Apparent resistance to brodifacoum in *Rattus rattus* in a New Zealand site with no history of anticoagulant-based rodent control

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ABSTRACT

Context. Anticoagulants have been used in New Zealand for decades, but few data are available on the sustainability of these toxins for rodent control. It is important to regularly monitor for resistance in long-term brodifacoum-use areas and establish a database for future references. **Aims.** This study aimed to estimate the effective dose (ED_{50}) of brodifacoum for ship rats from an area of New Zealand with no history of brodifacoum use, in order to establish a blood-clotting response test for assessing resistance in rodent populations from other areas. **Methods.** A ranging study was conducted whereby successive groups of ship rats were administered brodifacoum doses that were increased or decreased progressively, until an International Normalised Ratio (INR) of 3.6 was reached. Linear regression was used to model the relationship between dose and INR, and ED50 dose was estimated using the resulting model. **Results.** None of the rats appeared susceptible to brodifacoum at previously reported LD_{50} exposures for this species. The ED_{50} of brodifacoum was estimated to be 2.88 mg/kg for males and 3.81 mg/kg for females. These values are 6–8 times greater than the previously published lethal dose values for ship rats in New Zealand. **Conclusions.** Blood-clotting inhibition was detected in the rats only following high doses of brodifacoum, which may indicate resistance within the sampled population. **Implications.** Relatively low susceptibility, or resistance, to brodifacoum in New Zealand ship rats may be mediated by spatial connections between areas with different histories and patterns of anticoagulant rodenticide use.

Keywords: anticoagulants, blood clotting response test, brodifacoum, effective dose, pest management, *Rattus rattus*, resistance, rodent control.

Introduction

Anticoagulant resistance in rodents was discovered soon after first-generation anticoagulant rodenticides (FGARs) were introduced, when warfarin treatment failed to control Norway rat (*Rattus norvegicus*) populations in Scotland (Boyle 1960). Subsequently, there were many reports throughout Europe of resistance to warfarin as well as other first-generation anticoagulants like coumatetralyl and diphacinone (Bentley 1968; Lund 1969; Greaves and Rennison 1973). With declining efficacy of FGARs against resistant rodent populations, more potent second-generation anticoagulants (SGARs) were marketed in the 1970s (Hadler and Shadbolt 1975). Bromadialone and diphanacoum were the first to be marketed and were initially found to be effective against rats resistant to FGARs (Hadler *et al.* 1975; Marsh 1977). However, resistance to these compounds was subsequently reported (Redfern and Gill 1978; Greaves and Cullen-Ayers 1988; Quy *et al.* 1995). Three other SGARs (brodifacoum, flocoumafen and difethialone) were introduced to the market as 'resistance breakers', being effective against rodent populations resistant to FGARs and other SGARs (Buckle *et al.* 2013). Although brodifacoum and flocoumafen have proven to be very effective in rodent control around the world, continuous long-term use of any anticoagulant rodenticide may lead to selection of resistance-conferring mutations by eliminating the susceptible individuals of the population. Many studies

have confirmed resistance to anticoagulants throughout the world. For example, anticoagulant resistance has been widely documented in Europe (Rost *et al.* 2004; Pelz *et al.* 2005), the United States (Jackson and Ashton 1979), Indonesia (Andru *et al.* 2013), Japan (Ishizuka *et al.* 2007) and China (Liang 2005). Most of the documented literature on resistance to anticoagulants in rats concerns Norway rats (Li *et al.* 2004; Rost *et al.* 2009; Rymer 2017). Less work has been done on potential resistance in ship rats (black rats, *Rattus rattus*), because they are not a major pest species in countries where extensive research on anticoagulant resistance has been done (Berny *et al.* 2018). Resistance to FGARs like warfarin and the more potent SGARs, bromadialone and difenacoum, has been reported in ship rats in Denmark, France, USA, Germany, UK and Japan (Desidiri *et al.* 1978; Lund 1984; Myllymäki 1995; Ishizuka *et al.* 2007).

