Residual Concentrations and Persistence of the Anticoagulant Rodenticides Brodifacoum and Diphacinone in Fauna

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Abstract of a thesis submitted in partial fulfilment

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Residual Concentrations and Persistence of the Anticoagulant Rodenticides Brodifacoum and Diphacinone in fauna

P.M. Fisher

Brodifacoum is a highly effective anticoagulant rodenticide that presents a secondary hazard to some non-target wildlife. The high acute toxicity of brodifacoum to mammals and birds, and its prolonged persistence in liver predicates secondary risk to predators and scavengers of poisoned rodents. Hence there is a need to improve ability to monitor and predict hazards of brodifacoum to non-targets, and optimise use patterns accordingly. Use of a less persistent anticoagulant rodenticide, diphacinone, is an alternative approach currently under investigation in New Zealand. This thesis describes a series of laboratory and pen studies that address information gaps relevant to the assessment of non-target hazards in continued use of brodifacoum, and of using diphacinone as an alternative.

Non-lethal techniques for determining sublethal brodifacoum exposure in birds was investigated in chickens. Elevation of prothrombin time was a less reliable index than residual concentrations in tissues. Samples requiring less invasive procedures, such as dried blood spots or faeces, have potential to detect recent sublethal brodifacoum exposure and refinement of these indices could be useful in proactive monitoring of avian wildlife. Residual brodifacoum in eggs of sublethally-exposed hens raised further questions regarding wider non-target hazard and adverse effects on development of fertile eggs or chicks. A laboratory trial with rats found a positive correlation between residual brodifacoum concentrations in liver and the amount of brodifacoum ingested as bait. An estimated 14-22% of ingested brodifacoum was excreted in rat faeces in the period

between ingestion of a lethal dose and death, indicating another potentially significant environmental pathway for brodifacoum transfer.

In considering diphacinone as a less persistent alternative rodenticide to brodifacoum, evaluation of residual concentrations and persistence in pig tissues was required to estimate secondary hazard to human consumers and adequate with-holding periods for hunting feral pigs in areas where diphacinone was applied. A pen trial showed that domestic pigs were more susceptible to diphacinone toxicity, and thus primary poisoning risk, than previously estimated. Hepatic half-life of diphacinone in pigs was approximately 14 days, indicating reduced persistence in comparison to brodifacoum and enabling estimates of with-holding periods for hunting feral pigs from areas where diphacinone baits were applied. To investigate potential hazards of diphacinone use to invertebrates a trial using tree weta, a native New Zealand invertebrate, was undertaken. Weta readily ate diphacinone wax block baits with no mortality or weight loss evident, indicating low susceptibility. Residual whole-body diphacinone concentrations did not increase with the amount of diphacinone bait eaten. A simple, deterministic risk assessment suggested that, as a single secondary exposure, the maximum diphacinone concentration measured in weta would present a low risk to non-target birds.

Given international recognition of the high secondary hazard and corresponding restrictions on use of brodifacoum, continued availability of brodifacoum to non-licensed users and sustained field applications for possum and rodent control in New Zealand is an exceptional use pattern. New data in this thesis suggest that baiting strategies that minimise the amount of brodifacoum available in the environment are important and regulatory review of some New Zealand brodifacoum applications should address this. In parallel, development of diphacinone as an alternative to brodifacoum should continue, as new data here confirms lower persistence in mammalian liver than brodifacoum, and also indicates low toxicity to invertebrates. However further investigation of multiple-exposure hazard and potential sublethal effects of diphacinone on non-target mammals and birds is warranted before extensive and sustained field applications of diphacinone are undertaken.

Keywords: anticoagulant, brodifacoum, diphacinone, elimination half-life, invertebrates, liver, non-target wildlife, pigs, residues, rodents, rodenticide, secondary poisoning, sublethal effects

Contents

List of Tables

List of Figures

Chapter 1: Brodifacoum as a vertebrate pesticide and the need to monitor non-target wildlife

