Assessing anticoagulant resistance in rats and coagulation effects in birds using small-volume blood samples

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Cover: Ship rat (*Rattus rattus*) eating fantail chicks at nest.
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CONTENTS

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ABSTRACT

The second-generation anticoagulant brodifacoum is currently used for possum and rodent control on the New Zealand mainland, and for rodent eradication on offshore islands. To investigate whether these uses have produced undesirable effects in localised wild rat populations (anticoagulant resistance) or in birds (effects on blood coagulation times), blood clotting response tests utilising small-volume sampling were established using albino laboratory rats (*Rattus norvegicus,* Wistar) and domestic chickens (*Gallus domesticus*). These tests were then used on blood samples taken from wild rats (*R. norvegicus*) and kiwi (*Apteryx australis mantelli*). During the development of the blood-clotting test in laboratory rats we observed a reduced anticoagulant response after a second dose of 0.1 mg/kg of brodifacoum, compared with the initial exposure. There was no evidence of anticoagulant resistance in rats from areas where there was a history of 2–5 years' use of brodifacoum. Statistical differences were found in the clotting times of kiwi from areas with a history of anticoagulant use and those from areas where brodifacoum has not been used, but the clotting times were the opposite of what would be expected if they were interpreted on the basis of exposure history alone. These results highlight uncertainties regarding resistance, sub-lethal effects, threshold concentrations in the liver and the toxicodynamics of anticoagulants in mammals and birds.

Keywords: anticoagulant, brodifacoum, rodents, non-target species, kiwi, resistance, birds, blood sampling, coagulation.

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1. Introduction

In New Zealand, introduced pest animals such as brushtail possums (*Trichosurus vulpecula*) and rodents (*Rattus rattus*, *R. norvegicus*, *R. exulans* and *Mus musculus*) have a severe impact on indigenous flora and fauna. Control of possums and rodents currently relies on the use of a range of vertebrate pesticides, including first- and second-generation anticoagulant compounds. First-generation anticoagulants, such as warfarin and pindone, were developed in the 1950s. More potent second-generation anticoagulants, such as brodifacoum and flocoumafen, were developed from the 1970s onwards in response to the development of rodent resistance to some of the first-generation anticoagulants (Gratz 1973). While continued use of first-generation anticoagulants may lead to resistance, this can be managed by integration with other control methods such as traps, non-anticoagulant rodenticides such as cholecalciferol and intermittent or pulsed baiting strategies (e.g. MacNicoll 1993; Bailey & Eason 2000). Continued use of second-generation anticoagulants, e.g. brodifacoum, may have a lower risk of inducing resistance, but this advantage is offset by the persistent nature of these compounds, with increased potential for adverse effects on non-target species, especially sub-lethal or lethal poisoning through secondary exposure (Eason et al. 2002). Anticoagulant use patterns in New Zealand differ from those in other parts of the world, where anticoagulants are generally only used for control of commensal rodents in and around buildings. Recent data have shown that game species and native birds in New Zealand have been contaminated with brodifacoum (Eason et al. 1996, 2002). While brodifacoum has proved especially effective in achieving pest control targets in New Zealand (e.g. Innes et al. 1995), there is a need to balance the risks and benefits of broad-scale field application of anticoagulants. In the short to medium term, the need for effective toxic baits for rodent and possum control on the mainland is ongoing and likely to increase. Concomitant to the use of anticoagulants for field pest control, it is important to develop and establish techniques that will allow monitoring of rodent populations for anticoagulant resistance and of non-target species for evidence of exposure and any resultant adverse effects.

2. Background

Anticoagulants can be classified according to chemical structure as indandiones (e.g. pindone, diphacinone) or coumarins (e.g. warfarin, brodifacoum), and also as first- or second-generation compounds, according to the timing of their development. All of these compounds act by interfering with the normal synthesis of vitamin K-dependent blood-clotting factors in the liver (Bell & Caldwell 1973; Zimmerman & Matschiner 1974; Thijssen et al. 1986). Vitamin K (hydroquinone form) is a co-factor for a carboxylase enzyme in the production of proteins such as blood clotting factors II, VII, IX, and X. In this carboxylation

cycle, vitamin K is oxidised (VKO) and is then unavailable until recycled back to vitamin K hydroquinone by the enzyme vitamin K epoxide reductase (VKOR). It is this enzyme that is inhibited by the action of coumarin anticoagulants, which bind strongly to the enzyme, leaving it unavailable for VKO. Recycling of Vitamin K is required to maintain normal levels of the different blood clotting factors, which mediate the coagulation response to injury. When anticoagulants inhibit the recycling of Vitamin K to the extent that levels of blood clotting factors are depleted, fatal haemorrhaging may result from the inability of blood to clot normally.

Resistance may be defined as an inheritable trait that allows animals to survive a dose that would otherwise kill 99% of susceptible individuals. Resistance will become prevalent in populations that are strongly selected by sustained application of a toxicant, which will kill non-resistant individuals and allow surviving resistant animals a greater genetic input to future generations. Different 'types' or mechanisms of anticoagulant resistance have been described in rodents (MacNicoll 1993; Thijssen 1995). In one type, VKOR has become modified so it remains functional but has a reduced affinity for the anticoagulant or the anticoagulant is more easily displaced from VKOR by VKO (Bell & Caldwell 1973; Zimmerman & Matschiner 1974). Resistance to warfarin has been widely documented in United Kingdom rodent populations; in some cases rodents freely feed on toxic bait without harmful effects (MacNicoll 1985, 1993; Quy et al. 1995). The development of resistance in New Zealand populations of target rodents has not been investigated, but the possibility it might occur highlights the importance of establishing a consistent assessment methodology, proactive monitoring and a database addressing spatial exposure of target rodent populations to anticoagulants (Bailey & Eason 2000).