Anticoagulants have been used in New Zealand for decades, including for the extensive control of ship rats, which are a major invasive predator in New Zealand forests. However, very little research has been done on anticoagulant resistance in ship rats or other rodents in New Zealand. The New Zealand Department of Conservation has used brodifacoum extensively for island rodent eradications, but stopped using it for mainland control operations in 2002 due to concerns about its persistence in the environment (Eason *et al.* 2002). However, it continues to be used extensively on the mainland by regional councils, community groups and private landowners. 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 potential anticoagulant resistance in ship rat populations in areas with a history of long-term exposure to anticoagulants.

Anticoagulant resistance was suspected in parts of Auckland and on Raoul Island in 1978 but no investigation took place. However, rats were eradicated successfully in Raoul Island using brodifacoum by aerial application in 2002 (Wodzicki 1978). The first evidence of heritable genetic resistance was established in New Zealand in 2017, with identification of a Tyr25Phe mutation in eight out of 482 ship rats screened (Goulois *et al.* 2016). Although this mutation is associated with resistance to all FGARs and some SGARs (Goulois *et al.* 2016), demonstration of phenotypic changes is required to conclusively document anticoagulant resistance in New Zealand rodent populations. Novel mutations affecting anticoagulant resistance like Ala14Val and Ala26Val have also been identified in ship rats in New Zealand (Cowan *et al.* 2017), but they need to be assessed using lethal feeding period (LFP) tests or blood-clotting response (BCR) tests to assess the degree of resistance it confers towards each anticoagulant compound used in New Zealand. If the mutations confer little practical resistance,

anticoagulants can still be effective as a control strategy. Similarly, if these mutations cause resistance to FGARs only, SGARs may still be an option for effective control. The information obtained from LFP and BCR tests is therefore crucial for making practical decisions about control of resistant ship rat populations.

The standard BCR test (Prescott *et al.* 2007) for Norway rats and house mice (*Mus musculus*) involves comparing blood coagulation times before and after administration of a sublethal dose of anticoagulant. Animals that show considerably increased blood-clotting times after administration of a dose of anticoagulant are considered susceptible (Erica Gill *et al.* 1993). This test does not rely on poisoning mortality of animals, as is the case for LFP tests, and is therefore considered more humane, has fewer ethical constraints, and can be performed in 24 h. BCR tests are very sensitive and can detect even small differences in the susceptibility of rodent species to specific anticoagulant rodenticides (Prescott and Buckle 2000).

The sublethal dose administered to animals to test for blood-clotting responses is called an effective dose (ED_{50}). For example, an ED_{50} dose will not kill the animal, but will produce a critical measurable change in blood-clotting time in 50% of individuals (Prescott *et al.* 2007). Once the ED50 has been calculated for a species in the absence of anticoagulant exposure, it can be tested whether the ED50 is significantly higher in populations with a history of exposure, giving evidence of resistance. The ED50 value may or may not differ significantly between males and females (Prescott *et al.* 2007).

The initial aim of our study was to determine the effective dose (ED_{50}) of brodifacoum for a New Zealand population of ship rats with no known exposure to brodifacoum. This would then provide a baseline that could be used in BCR tests to test for resistance in ship rats in parts of New Zealand with long-term brodifacoum use. Because there are no laboratory-strain ship rats available in New Zealand, we captured wild ship rats from an area with no documented history of anticoagulant use and conducted a ranging experiment to determine the brodifacoum dose that produced a blood-clotting response in 50% or males and females.