1.1 Context of thesis

Anticoagulant poisons have an ongoing history of worldwide use for the control of pest vertebrates, particularly rodents. Balancing benefits of rodent control and the potential costs of applying anticoagulant rodenticides to the environment remains a challenge. This can be addressed by risk assessments, which require data regarding the movement and degradation of anticoagulants in different environmental media, and their effects on non-target organisms including humans. This thesis comprises a series of studies initiated by the climate of vertebrate pest management in New Zealand (NZ) but also have relevance to international uses of anticoagulant rodenticides. In NZ, application of brodifacoum for control of introduced vertebrate pests has been shown to create a persistent secondary hazard to some non-target species. For government agencies, private landholders and other resource managers that retain brodifacoum as a tool for effective pest control there is an interim need for better understanding of how to minimise the non-target risks. Replacing brodifacoum with an equally effective but less persistent alternative anticoagulant has been identified as a progressive priority for NZ vertebrate pest management. Figure 1.3 outlines the thesis structure as a sequence of review, potential options for anticoagulant use and corresponding research questions that were investigated in laboratory studies. While many other research questions are also relevant, the range of specific studies completed here were identified as 'data gap' priorities for a current management situation in NZ, where the results would be applied to on-ground applications of anticoagulants and policies regarding their use. In some cases this was research directed by management agencies (e.g. NZ Department of Conservation). Literature review and dialogue with pest managers identified the importance of avian and invertebrate species in the NZ context of non-target native wildlife, and also the issue of potential contamination of feral pigs that are hunted for human consumption.

1.2 Use patterns of brodifacoum for vertebrate pest control

In common with a range of toxic anticoagulant compounds used as rodenticides, brodifacoum inhibits the synthesis of vitamin K-dependent blood clotting factors in the liver (e.g. Thijssen 1995) by binding to the active site of vitamin K epoxide reductase (VKER) (Gebauer 2007). When this inhibition occurs over a sufficient time, blood will fail to coagulate in response to injury and typical clinical signs of anticoagulant toxicity are haemorrhage and anemia, with death

through massive haemorrhage occurring several days after a lethal exposure (Pelfrene 2001). This delayed onset of toxicity is an important factor in the effectiveness of anticoagulant rodenticides, as rodents are less likely to associate ingestion of bait with onset of illness, and then avoid ingestion of a lethal amount (Kaukeinen & Rampaud 1986). Brodifacoum is a synthetic compound, developed in 1976 as a rodenticide (British Crop Protection Council 2000). As a second generation anticoagulant (see Chapter 4.1), brodifacoum has high oral toxicity and a lethal dose is usually ingested by target rodents in a single feed of bait, making it a very effective rodenticide (Kaukeinen & Rampaud 1986). Worldwide the economic, environmental and human health benefits of using anticoagulant rodenticides is undoubted. The use patterns of brodifacoum for the control of rodents and other vertebrate pests are briefly reviewed here.

1.2.1 Commensal rodent control

Brodifacoum is used in many countries to manage commensal rodents that share habitat and food sources with humans, particularly Norway rats (*Rattus norvegicus*), ship rats (*R. rattus*) and house mice (*Mus musculus*). Proprietary bait formulations containing brodifacoum are registered in various countries (Kegley *et al.* 2007), with application generally limited to 'indoor' use, as defined by proximity to human habitation and infrastructure e.g. farms, sewers, factories and warehouses. To minimise exposure of humans or non-target wildlife to bait, label instructions may specify use only inside or within a nominated distance of buildings, or use of fixed baits in tamper-proof bait stations. In some countries bait formulations containing brodifacoum are available to the public 'over the counter' for household rodent control while in others, such as the United Kingdom, brodifacoum use is restricted to indoor use by licensed or professional applicators (Quy *et al*. 1998). The ongoing development of resistance to second-generation anticoagulants in British rat populations (Smith 2007) has recently required special permission for the outdoor use of brodifacoum, as the most toxic of the anticoagulants, to effectively control intractable Norway rat populations (Meyer 2007).

1.2.2 Controlling field populations of vertebrate pests

The scale of field application of vertebrate pesticides in NZ contrasts with other countries (e.g. Green 2004), reflecting policy imperatives for immediate mitigation of severe impacts of introduced mammals in the general absence of (non-target) terrestrial native mammals. Introduced species such as ship rats, Norway rats, house mice and brushtail possums (*Trichosurus vulpecula*) pose serious threats to indigenous NZ species and biodiversity values

(Anonymous 2000) and possums threaten agricultural production as vectors of bovine tuberculosis (Animal Health Board Incorporated 2001). Broadscale field application of toxic baits will probably remain an important component of regional NZ vertebrate pest management strategies in the short to medium term, until more acceptable and effective control methods (e.g. fertility control, Cowan 2000) become operationally available.