Second-generation anticoagulants have been shown to persist for prolonged periods in live animals, particularly in liver tissue (WHO 1995). Residues of brodifacoum have been detected in kiwi (Robertson et al. 1999), and a range of other native New Zealand birds and introduced mammals (Eason et al. 2002). However, the prevalence and effects of cumulative residue burdens on the survival and long-term health of non-target wildlife are not well understood. To date, most anticoagulant residue data from native New Zealand birds have been derived from opportunistic sampling of tissue, particularly liver, from those found dead in field situations. Because of the high conservation status of many native birds, destructive sampling to collect liver tissue for residue analysis is not an acceptable practice. From an ethical perspective, less invasive sampling procedures are always preferable when working with animals.

Traditionally, anticoagulant (rodenticide) effects are measured by feed-testing or by blood clotting response testing (BCRT). The latter involves blood sampling and coagulation time assay of captive wild animals before and after an anticoagulant dose. After dosing, animals that show substantially reduced clotting activity are considered susceptible, while those showing smaller changes in clotting times after dosing are considered resistant (Kerins et al. 1993). BCRT is faster and has fewer ethical constraints than feed-testing and is widely used to monitor resistance, with considerable international effort expended in recent years to standardise BCRT methodologies. However, BCRT can require relatively large-volume blood samples, with associated invasive and (in the case of small animals and birds) destructive sampling procedures.

Because we aimed to investigate adverse effects of anticoagulant exposure in native birds (as well as resistance in rodents), we aimed to develop a technique that required relatively small volumes of blood which would minimise the invasiveness of sampling. Comparison of blood clotting parameters in laboratory or captive birds can then be made with those from birds sampled from wild populations, and results from wild populations could then be related to what is known about anticoagulant use in the area.

There are some pronounced differences between the clotting mechanisms of different classes of animals, although some major clotting factors (e.g. prothrombin, Factor II), involved in avian blood coagulation appear to have a role similar to that which they have in the coagulation of mammalian blood (Griminger 1986). The prothrombin time (PT) is a test that provides a measure of the extrinsic blood coagulation system, and is commonly used to indicate clinical effects of oral anticoagulants (Poller & Hirsh 1996). Coagulation of avian blood seems to be especially dependent on the extrinsic clotting system, which involves the release of tissue thromboplastin, and a certain degree of species-specificity exists for brain tissue thromboplastin (Factor III) (Griminger 1986). Accordingly, it was important to establish separate test reagents and systems in model species of mammals (laboratory rats) and birds (chickens). Here we report the development of new methods for sampling and monitoring anticoagulant exposure, and present preliminary data on resistance in rodents and sublethal effects in birds.

3. Objectives

The first objective of this study was to develop and calibrate small-volume blood-sampling techniques and specific blood-clotting-response-testing (BCRT) in laboratory rats and captive chickens as model species. The second objective was to apply the respective BCRT methods established in the laboratory to assessments of anticoagulant resistance in wild rodents, and to assessments of the effects of potential field anticoagulant exposure on native birds (kiwi).

4. Methods

4.1 DEVELOPMENT OF SMALL-VOLUME BLOOD-SAMPLING TECHNIQUE IN LABORATORY RATS

4.1.1 Animal housing and calibration of clotting activity

Adult male laboratory rats (*Rattus norvegicus*, Wistar) were housed in the Landcare Research Animal Facility, Lincoln, in standard solid polypropylene rat-cages (44.5 × 28 × 20 cm) in temperature-controlled rooms (19 ± 5°C) under

natural-day-length fluorescent lighting. All animals had free access to water and rat-and-mouse cereal feed pellets (Weston Animal Nutrition, Rangiora). Rats were acclimatised for at least 14 days prior to beginning any procedures. As there are indications of sex-linked determinants in response to anticoagulants in rats (MacNicoll 1993), only male rats were used in this trial.

A calibration curve for blood clotting times in laboratory rats was required for later use in standardising the clotting time response measured in whole (small-volume) blood samples taken from laboratory rats treated with brodifacoum. The curve shown in Fig. 1 (data in Appendix 1a) was generated using plasma sampled from untreated rats, because with dilution of plasma, the concentration of active clotting factors decreases and reduces the ability of blood to clot, similar to the effect of anticoagulants on blood. Whole blood (up to 5 mL) was sampled from eight rats that were deeply anaesthetised and sampled by heart puncture. Blood samples were taken in citrate tubes and centrifuged as soon as practical after sampling (2500×*g* for 15 min at 4°C). The plasma was pipetted into eppendorf tubes and frozen at –80°C if it could not be tested within the next 2 hours. Plasma samples were thawed in a hot water bath at 37°C, and dilutions of plasma prepared with phosphate-buffered saline (pH 7.4). Dilutions were tested for prothrombin time (PT) using a reagent kit (Dade Innovin) and an Amelung coagulometer. Data (Appendix 1a) were converted from time to clotting (seconds) to percentage clotting activity (PCA) relative to plasma dilutions, to produce a standard curve (Fig. 1). The curve was used to convert clotting times measured in the following trial to percentage clotting activity (PCA).