Material and methods

Study site

We captured wild ship rats from the north-west region (40°58′34.66″S, 175°1′35.45″E) of Akatarawa forest (Fig. 1) in New Zealand, a site where there was no prior anticoagulant history and the pest control history was well documented (Table 1). Akatarawa Forest is in the steep hill country of the Akatarawa Ranges, covering 15 500 ha, and is managed by Greater Wellington Regional Council (GWRC). Trapping was carried out in the north-west region of Akatarawa because
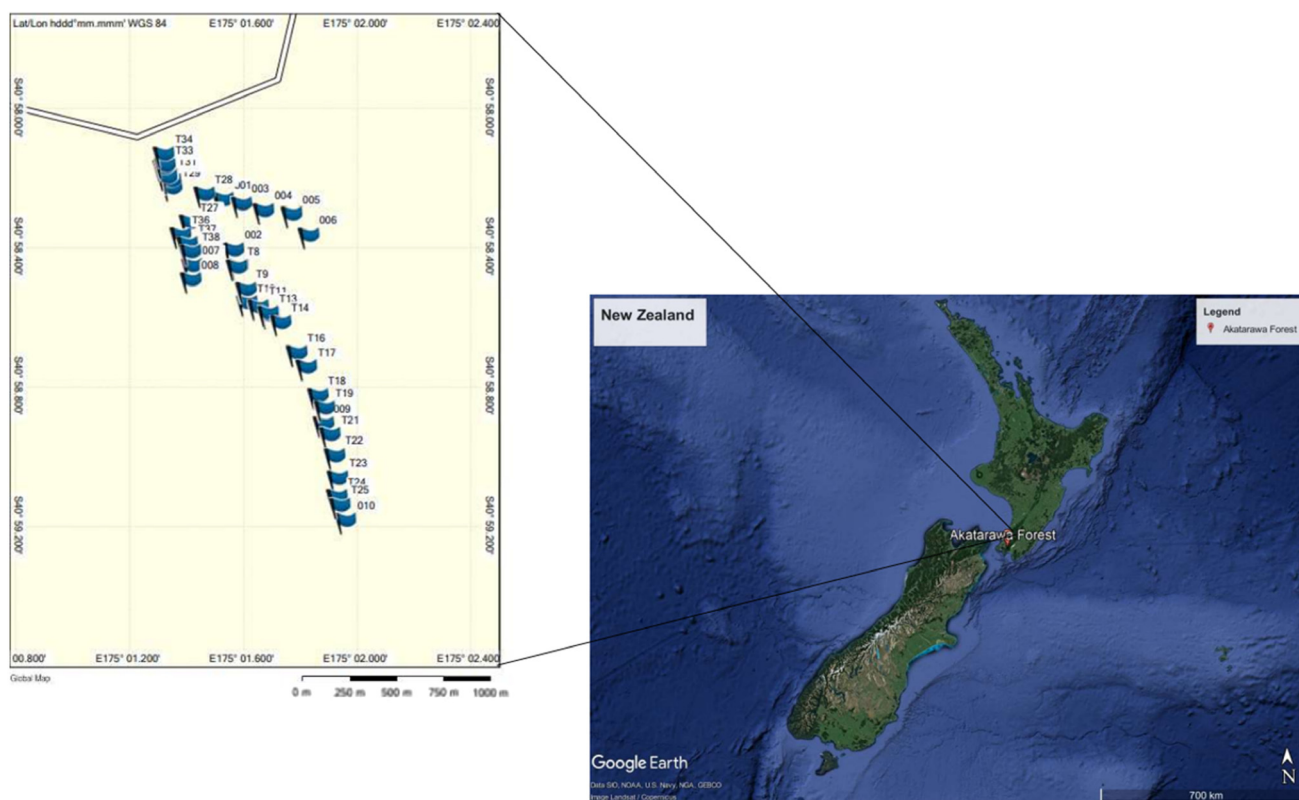
Fig. 1. Map of New Zealand showing location of Akatarawa forest along with expanded map of north-west region depicting location of individual traps placed about 100 m from each other.

Table 1. Small mammal pest management history at Akatarawa forest as provided by Greater Wellington Regional Council.

Year	Poison used	Targeted area
1995–97	Sodium fluoroacetate	Selected parts of the forest
2000–01	Cholecalciferol and brodifacoum	An initial knockdown using cholecalciferol and brodifafoum 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
2007	Sodium fluoroacetate	Entire forest
2013	Sodium fluoroacetate	Entire forest

there was temporary use of anticoagulants in the south-east region (18 km away) in 2000–01 (Table 1). Brodifacoum has also been used in the neighbouring private lands by GWRC.