In particular, brodifacoum use in NZ differs from most other countries. 'Over the counter' anticoagulant baits for commensal rodent control are available but bait formulations (0.005% or 0.002% brodifacoum by weight) are also registered for field use against rodents and brushtail possums. Earlier field uses against rabbits (*Oryctolagus cuniculus*) and wallabies (*Macropus rufogriseus*) have been discontinued (Bell 1983). Hoare & Hare (2006a) provide a recent overview of brodifacoum use in NZ; bait station deployments of brodifacoum can cover considerable mainland areas (up to 300,000 ha) and may be sustained for a number of years in key areas. A 'controlled substances' licence is not required for purchase and field application of brodifacoum (National Possum Control Agencies 2006), so of the estimated 6 kg brodifacoum (as active ingredient in bait) sold annually, approximately 50% is used by professional pest contractors, 30% by regional councils and 5% by private landowners (Hoare & Hare 2006a). The NZ government's Department of Conservation (DOC) uses the remaining estimated 15% of brodifacoum, but restrict sustained brodifacoum use over public conservation land, because of concerns for secondary effects on non-target wildlife (Hoare & Hare 2006a) - current DOC applications of brodifacoum are mostly for the eradication of introduced rodents from islands.

1.2.3 Rodent eradications from offshore and mainland islands

Over the last two decades, broad-scale aerial application of brodifacoum bait has developed as an important conservation tool, instrumental in successful eradication of invasive rodents from an increasing number offshore islands around NZ (Towns & Broome 2003) and elsewhere (Howald *et al.* 2007). Aerial application is also integral to the establishment of "mainland island" sanctuaries in NZ, through complete and rapid removal of vertebrate pests from areas protected by exclusion fencing (e.g. Maungatautari Ecological Trust 2004). The 'single-feed' efficacy of brodifacoum against rodents is an important criterion in island eradications - Pestoff® Rodent Bait 20R (20 ppm brodifacoum), a cereal pellet formulation, is registered for these uses in NZ, with label instructions limiting aerial and/or hand broadcast to non-stocked offshore islands, or within areas enclosed by an effective pest-proof fence (Anonymous 2006). In contrast to

commensal rodent control and sustained field control operations using brodifacoum, eradication operations are deliberately engineered to be 'one-off' bait applications, entailing clearly defined quantities, rates and timeframes for bait application. As outlined in Chapter 4.1, the time over which brodifacoum is available in the environment is an important consideration when weighing potential risks to non-target wildlife.

1.3 Brodifacoum in the environment and hazard to non-target wildlife

The comparative pharmacokinetics of anticoagulants are discussed in more detail in Chapter 4. In brief, after ingestion and absorption, the persistence of an anticoagulant in tissues is influenced by the dose ingested and relative affinity of the compound for specific, high-affinity, saturable binding sites for anticoagulants (Parmar & Batten 1987). Residual concentrations of brodifacoum have been shown to persist for prolonged periods in the livers of live mammals (Eason *et al.* 1996: Laas *et al.* 1985; Fisher *et al.* 2003). On this basis, liver tissue has been the focus for detecting exposure to anticoagulants. Bachman & Sullivan (1983) predicted that serum and liver concentrations of brodifacoum would bioaccumulate in rats with repeated daily exposures. Bioaccumulation of flocoumafen, another persistent second-generation anticoagulant, has been demonstrated in rats (Huckle *et al.* 1988). Thus it is considered likely that brodifacoum would bioaccumulate in an 'environmental reservoir' of mammalian, and probably avian, liver with repeated exposure.

An effective vertebrate pesticide requires high acute toxicity to the target species but brodifacoum is a broad spectrum toxicant, posing an unwanted hazard for non-target mammals and birds that ingest bait (primary exposure) or tissues of animals poisoned by brodifacoum (secondary exposure). Brodifacoum presents a relatively high risk of secondary poisoning in comparison to other rodenticides (Erickson & Urban 2002), through a relatively prolonged residual persistence especially in liver. Over the last two decades, reports of secondary poisoning and the presence of residual brodifacoum in tissues of non-target wildlife, particularly predatory or scavenging mammals and birds appear to be increasing – these are briefly reviewed here.