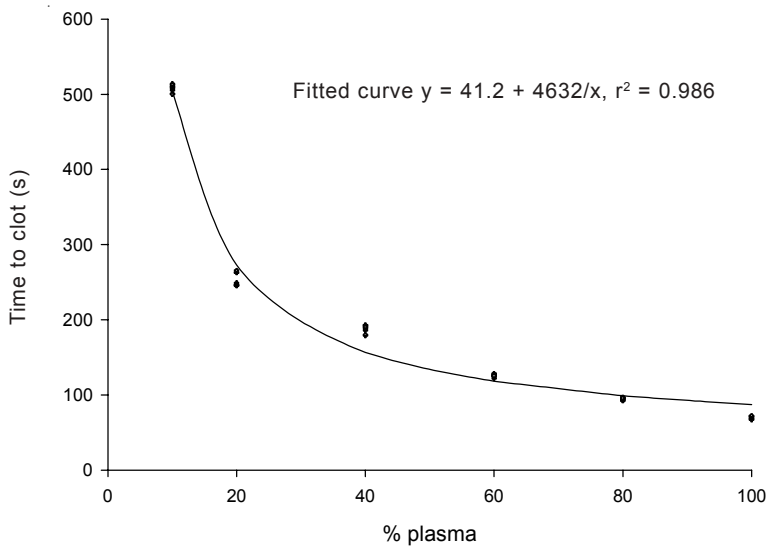


Figure 1. Standard curve of clotting times for male laboratory rat plasma diluted with phosphate-buffered saline (pH 7.4). Raw data for the standard curve is shown in Appendix 1a.

4.1.2 Clotting response of laboratory rats to brodifacoum dose

Baseline blood samples were taken from rats prior to dosing by oral gavage with either brodifacoum (0.1 mg/kg) or control solution (approximately 1 mL of monopropylene glycol), and rats were then blood-sampled at intervals after dosing (Table 1). The sublethal dose of brodifacoum (~LD_{15}) was not expected

TABLE 1. DOSING AND SAMPLING REGIME FOR DEVELOPMENT OF SMALL-VOLUME BLOOD-SAMPLING TECHNIQUE IN LABORATORY RATS.

GROUP ID	NO. OF ANIMALS, TREATMENT	SAMPLING DAY, METHOD
Group 1	6 rats, control solution	Day 0, tail vein sampled then vena cava
Group 2	6 rats, brodifacoum 0.1 mg/kg	Day 1, tail vein sampled then vena cava
Group 3	6 rats, brodifacoum 0.1 mg/kg	Day 2, tail vein sampled then vena cava
Group 4	6 rats, brodifacoum 0.1 mg/kg	Day 4, tail vein sampled then vena cava
Group 5	6 rats, brodifacoum 0.1 mg/kg	Day 8, tail vein sampled then vena cava
Group 6	12 rats, brodifacoum 0.1 mg/kg on Day 0 with a repeat dose on Day 64	Days 0, 2, 4, 8, 12, 16, 20, 34, 64, 65, 66 68, and 72 by tail vein microsampling

to kill the rats, but to produce measurable changes in blood clotting times. This was to enable comparison of the clotting times of blood samples taken from either the tail vein (small-volume) or vena cava, with samples taken at intervals after dosing and samples taken after a repeat sublethal dose of brodifacoum (Table 1). Blood samples were taken first using the small-volume sampling technique, followed by terminal bleeding from the vena cava from the same rat, or by small-volume sampling alone. For terminal bleeding, rats were deeply anaesthetised using halothane/oxygen and exsanguinated from the inferior abdominal vena cava (up to 5 mL). Samples were collected in syringes containing 20 mL of a 0.22-M sodium citrate solution.

Clotting time of whole blood samples was determined by assaying samples in duplicate using a commercial prothrombin-time assay kit (Dade Innovin) and an Amelung coagulometer (Sigma Diagnostics, USA). Recombinant-tissue-factor-assured, consistent, International Normalised Ratio (INR) values were used over different batches of thromboplastin used in the tests. Clotting times for each sample were expressed as PCA, i.e. percentage of clotting time derived from the calibration curve (Fig. 1, Appendix 1a). Samples that did not clot were allocated the value of 999 seconds.

4.2 RESISTANCE TESTING IN WILD RATS

4.2.1 Animal housing and calibration of clotting activity

Adult wild rats (*R. norvegicus*) were live-trapped from eight field sites throughout New Zealand with different histories of anticoagulant use. Sites were classified as having either 'exposure' or 'no exposure' to anticoagulants (Table 2). 'Exposure' sites were those where regional Department of Conservation staff confirmed a history of broad-scale brodifacoum use for vertebrate pest management, and no-exposure sites were those where no extensive brodifacoum use had occurred. If transport of the rats to the Landcare Research Animal Facility, Lincoln, took longer than 24 h, feed and water were provided. The disease status of the rats was assessed and they were acclimatised for at least 2 weeks to individual laboratory housing as described above. A calibration curve for plasma clotting times in wild rats was determined as described for laboratory rats. Data (Appendix 1b) were converted from time to clotting (seconds) to percentage clotting activity (PCA) relative to plasma dilutions, to produce a standard curve (Fig. 2).

TABLE 2. RESULTS OF BLOOD CLOTTING RESPONSE TIME (FACTOR-VII) TESTS IN WILD RATS (*Rattus norvegicus*) CAPTURED FROM 'EXPOSURE' OR 'NO EXPOSURE' FIELD SITES AND DOSED WITH 0.25 mg/kg BRODIFACOUM.