Trapping and maintenance

Forty single-catch iron-mesh rat traps were set up close to Maungatotukutuku access point. To avoid catching rats from surrounding areas, no traps were placed within 1 km of the forest boundary. The rat population was at low density because of ongoing management using the non-anticoagulant toxicant sodium fluoroacetate (1080), hence the 12 nights of trapping effort (40 traps set per night) it took to catch the 36 rats used in the experiment (Table 2). Traps were placed 100 m apart and baited with cheese and peanut butter. Traps were checked every morning, and captured animals were transported in their traps to Massey University, Palmerston North within 24 h. On arrival at Massey University, each animal was transferred from its trap to an individual 30 cm × 30 cm × 25 cm rat cage, and was acclimatised for 15 days before the commencement of the experiment.

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 euthanased by cervical dislocation while under general anaesthesia (isoflurane). Cages were provided with sawdust for bedding and shredded newspaper for nesting material, and food and water were provided *ad libitum*. Rats were provided with rat cereal feed pellets (Diet 86, manufactured at the feed mill at Massey University). The laboratory was maintained at 20 ± 2°C on a 12-h light/dark cycle.

Vitamin K is an important component of the blood-clotting system in mammals. Deficiency of vitamin K can lead to

Table 2. Live rat trapping at Akatarawa forest with trapping dates and number of ship rats caught (one of the captured females died while in captivity).

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 November 2016	29 November 2016	5	22	12	10	0
Total		12	39	21	16	2

prolonged blood-clotting time. Similarly, excess of vitamin K can act as antidote to brodifacoum (Prescott *et al.* 2007). The level of vitamin K in ship rats captured from wild is unknown, and therefore it is important to provide rats with the appropriate amount of vitamin K for a sufficient acclimatisation period. The diet provided contained the minimum amount of vitamin K required by a rat (1 mg/kg per diet; Fu *et al.* 2007) to avoid vitamin K deficiency, and any initial excesses of vitamin K in their bodies was expected to have been utilised during the 15-day acclimatisation period.

Brodifacoum dose formulation

Successive doses of brodifacoum were formed by dissolving the required amount of brodifacoum in polyethylene glycol 200 (PEG200) in a beaker. The brodifacoum and polyethylene glycol (PEG) 200 were obtained from Sigma Aldrich Company Ltd, Auckland, New Zealand. The solution was shaken and heated to 60°C in a laboratory incubator overnight to aid dissolution of brodifacoum and achieve the required concentrations. The beaker was covered with aluminium foil to avoid light throughout the procedure. This methodology is standard for preparing brodifacoum formulations, which have been shown to be thermally stable below 150°C with no decomposition or transformation (https://circabc.europa.eu/sd/a/d88b968d-e5b8–4b4e-84bb-79a8b41acc4b/2010%2001%2017%20Final%20combined%20AR%20Brodifacoum_rev%20_2_.pdf).

Experimental design

The experiment involved a ranging study whereby ship rats of each sex were divided into groups of three, including a control group and multiple treatment groups. Rather than deciding the treatment groups *a priori,* the doses for each successive treatment were adjusted up or down until the effective dose was determined with sufficient precision (see statistical analysis). Such procedures reduce the number of animals used (Bruce 1985).

Published LD_{50} values of brodifacoum for ship rats include 0.46 mg/kg (O'Connor and Booth 2001) to 0.77 mg/kg (Mathur and Prakash 1981). The ED is a sublethal 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, i.e. the dose that kills 50%

individuals in a susceptible population. Based on the highest published LD_{50} dose of brodifacoum for ship rats 0.77 mg/kg, the first treatment groups of each sex were 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) of 3.6 was reached.