1.3.1 Primary and secondary exposure pathways

Abundant literature concerning accidental poisoning of domestic animals following anticoagulant application for commensal rodent control (Robben 1998; Petterino *et al.* 2004) illustrates the primary non-target hazard of toxic bait. As discussed (section 1.1.1) non-target access to bait can be minimised by physical exclusion or reducing the attractiveness / palatability of bait. However in other instances e.g. broadcast applications, improper bait placement or when rodents cache baits, it becomes more likely that some non-target species (including invertebrates) will encounter bait and find it edible. Field applications of brodifacoum in NZ have resulted in primary poisoning of a range of non-target bird species, as summarized by Eason *et al.* (2002) and in Figure 1.1. Because brodifacoum is not mobile in water, air, soil or plants in the context of bait applications (Eason and Wickstrom 2001) primary exposure to bait is the predominant precursor to secondary routes of environmental exposure via tissues of living animals or carcasses.

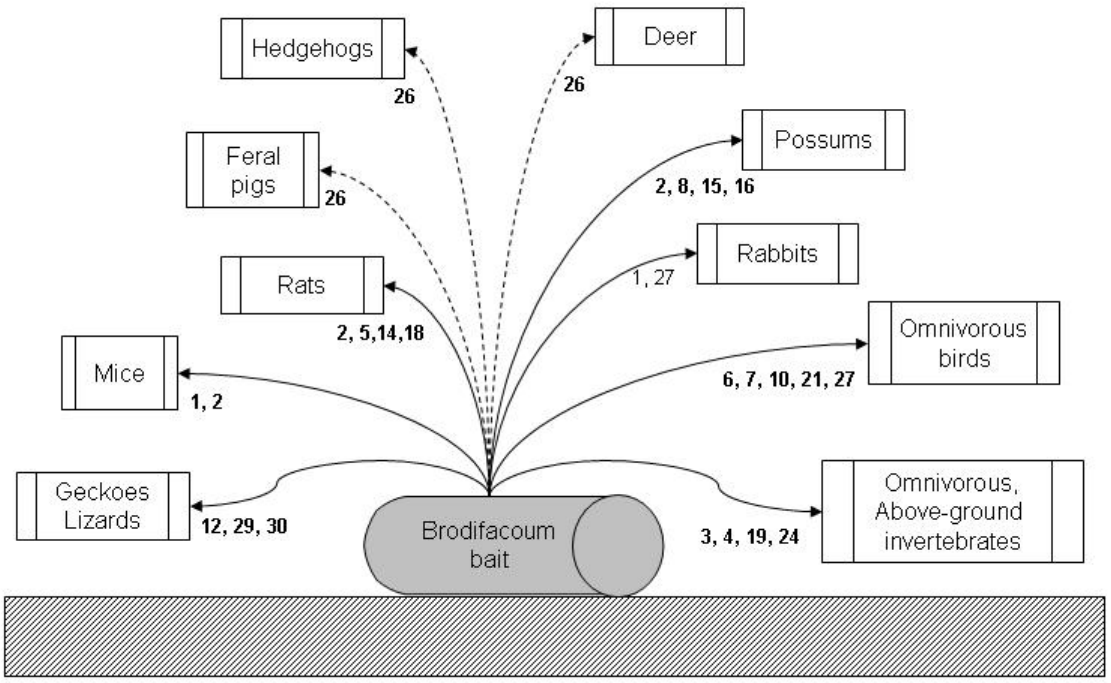


Figure 1.1 Primary exposure to cereal-based brodifacoum baits (station or aerial broadcast applications). Solid lines indicate documented instances of bait uptake, presence of residues, field mortality or pen-based studies indicating exposure, with corresponding numbered references cited in Appendix 1a. Dashed lines indicate a strong inference of primary exposure due to dietary habits and/or analysis of residual brodifacoum in the species.