CAPTURE SITE	ANTICOAGULANT HISTORY	NO. RATS TESTED	DATE TESTED IN 2001	MINIMUM CLOTTING TIME AFTER DOSING (SECONDS)
West Coast site 1	No exposure	10	15 May	8 rats no clotting, 1 at 771 s, 1 at 903 s (< 10% PCA)
West Coast site 2	No exposure	13	10 May	11 rats no clotting, 1 at 799 s, 1 at 879 s (< 10% PCA)
DOC Northland	Exposure	2	18 October	No clotting
DOC Hawkes Bay	Exposure	9	22 August	8 rats no clotting, 1 at 622 s (< 10% PCA)
Lincoln dump	Exposure	3	21 May	2 rats no clotting, one at 884 s (< 10% PCA)
St Arnaud	Exposure	1	18 October	No clotting
Springs Junction	Exposure	8	10,14 May	7 rats no clotting, one at 842 s (< 10% PCA)
Lewis Pass	Exposure	8	15,16 May	No clotting

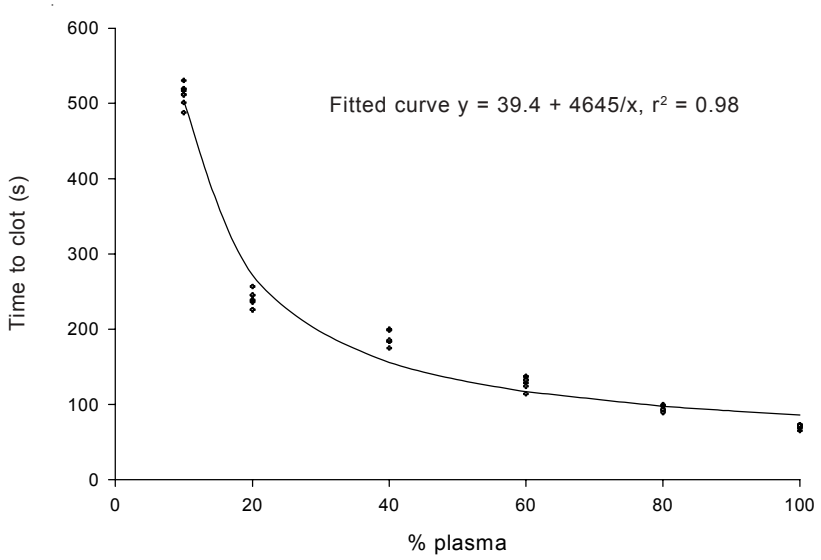


Figure 2. Standard curve of clotting times for wild rat plasma diluted with phosphate-buffered saline (pH 7.4). Raw data for the standard curve is shown in Appendix 1b.

4.2.2 Clotting response of wild rats to brodifacoum dose

Baseline blood samples were taken from wild rats (under light anaesthesia for these procedures) prior to dosing by oral gavage with 0.25 mg/kg brodifacoum in a solution of alkalinised water. This was expected to be close to a LD_{50} dose of brodifacoum for this species (after Godfrey 1985). After dosing, rats were returned to their housing and allowed to recover consciousness. One day later they were deeply anaesthetised and blood sampled, before being euthanased by cervical dislocation. Whole blood samples were assayed for factor-VII clotting time (commercial factor-VII testing kit, Dade Innovin). In comparisons of non-resistant and resistant rodents by Kerins et al. (1993), the 24-h prothrombin percentage clotting activity falls to below 10% of normal plasma in non-resistant animals and to about 50% in resistant animals. These values were used as criteria for 'resistance' in this trial.

4.3 DEVELOPMENT OF AN AVIAN PROTHROMBIN TIME (PT) TEST

4.3.1 Bird housing and calibration of clotting activity

Point-of-lay female chickens (*Gallus domesticus* brown Hyline strain) were sourced from Tegel Chickens, Christchurch, and housed in the Landcare Research Animal Facility, Lincoln in February 2001. Each bird was leg banded for identification, weighed, and housed separately. They were given free access to layer mash and water. Feed intake was assessed daily and weights noted at each handling.

A calibration curve for blood clotting times in chickens was required for later use in standardising the response measured in whole blood samples from chickens treated with brodifacoum. Plasma from ten untreated chickens was tested for PT in dilutions, and the data (Appendix 1c) were converted from time to clotting (seconds) to percentage clotting activity (PCA) relative to plasma dilutions, to produce a standard curve (Fig. 3). The curve was used to convert clotting times measured in the following trial to percentage clotting activity (PCA).

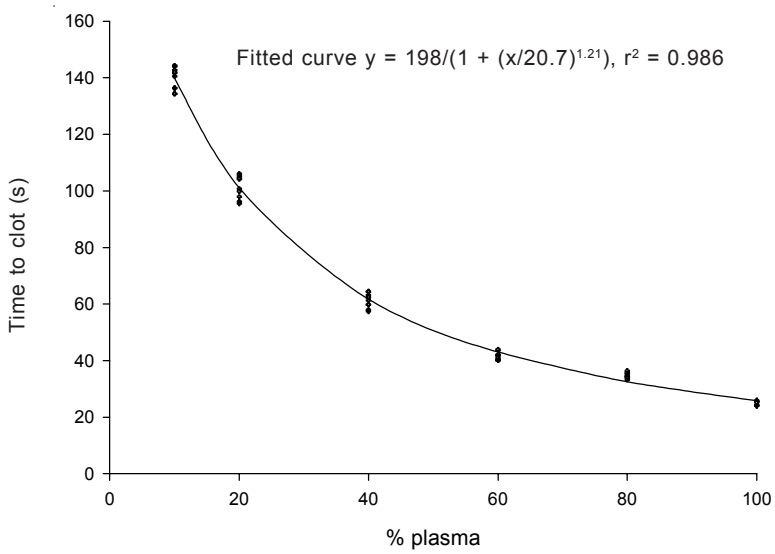


Figure 3. Standard curve of clotting times for chicken plasma diluted with phosphate-buffered saline (pH 7.4). Raw data for the standard curve is shown in Appendix 1c.

4.3.2 Clotting response of chickens to brodifacoum dose

Because of the likelihood of differences in the activity of brain tissue thromboplastin between birds and mammals, an avian thromboplastin solution was prepared as required for use in this trial with chickens (instead of the mammal-derived reagent provided in test kits). Following the methods of Doerr et al. (1975), twenty fresh chicken brains were homogenised, strained, dehydrated by addition of acetone, and stored in 1-g aliquots at –80°C. An extract was made by incubating 1 g of powdered brain tissue with 20 mL of 0.025 M $CaCL_2$ (with stirring) for 1 h at 37°C. The resulting suspension was centrifuged for 10 min at 500×*g* and the supernatant decanted. This extract was further diluted 1:7 with isotonic saline, the resulting mix giving a mean clotting time of 25 s for normal chicken blood.