The INR in this context is derived by calculating the ratio of the post-treatment clotting time to the pre-treatment clotting time, which is then multiplied by the International Sensitivity Index (ISI). A ship rat is considered to be a responder (susceptible) to a given dose of anticoagulant when it has an INR $\geq$ 3.6 (3.5–3.8) (Garg and Singla 2015). 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 with international reference tissue factor. An ISI ranging between 0.9 and 1.4 indicates a highly sensitive reagent. A CoaguChek® XS Plus analyser (Roche Diagnostics International Ltd) was used to determine blood-clotting times of animals before and after treatment, with an allocated ISI value of 1.

Pre-treatment

After the 15-day acclimatisation period, 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* 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 measures clotting times within the range of of 9.6–96 s; therefore, for rats with clotting times less than 9.6 s, 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 PEG. The control group of rats was administered with PEG200 only. In all cases the total volume injected was 0.08 mL per 10 g bodyweight of rat. The rats were returned to their cages

while they were still under influence of anaesthesia, and recovered 2–3 min later.

Post-treatment

Up to 20 µL of blood was collected from each rat 24 h after administering the dose of brodifacoum. Rats were anaesthetised as described above, then the post-treatment blood sample was collected *via* cardiac puncture with a 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

Table 3. Blood-clotting responses of ship rats from Akatarawa forest to different doses of brodifacoum (shown as mg brodifacoum per kg of rat bodyweight).

Sex	Bodyweight (g)	Dose (mg/kg)	Pre-treat. BCR (s)	Post-treat. BCR (s)	INR (s.e.)
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

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 the standard error (s.e.) around the INR.

sampled 24 h after the PEG was administered, but at this stage 20 μL of blood was 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. All procedures involving the use of animals were approved by the Massey University Animal Ethics Committee (Protocol 15/97).

Statistical analysis

Probit analysis is used commonly to calculate the ED_{50} of different rodenticides (Prescott *et al.* 2007; Akçay 2013). In probit analysis, each animal's blood-clotting times are reduced to a binary variable, indicating whether it is considered to be a responder. The approach is therefore wasteful of data, and inappropriate for a sample of 36 animals. We 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 and 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 multiple imputation, and because it allowed the effective dose to be easily calculated as a derived parameter. Parameter estimates are reported ±1 s.e.

Results

BCR test

The 36 rats used in the experiment were healthy mature adults. The average weight of males was 169 g (range = 142–219) and the average weight of females was 148 g (range = 130–162).

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 3). No clinical signs of lethargy, distress or haemorrhage were identified before the end of the 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 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 considered a threshold response for susceptible ship rats to a given anticoagulant dose (Fig. 2). The dose of brodifacoum was increased successively to 0.8, 1.0 and 2.0 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 ranging between 3.63 and 4.00. 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 to 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.

Estimation of effective dose

The data indicated that it was reasonable to assume a linear relationship between INR and dose (Fig. 3). Because there was no apparent effect of the PEG, 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. 3). 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.

Discussion and conclusions

In the present study, successive doses of brodifacoum were administered to each group of rats, ranging from 0.72 to 3.00 mg/kg in male ship rats and 0.72 to 4.00 mg/kg in female ship rats, until an INR ≥ 3.6 was obtained. From this experiment, the ED_{50} of brodifacoum for ship rats at Akatarawa forest was estimated to be 2.88 mg/kg for males and 3.81 mg/kg for females. The effective dose is a sublethal dose that produces a critical measurable response

Table 4. Lethal and effective doses of brodifacoum for Norway rat, ship rat and house mouse.

