle basis for each of the 2 tiles in the MiSeq run as a heatmap using a black body radiation scale. They are virtual tiles that are captured by the camera for analysis. Good data is white, bad data goes through orange to black. As an additional check, the tile fraction (calculated as the number of reads in each tile over the total number of reads) is shown next to the tile name. An example from your dataset is shown below:

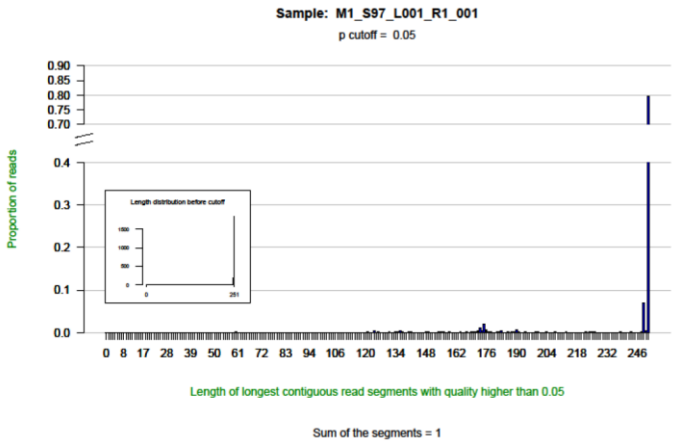

*Quality:*

The same data as above is shown here, but as a graphical chart on a cycle by cycle basis. The qualities for individual tiles are shown as thin lines and the average by a red dot. An example from your dataset is shown below:



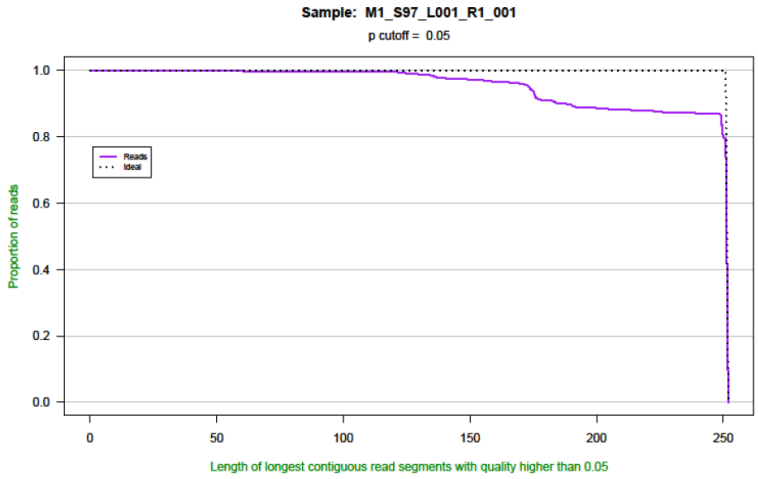

*Histogram:*

This plot shows distribution of the longest contiguous sequence where the base quality is better than the value chosen for analysis. The default value is 0.05, approximately equal to a Phred score of ~13. An example from your dataset is shown below:

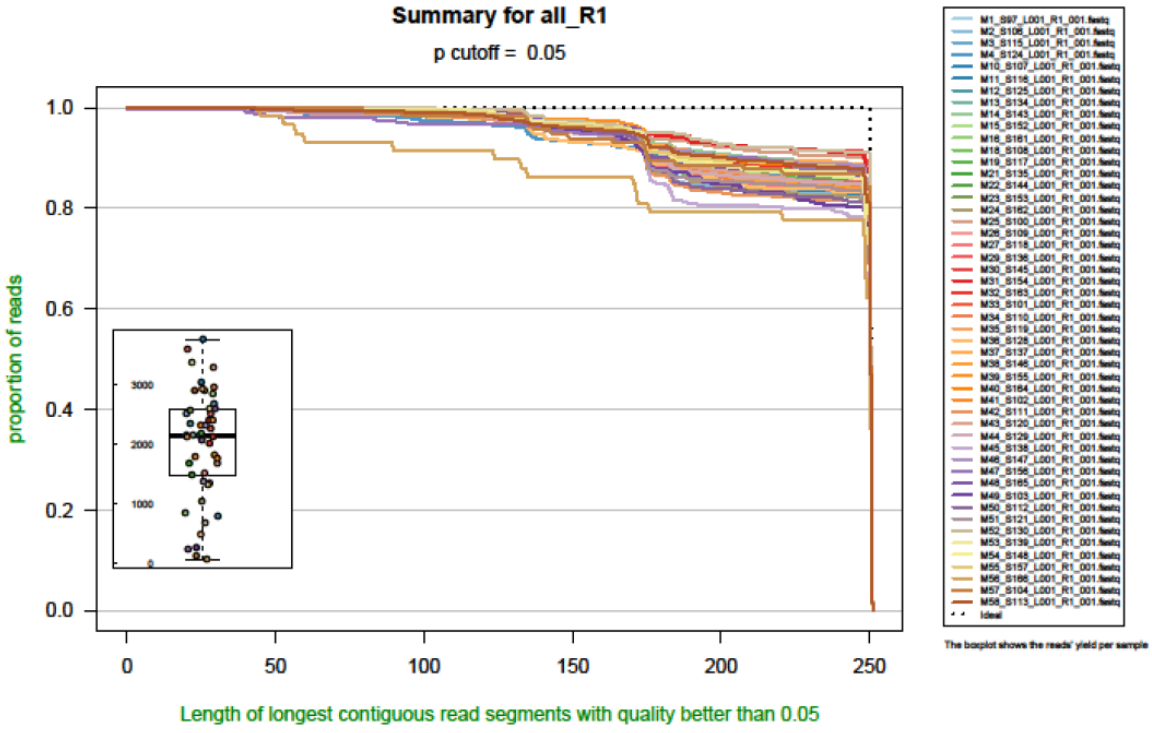



*Cumulative:*

A line graph showing the cumulative frequency of trimmed read lengths, with the perfect result shown as a dotted line. This graph allows you to see what fraction of your data is at a given length or longer. An example from your dataset is shown below:

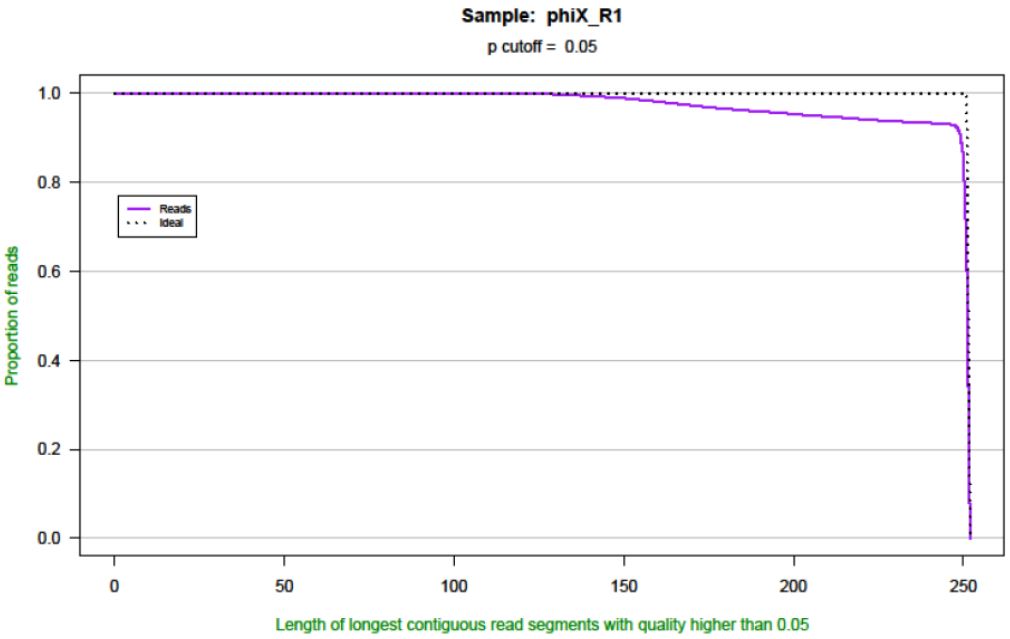



*Summary (folder):*
This folder contains two graphs - one for read 1, the other for read 2. Each graph shows all the *cumulative* plots across all samples for that read, and a small boxplot showing the reads' yield for each sample. The graph for read 1, subset 1 is shown below:




*PhiX_quality:*
Using the results of the mapping of the 'Undetermined' sequences against PhiX as described in section 2.2.2, the sequences that do map to the PhiX genome are now being quality checked, in order to assess the overall run quality independently of the sample quality. In this folder you can find the results of the SolexaQA++ analysis for these PhiX-aligning reads. To perform this task, we reconstructed the .fastq files from

the .bam file in the previous step, again using the SamToFastq.jar program from the Picard suite, and ran it through SolexaQA++. The same kinds of graphs described in section 2.2.4.1 are produced; as an example, the *cumulative* graph for read 1 is shown below:




*FastQC:*

Website: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

The process is run on the file, and the standard output (in the folder) viewable in a web browser is provided. A zip file is also provided. For example, clicking on the fastqc_report.html file inside any folder will open up a page in a web browser with the information from the processing of the sequences. This is run on the sequences in the 'fQsequences' folder.

FastQScreen:

Website: http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/

As mentioned above, the sequences are screened against a set of sequences for checking the level of any potential contamination. Currently, this means screening against *E. coli*, PhiX, yeast, Illumina adapters and cloning vectors. Again a text file and a graphical png file results from this process result for each sequence file, as shown below for some of your samples:

**Figure 2: example fastQscreen output**