Prior to dosing with brodifacoum, chickens were restrained without anaesthesia for blood sampling from the wing vein (e.g. UFAW 1987), and 0.5 mL blood samples were collected into syringes containing 50 mL of 0.22 M sodium citrate. Clotting time was determined by assaying these samples in duplicate using the avian thromboplastin solution and an Amelung coagulometer (Sigma Diagnostics, USA). Clotting time was expressed as PCA (percentage clotting activity derived from the standard curve of diluted control plasma). Chickens were randomly allocated into treatment groups for dosing with brodifacoum: either 3 mg/kg ('high dose', n = 10), 1.5 mg/kg ('medium dose', n = 8), 0.75 mg/kg ('low dose', n = 6) or 0 mg/kg ('control', n = 6). After dosing by oral gavage (maximum dose volume of 1 mL), chickens were checked at least twice daily during the first week for overt signs of toxicosis, and food and water intake was also monitored. On days 7, 14, 21 and 28 after dosage, birds were again blood sampled from the wing vein and the samples assayed for PCA. After the final blood sample was taken, all surviving chickens were euthanased by cervical dislocation while under halothane/oxygen anaesthesia.

4.4 BASELINE AND FIELD ASSESSMENTS OF PROTHROMBIN TIME IN NORTH ISLAND BROWN KIWI

North Island brown kiwi (*Apteryx australis mantelli)* from four different sites were sampled. Sites were classified as either 'exposure' or 'no exposure': the two 'exposure' sites (Rewarewa and Trounsen) were those where DOC staff confirmed a history of broad-scale brodifacoum use for vertebrate pest management, and thus potential for resident kiwi to have been exposed. The two 'no exposure' sites (Ripanui and Orana Park Zoo) were those where no extensive brodifacoum use had occurred.

Blood sampling was carried out by trained staff from DOC or Orana Park Zoo. Where possible, sampling of wild kiwi for this trial coincided with planned DOC sampling for DNA identification of kiwi in field sites. Kiwi were restrained without anaesthesia and a 200-mL blood sample was taken from the ankle vein, which was swabbed with a sterile isopropanol wipe before sampling and gentle pressure applied after sampling to encourage clotting to occur. No kiwi exhibited extended bleeding from the sample site. Samples were collected into syringes containing 20 mL of 0.22 M sodium citrate, and stored on ice to reduce degradation of clotting factors, which could have artificially increased clotting time. Clotting times were determined within 48 h of sampling, with samples assayed in duplicate using the reagents and methods described above for chickens. Because it was impractical to obtain larger volume whole blood samples required to establish a calibration curve for plasma clotting times in kiwi, the calibration curve established for chickens was used to derive PCA values from the clotting times measured in kiwi.

5. Results and discussion

5.1 CLOTTING RESPONSE OF LABORATORY RATS TO BRODIFACOUM DOSE

Baseline (Day 0) clotting times for vena cava blood samples were consistently faster (mean ± standard error, 15.7 ± 0.9 s) than those taken from the tail vein (68.8 ± 3.4 s). When clotting times were converted to PCA relative to the standard curve of clotting times for dilutions of laboratory rat plasma (Fig. 1.), in some cases the values were above or below the range of the curve, producing PCA values < 10% or > 100%. For the purposes of comparison, mean PCAs for tail vein samples (Fig. 4a) and vena cava samples (Fig. 4b) were calculated even if they fell beyond the range described by the standard curve. Tail vein samples showed the expected decrease in PCA (i.e. increased clotting times) after a sublethal dose of brodifacoum, with some samples taken from Day 2 onwards failing to clot at all (allocated a clotting time of 999s).

Samples taken from the vena cava (Fig. 4b) showed consistently higher PCA than those taken from the tail vein, with raw clotting times generally outside the range obtained in the standard curve. However, vena cava samples showed the same general trend, with decreased mean PCA at Day 2, which remained lower than baseline through to Day 8. Considerable differences between the clotting times of blood sourced from the extremities (tail vein), and those from the blood vessel closest to the liver (vena cava) may have been due to the relative depletion of clotting factors from blood as it returns from peripheral tissues. There may also have been some influence of deep anaesthesia for vena cava sampling on the clotting characteristics of the blood samples. We attempted to correlate tail vein to vena cava blood clotting times, but because of the number of tail vein samples that did not clot, the correlation between vena cava and tail vein clotting times was poor (r^2 ~0.3).

Differences in blood clotting parameters can occur dependent on which blood sampling site on the animal is used. This was evident in the difference in the clotting times of blood samples taken from the vena cava and tail vein of the same rats (Figs 4a & 4b), and highlights the importance of consistency in location and technique of blood sampling for anticoagulant response testing. In this instance, with laboratory rats, small-quantity venous blood sampling from the tail vein adequately described the response in blood clotting times to a single dose of brodifacoum, within the values for clotting times described by a standard curve (Fig. 1). Baseline raw clotting times of vena cava samples were approximately 16 s, compared with a range of 8.9–13.9 s reported in heart samples taken from Wistar laboratory rats (Karges et al. 1994). However, it is difficult to compare PCA values between studies because different reagents and strains of animals are used. For example, Kerins (1999) described mean baseline PCA values in laboratory rats sampled from the tail vein of between 103.7 and 150.0%, indicating considerable variability.