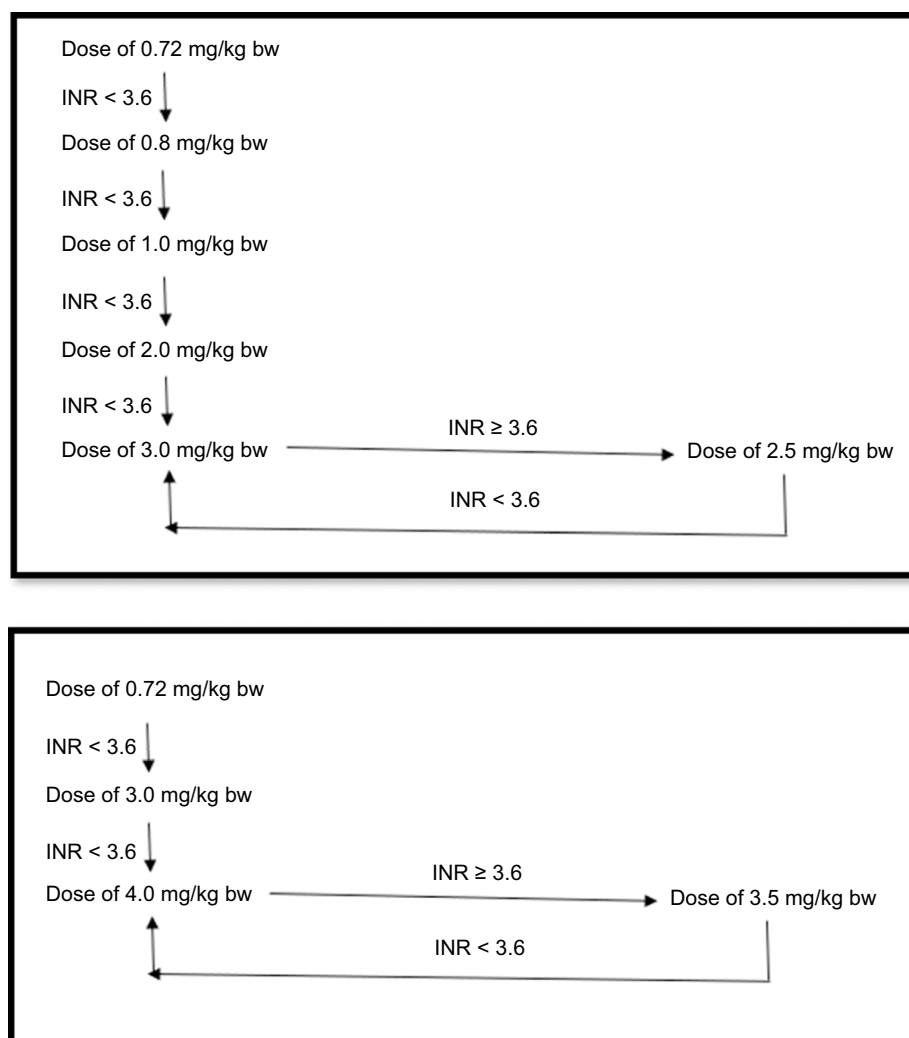| Species of rodent	LD_{50} value	ED_{50} value
Wild Norway rat	0.17–0.56 (Redfern *et al.* 1976; O'Connor and Booth 2001)	0.22 (Prescott *et al.* 2007)
Wild house mouse	0.40–0.52 (Redfern *et al.* 1976; O'Connor and Booth 2001)	0.35–0.39 (Prescott *et al.* 2007)
Ship rat	0.46–0.77 (O'Connor and Booth 2001; Mathur and Prakash 1981)	2.88–3.81 (present study)

Fig. 2. 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.

to an administered quantity of anticoagulant; therefore, it is always a less-than-lethal dose (LD_{50}) of brodifacoum, i.e. the dose that kills 50% individuals in a susceptible population. Reported LD_{50} doses of brodifacoum vary in rodent species populations around the world, and may be affected by several 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 for ship rats (Dubock and Kaukeinen 1978; Fisher 2005), which is lower than the ED50 values found in this study, possibly indicating a relative tolerance, or outright resistance, to brodifacoum in our study population.