Oral exposure to brodifacoum may be lethal or sublethal, depending on the amount ingested and the susceptibility of the animal to anticoagulant toxicity. Studies with captive birds and animals in artificial feeding situations have demonstrated the potential for secondary mortality to occur especially in the case of second-generation anticoagulants (e.g. Joermann 1988) and this has been confirmed by a number of studies. In NZ, stoats (*Mustela erminea*), ferrets (*Mustela furo*) and feral cats (*Felis catus*) were killed following brodifacoum bait application (Alterio 1996; Alterio and Moller 2000) although in these cases the 'non-target' species were also considered pests, so their mortality was regarded as a desirable side-effect of control operations targeting rabbits and possums. Figure 1.2 summarises some of the pathways of secondary exposure to brodifacoum that have been reported for NZ species.

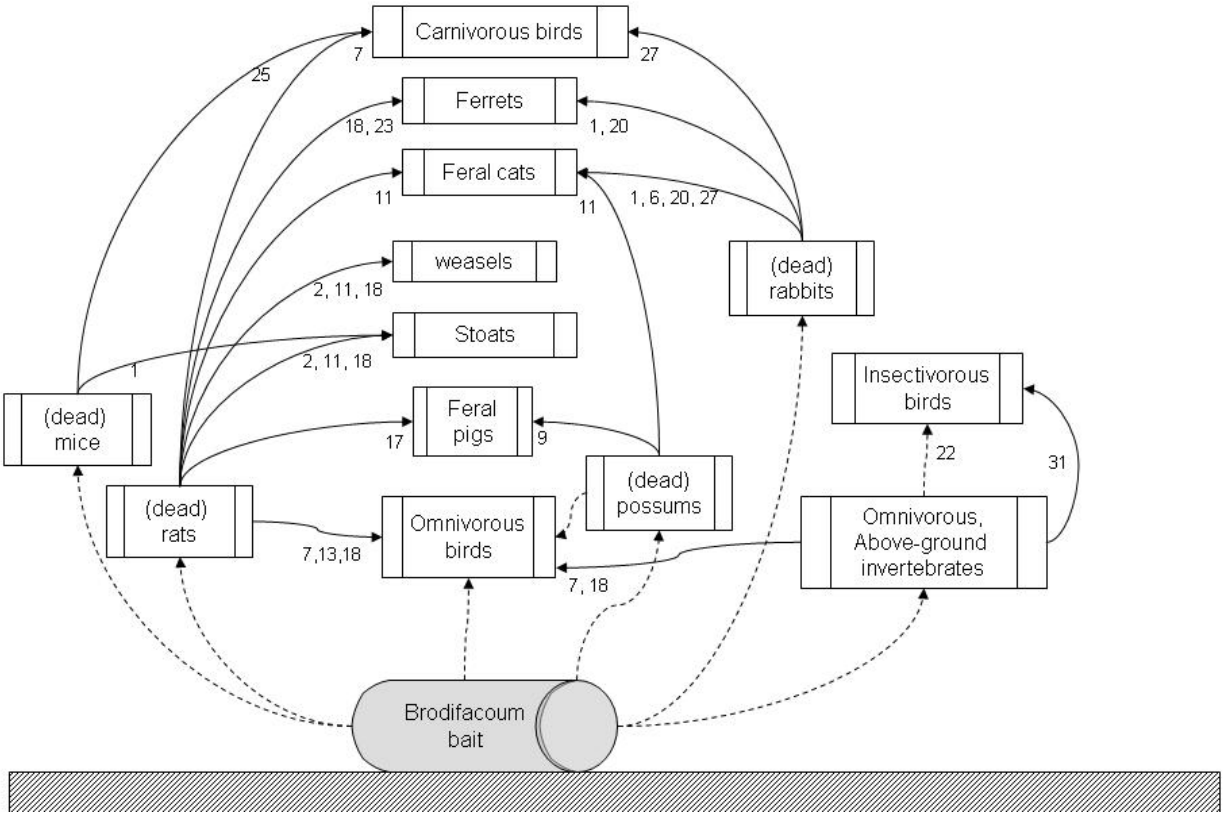


Figure 1.2 Secondary exposure pathways for brodifacoum. Solid lines indicate documented instances of preying on or scavenging animal tissues containing brodifacoum, presence of residues, field mortality or pen-based studies indicating exposure, with corresponding numbered references cited in Appendix 1a.