Figure 4a. Mean percentage clotting activity (PCA) of small-volume (200 µL) blood samples taken from the tail vein of laboratory rats following a sublethal (0.1 mg/kg) dose of brodifacoum. Error bars indicate the standard error of the mean PCA values.

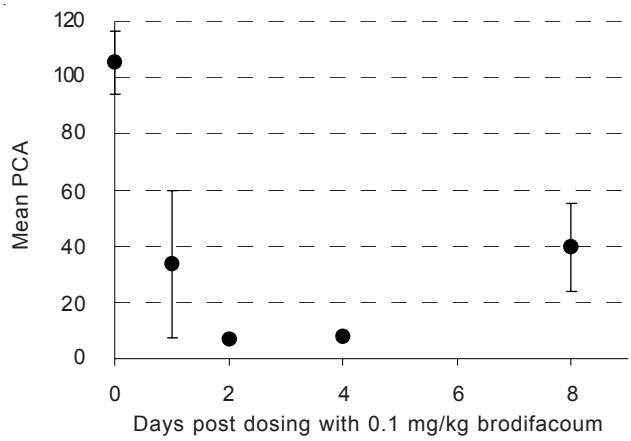
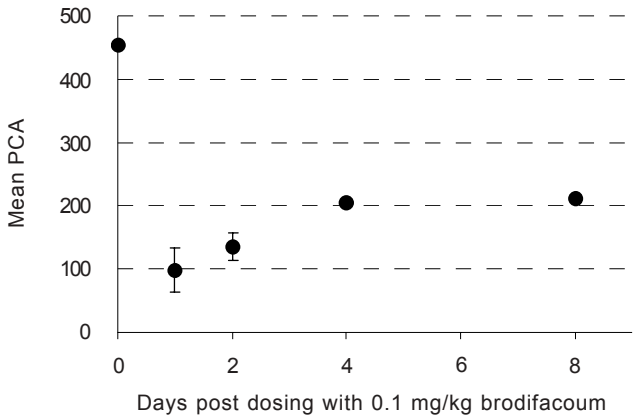


Figure 4b. Mean percentage clotting activity (PCA) of blood samples taken from the vena cava of anaesthetised laboratory rats following a sublethal (0.1 mg/kg) dose of brodifacoum. Error bars indicate the standard error of the mean PCA values.



The group of twelve rats that underwent a repeat dosing with 0.1 mg/kg brodifacoum on Day 64 (Table 1) showed a difference in clotting response to the first and second doses. Figure 5 shows that baseline (pre-dose) tail vein samples had a mean PCA approximating 100% (i.e. normal clotting), with considerable increase in mean clotting times (i.e. decreased PCA) at Days 1 and 2 after the first dose. By Day 34 after the first dose, the clotting times were slightly, but not significantly, below those of rats not exposed to brodifacoum. The second dose on Day 64 was followed by increased clotting times to only the equivalent of 50% PCA, compared with < 10% following the first dose. The elevation of clotting times following the second dose also appeared to be shorter-lived than those following the first dose (Fig. 5). The reduced response in clotting time of laboratory rats to a second dose of brodifacoum highlights gaps in information on the toxicodynamics of brodifacoum. However, it seems likely that a dose that would be lethal to naive animals (which did not have increased thrombin levels) might not be lethal to previously exposed animals. 'Overcompensation' in clotting times of rats that received repeated doses of difenacoum, thought to be due to increased thrombin levels, was noted by Kerins et al. (1999). While it is uncertain whether the response to second dose observed here was overcompensation, these results may have implications for the development of tolerance in rodents receiving small repeated doses of brodifacoum. This warrants further investigation by confirming the effect on blood clotting time of a range of repeat doses of anticoagulant.