The highest published LD_{50} value of brodifacoum for ship rats, 0.77 mg/kg, was 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 in a brodifacoum-free area on the west coast of the South Island (O'Connor and Booth 2001). The west coast of the South Island is more remote in terms of human habitation than Akatarawa forest, from which rats were captured for the present study. The effective dose estimated for male ship rats in this study (2.88 mg/kg) is nearly 3.7 times the highest published lethal dose of brodifacoum, and 6.2 times the published lethal dose in New Zealand. In females, the value of 3.81 mg/kg is nearly five times the highest published lethal dose in India and 8.2 times the published lethal dose in New Zealand. The calculated effective doses in this study are very high compared with the two published LD_{50} values.

In the present study, all the ship rats with INR values < 3.6 at a given dose were categorised as non-responsive to standard doses of anticoagulants based on Garg and Singla's (2015) research, indicating this was a suitable criterion for
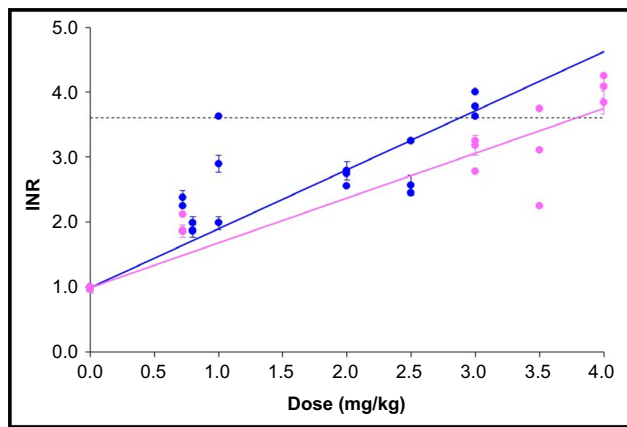
Fig. 3. Linear regressions for the relationship between brodifacoum dose (mg/kg of bodyweight) and proportionate increase in blood-clotting time (INR) for male (blue) and female (pink) ship rats from Akatarawa forest. The dotted line shows the INR level at which a ship rat is considered to be a responder (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.

R. rattus. Prescott *et al.* (2007) earlier suggested that an INR > 5.0 was a suitable indicator of responsiveness to an anticoagulant dose in rodents (Norway rats and house mice). However, none of the rats in our study were given a high enough dose to achieve an INR > 5.0, so our conclusions are not sensitive to this difference in cut-off INR value. In any case, our estimated ED_{50} values were clearly still higher than initially expected, based on known LD_{50} values of brodifacoum for ship rats.

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 BCR test on ship rats was for detecting resistance to bromadialone in India (Garg *et al.* 2017). However, in that study, the dose used for the BCR test was calculated based on a published lethal dose rather than conducting an experiment to determine the effective dose. Therefore, we do not have any data to compare the effective dose and lethal dose for any anticoagulant in ship rats, but we can make comparisons with published data for LD_{50} and ED_{50} values of brodifacoum in Norway rats and house mice (Table 4). Both species are major pests in most parts of the world where anticoagulant resistance has been studied extensively. There are published effective doses of brodifacoum available for Norway rats from studies around the world, and those effective doses are far less than the highest published lethal dose. Similarly, for house mice, published effective doses are always lower than the published lethal doses.

The second dose administered to the rats, 0.8 mg/kg, exceeded the highest published lethal dose of 0.77 mg/kg. Therefore, all the animals were expected to respond to this dose and produce an INR ≥ 3.6. A recent study undertaken on Lord Howe Island in Australia, in preparation for a rodent eradication programme, found doses of 0.8 mg/kg administered to ship rats achieved 100% mortality, irrespective of the long history of use of FGARs and SGARs on the island since the 1960s. Although there is no formal record of brodifacoum use by residents on the island, it is thought to have exceeded 400 kg per annum since 2000 (Wheeler *et al.* 2019). 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 INR ≥ 3.6.

Susceptibility may vary among populations of ship rats due to differences in historic exposure to the toxin, but also to natural variation. Wild rodents are expected to be more variable in their responses than laboratory strains (Prescott *et al.* 2007), thus laboratory strains are generally preferred for evaluating the effective dose of a poison. Due to the absence of laboratory ship rats in New Zealand, we attempted to measure the effective dose for susceptible ship rats using a wild population with no known history of anticoagulant exposure. The apparently high level of anticoagulant tolerance or resistance in this population means we were unable to confirm that our estimates represented baseline ED50 values for susceptible ship rats in the New Zealand context.