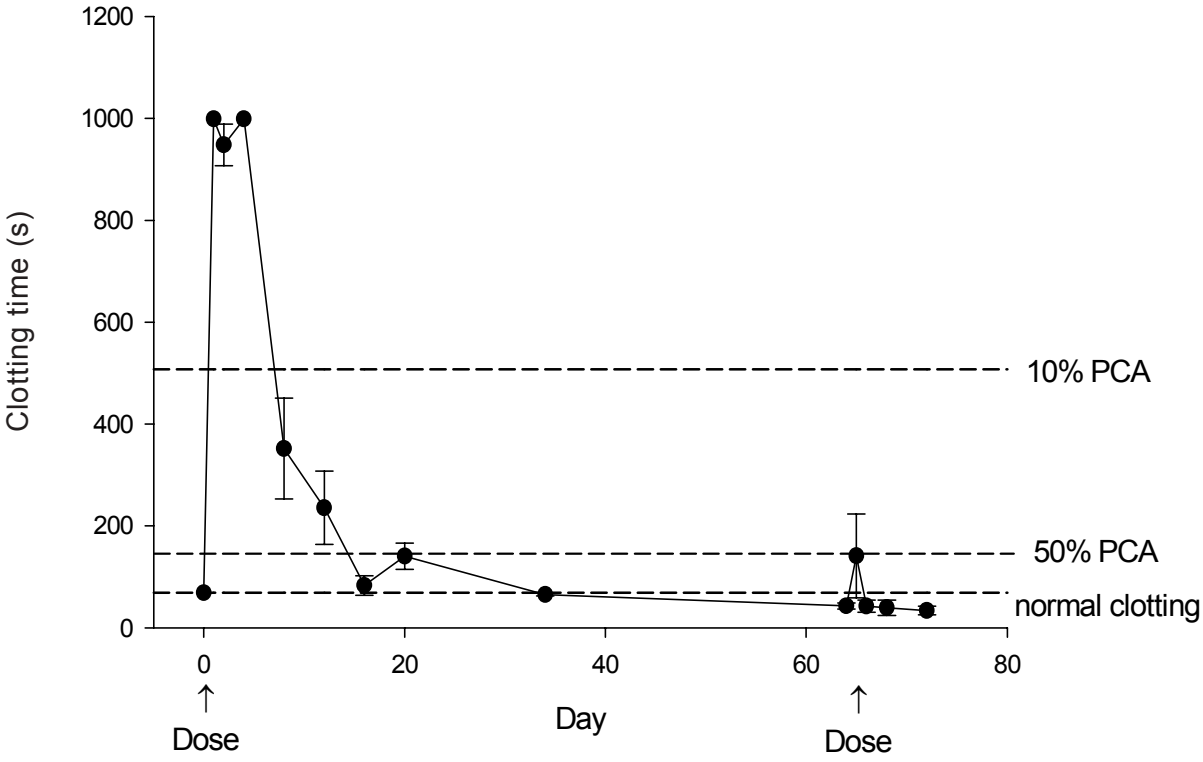


Figure 5. Mean percent clotting activity (PCA) for tail vein samples taken from rats dosed with 0.1 mg/kg brodifacoum on Days 0 and 64. Bars represent standard error.

5.2 RESISTANCE TESTING IN WILD RATS

When clotting times were converted to PCA relative to the standard curve of clotting times for dilutions of wild rat plasma (Fig. 2), we found none of the wild rats tested exhibited PCA values greater than the 10% threshold indicative of resistance (Table 2). However, this result only indicates that no resistant individuals were sampled, i.e. resistant individuals may be have been present at low density and not adequately sampled by trapping. Resistance to a second-generation anticoagulant (brodifacoum) has been reported in wild *Rattus norvegicus* in the United Kingdom (Gill et al. 1992). Although wild and laboratory-reared *Rattus norvegicus* were used in this study, *R. rattus* is probably more common in New Zealand forests than *R. norvegicus* and *R. exulans*. *Rattus rattus* is recognised as having significant conservation impacts and is more commonly subject to poison baiting to manage its impacts than *R. norvegicus* and *R. exulans*. It will be important to consider the possibility of rodent species differences and of resistance developing in populations of all three rat species, if they are subject to ongoing control with anticoagulant poisons. The methods described here could be applied to resistance testing in populations of other wild rodents in New Zealand.

5.3 DEVELOPMENT OF AN AVIAN PROTHROMBIN TIME (PT) TEST

The high dose of brodifacoum (3 mg/kg) was lethal to all ten chickens dosed. There was no significant difference in the mean number of eggs laid per chicken per day over 32 days in any of the dose groups (high dose group = 0.70, medium dose group = 0.73, low dose group = 0.78 and control group = 0.71). When clotting times were converted to PCA relative to the standard curve of clotting times for dilutions of chicken plasma (Fig. 3), decreases in PCA of chickens appeared to be dependent on the sublethal dose of brodifacoum received (Table 3). Chickens exhibited a dose-dependent response in clotting times to a range of brodifacoum doses that was consistent with expected mode of action, indicating that the small-volume blood sampling technique was valid. In other studies using chicken-based substrates, mean prothrombin times in chickens were 38.6 s, 38.4 s and 9.9 s (Frost et al. 1999), as compared with the mean clotting time of 100% chicken plasma in this study of 25 s (Fig. 5). Another study of prothrombin time in birds, using species-specific brain thromboplastin reagents, found a mean time of 25 s in pigeons, and 18.2 s in kites (Tahira et al. 1977).

TABLE 3. BLOOD-CLOTTING TIMES, EXPRESSED AS MEAN PERCENT CLOTTING ACTIVITY (PCA), IN CHICKENS GIVEN DOSES OF BRODIFACOUM. THE CONTROL GROUP (0 mg/kg DOSE) REPRESENTS BASELINE VALUES.

SAMPLE DAY	BRODIFACOUM DOSE			
	0 mg/kg	0.75 mg/kg	1.5 mg/kg	3 mg/kg
0	103.1	103.5	102.7	103.1
7	103.9	6.1	3.2	0.0
14	102.3	18.5	5.1	2.0
21	101.9	42.3	28.4	27.3
28	104.3	112.4	66.1	All dead

5.4 BASELINE AND FIELD ASSESSMENTS OF PROTHROMBIN TIME IN NORTH ISLAND BROWN KIWI

There was a clear statistical difference in clotting times between kiwi from 'no exposure' and 'exposure' sites (*t*-test, $P = 4.74 \times 10^{-14}$) (Table 4). Kiwi from areas with a history of brodifacoum use had blood clotting times generally lower than those from areas with no history of brodifacoum use. This result was the opposite of what would be expected. It should be noted that the number of kiwi sampled here was small, and exposure (or not) of the kiwi to brodifacoum was only assumed from the history of the sites from which they were sampled. This approach was taken because an experiment that deliberately exposed kiwi to brodifacoum was considered unacceptable. Since we did not analyse the blood-sampled kiwi for brodifacoum residues—which would have helped to confirm exposure, had residues been detected—attribution of the effect on blood clotting to anticoagulant exposure can only be suggestive. Also, there is

TABLE 4. RESULTS OF PLASMA-CLOTTING-TIME TESTS IN NORTH ISLAND BROWN KIWI.

SITE	ANTICOAGULANT HISTORY	NO. KIWI SAMPLED (SEX)	DATE TESTED	MEAN CLOTTING TIME (SE) (SECONDS)
Ripanui	No exposure	3 (M)	16 May 2002	23.7 (0.21)
Orana Park Zoo	No exposure	4 (F)	12 June 2001	24.3 (0.22)
Rewarewa	Exposure	6 (M), 7 (F)	3 & 23 January 2002	18.0 (0.37)
Trounsen	Exposure	3 (M), 3 (F), 3 (unknown)	14 May 2002	20.1 (0.21)

no information available regarding the normal varaibility of blood clotting times in kiwi, and the effect of sex, age, season or other variables on blood clotting times—factors which varied amongst the sampled kiwi. It is also difficult to compare results in kiwi with what might be expected in mammal species, as a number of differences in the clotting mechanisms of avian and mammalian blood are recognised. Birds have generally slower coagulation mechanisms than mammals (Frost et al. 1999), avian blood lacks platelets, and while the major factors involved in avian blood coagulation appear similar to mammalian systems, it is likely that the coagulation of avian blood is more dependent on an extrinsic system involving the release of tissue thromboplastin (Griminger 1986). Also, there may be variability in clotting mechanisms between bird species. For example, clotting factors VII, IX, X, XI and XII were absent from ostrich plasma taken from wing veins and it was suggested that ostrich blood coagulation mechanisms may not be as intricate as those in other avian species (Frost et al. 1999). It is difficult to directly compare the clotting times obtained for kiwi here with those measured in birds in other studies because a wide variety of reagents and substrates have been used. Prothrombin times of ostrich plasma taken from wing veins were a mean of 90.1 s using chicken-based substrate and a mean of 73 s using an ostrich-based substrate (Frost et al. 1999), whereas in this study these times for kiwi, using a different chicken-based substrate, were approximately 20 s (Table 4).

It is not known how clotting in kiwi compares with that in other bird species, and how other factors such as sex, age, diet and stress might influence clotting times within a population of kiwi. Robertson & Colbourne (2001) calculated that, at worst, 8% of little spotted kiwi (*Apteryx owenii*) exposed to brodifacoum on Kapiti Island during a pest eradication campaign were killed by accidental (acute) poisoning. However, the specific effects of sublethal exposure of kiwi to brodifacoum, in terms of altered clotting times, long-term survival and reproductive fitness, remains unknown. Larger sample sizes, and an improved certainty of exposure history to brodifacoum (or other anticoagulants) are needed before the methods described here could be used as a reliable indicator of sub-lethal exposure in kiwi.

6. Conclusions and recommendations

The use of minimally invasive, small-volume (tail vein) blood sampling in rats appears to be practicable in the assessment of clotting time responses to anticoagulant exposure. Resistance to brodifacoum was not evident in the limited number of wild Norway rats tested; however, where anticoagulants are part of ongoing pest control strategies, field populations of target rodents should be tested for resistance at regular intervals. The lowered clotting times of laboratory rats to a second sublethal dose of brodifacoum highlights the paucity of information on the toxicodynamics of brodifacoum. Characterisation of the responses to repeated brodifacoum (or other anticoagulant) exposure is needed to better understand the potential effects of brodifacoum in both target and non-target species, and the effect of repeated, sublethal doses on the efficacy of brodifacoum against target rodents. The differences in clotting times of kiwi from areas determined as 'exposure' or 'no exposure' to brodifacoum were significant, and opposite to the result that would be expected if the exposure profiled was as assumed. The influence of variables such as sex, age and season on blood clotting times is unknown and brodifacoum residues in kiwi were not measured. Further investigation of the effect of brodifacoum exposure, and other variables such as sex, age and season on blood-clotting response times of kiwi, and other native birds, should be carried out. The small-volume blood sampling technique described here for birds would have application in such trials, and has the potential to reduce the invasiveness and ethical costs of sampling wildlife species.

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Appendix 1

Clotting times were converted from time to clotting (seconds) to percentage clotting activity (PCA) relative to dilutions of plasma from untreated rats or chickens. The tables below show the data generated from dilutions of standard plasma and used to produce a standard curve for laboratory rat, wild rat and chicken PCA, respectively

Appendix 1a. Raw data for standard curve of clotting times for laboratory rat plasma diluted with phosphate-buffered saline (pH 7.4).

% PLASMA	REP1	REP2	REP3	REP4	REP5	REP6	REP7	REP8	MEAN	SE
100	71.8	70.4	71.6	67.7	68.2	66.9	69.2	72.3	69.8	0.73
80	96.8	93.5	97.1	95.8	94.3	92.7	95.1	96	95.2	0.55
60	127.5	127.3	125.5	124.7	122.2	128.7	126	125.6	125.9	0.70
40	186	179.3	192.7	191.1	188.7	180.1	193	190.3	187.7	1.91
20	245.3	249.2	263.1	262.7	246.1	246.5	246.5	265.4	253.1	3.15
10	509.8	500.7	507.9	500.1	505.3	514.1	510.6	512.6	507.6	1.84

Appendix 1b. Raw data for standard curve of clotting times for wild rat (control) plasma diluted with phosphate-buffered saline (pH 7.4).

% PLASMA	REP1	REP2	REP3	REP4	REP5	REP6	REP7	REP8	MEAN	SE
100	70.1	71.2	73.2	67.5	68.4	71.1	65.3	72	69.9	0.9
80	98.5	97.1	93.1	95.7	90.5	89	99.5	93	94.6	1.3
60	128.2	113.9	132.4	113.8	136.9	132	137.3	124.1	127.3	3.3
40	184.1	200.1	184.1	180.6	198.8	174.6	199.3	185.8	188.4	3.4
20	244.9	256.8	225.9	222	236	239.3	237.7	245.6	238.5	3.9
10	530.4	501.1	519.4	521	516.7	487.8	511.9	511.2	512.4	4.6

Appendix 1c. Raw data for standard curve of mean clotting times for chicken (control) plasma diluted with phosphate-buffered saline (pH 7.4)

% PLASMA	MEAN CLOTTING TIME (SECONDS)	SE
100	25.0	0.24
80	34.8	0.31
60	41.9	0.41
40	61.6	0.78
20	101.6	1.26
10	140.1	1.23