One potential explanation for this resistance would be a high intake of vitamin K in diet in Akatarawa forest. Vitamin K is an antidote of anticoagulants and would result in no or low response to the administered brodifacoum dose. However, the acclimatisation period of 15 days should have led to utilisation of any extra vitamin K in the body, and the diet provided to ship rats during this period only contained the minimum amount of vitamin K required by rats.

Although the lack of response of ship rats to lower doses like 0.72–1.00 mg/kg could be explained based on natural variance in susceptibility to brodifacoum, it is unclear how a general low susceptibility or tolerance due to natural variance contributes to lack of response to substantially doses in the 2.5–3.0 mg/kg range. The lack of blood-clotting inhibition in response to such doses is plausibly due a high prevalence of some form of resistance to brodifacoum within the ship rat population at Akatarawa forest.

Although brodifacoum or any other anticoagulant was never used in the part of the forest from where rats were captured, it is used extensively in the neighbouring areas by private landowners as well as 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 resistance. Sodium fluoroacetate (1080) has been the only poison used in pest control operations in north-western Akatarawa forest, applied every 4–6 years since 2001. 1080 is an acute metabolic poison and has a mode of action different from anticoagulants. Because 1080

is a very effective rodenticide, there is a sharp decline in rat population in the forest each time it is used (Innes and Williams 1991; Innes *et al.* 1995). This may be facilitating a more rapid invasion of ship rats into the forest. The ship rat population in the area is known to recover quickly after the use of 1080 pellets, and in general, ship rat populations are known to recover in about 6–12 months (Barron *et al.* 2014). This time window provides 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). Also, ship rats are faster and more agile than Norway rats (Foster *et al.* 2011). Breeding in ship rats is polyoestrous, and 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, it seems possible that anticoagulant resistance is present in ship rats in the Wellington region. It is also likely that ship rats from areas of long-term brodifacoum use (20+ years) may have even higher resistance to brodifacoum than that observed in this study.

Further investigation needs to be done to confirm physiological and/or genetic basis for possible resistance in New Zealand ship rats. Genetic analysis of the rat tail samples preserved from this experiment may indicate whether the resistance to brodifacoum in ship rats at Akatarawa Forest is due to heritable resistance. If this is the case, rodent pest management by anticoagulants may become a challenge in near future. A rodent resistant to a more toxic anticoagulant is known to develop resistance to less toxic anticoagulants to which it may have even never been exposed (Pelz and Prescott 2015). This removes the option of using alternative anticoagulants to control rodent pest population 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 by 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 the higher effective dose found in females than in males. This may be due to two factors. First, basal activity of vitamin K-dependent blood clotting factors (II, VII and X) is higher in females than in 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

that may prove to be fatal. Higher activity of vitamin-K dependent blood clotting factors would reduce susceptibility of females to brodifacoum. Second, 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 bromadialone-resistant rats, different CYP450 isoforms have been known to express 5–80-fold higher in bromadialone-resistant female rats as compared with susceptible female rats, meaning the resistant females can metabolise bromadialone 5–80 times faster as compared with susceptible females. In contrast, these enzymes expressed only 1.7–3.0-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 future threat to effective rodent control in New Zealand because resistant female rats are likely harder to control than 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). Therefore, with 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.

At present, there is a need to consider poisons other than anticoagulants for pest 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. Although we were unable to evaluate the effective dose of a susceptible population, the estimated effective doses in this study are still useful for comparing with areas of known brodifacoum use (i.e. to assess whether the apparent level of resistance is even greater in these areas). We also recommend evaluating the effective dose using ship rats captured from isolated areas with no possible brodifacoum use, such as the few remaining populations on offshore islands, as a comparison with the ship rats used in this study.

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