From a wider international perspective, non-target wildlife mortality is also indicated by reports of toxicosis and death following known or strongly inferred secondary brodifacoum exposure, for example in owls (Mendenhall and Pank 1980), moreporks (*Ninox novaseelandiae*) (Stephenson *et al.* 1999), ravens (*Corvus corcorax*) (Taylor *et al.* 2000), bobcats (*Lynx rufus*) and mountain lions (*Puma concolor*) (Riley *et al.* 2007). In the United Kingdom, United States and Canada, brodifacoum use is restricted to commensal rodent control (indoor) applications yet predatory or scavenging wildlife species have been exposed. This is presumably through transfer of brodifacoum in live prey. Spurr *et al.* (2005) provide recent evidence of commensal applications of anticoagulant bait associated with occurrence of residues in non-target wildlife in the surrounding areas. Similarly Brakes and Smith (2005) demonstrate that routine commensal rat control using an anticoagulant (coumatetralyl) reduced local populations of non-target small mammals, demonstrating a significant route of secondary anticoagulant exposure for predators and scavengers. Borst and Counott (2002) document mortality from secondary brodifacoum exposure in captive bird species: two turkey vulture (*Cathartes aura*) chicks that died of toxicosis after being fed rodenticide-killed mice by the adult birds, previous case reports of small carnivorous birds (*Dacelo novae-guinae* and *Tockus deckeni*) killed after eating poisoned mice and a granivorous bird (*Rollulus roulroul*) that died, probably through contamination of its food by cockroaches that transported brodifacoum. Murray and Tseng (2008) report on diagnosis and treatment of secondary brodifacoum poisoning in a red-tailed hawk (*Buteo jamaicensis*). Dowding *et al.* (2006) provided strong field-based evidence of invertebrates as a source of secondary exposure, documenting mortality and brodifacoum residue in New Zealand dotterels (*Charadrius obscurus aquilonius*) that had apparently fed on invertebrates containing brodifacoum as the result of feeding on toxic bait.

1.3.2 Liver concentrations as an index of brodifacoum exposure

Retention of brodifacoum in liver provides an obvious focus for monitoring wildlife exposure and a growing literature documents the occurrence of residues of brodifacoum in a range of predatory and scavenging species worldwide. Appendix 1b presents three tables summarising liver brodifacoum concentrations measured in non-target birds and animals, grouped by scenario;

A. known or inferred to have been killed by brodifacoum poisoning (Table A)

B. exposure inferred from presence of liver residues, but could not be confirmed as lethal or sublethal (Table B)

C. sublethal exposure to brodifacoum (Table C)

Residue data in Table A were from studies involving deliberate searching for carcasses in areas where brodifacoum bait had been recently applied, or from human and veterinary clinical cases reported as brodifacoum poisoning. The presence of liver residues, sometimes associated with observations of haemorrhage, thus provided strong inferential evidence of lethal exposure. In general, the residue concentrations measured in this scenario are relatively high.

Residue data in Table B are from animals or birds collected as carcasses in general monitoring programmes e.g. as roadkills or from wildlife rehabilitation centres, in all instances from United Kingdom or the United States. Although brodifacoum exposure was confirmed by detectable liver concentrations, the timing and frequency of the exposure could not be retrospectively determined. There are proportionally more low concentrations of brodifacoum across this scenario, raising questions of whether the exposure was sufficient to have caused or contributed to mortality. In some instances, the presence of liver residues in association with pathologic observations of haemhorrage prompted diagnosis of anticoagulant poisoning as the cause of death (e.g. Hosea 2000; Stone *et al.* 2003; Hoops 2005).

Residue data in Table C are from live capture (thus exposure assumed to be sublethal) of animals or birds that were then killed to obtain liver tissue, often following brodifacoum bait applications. These are mostly from NZ field monitoring but also include captive studies and field monitoring from other countries. The residue concentrations in this scenario span those reported in Tables A and B, highlighting uncertainty about how diagnostic liver residue concentrations are of lethal exposure to brodifacoum. Kaukeinen *et al.* (2000) proposed a threshold liver concentration of 0.7 ppm i.e. concentrations below this in carcasses are not expected to have caused acute toxicity. However, Littin *et al.* (2002) measured liver concentrations as low as 0.33 ppm in brushtail possums that had died of brodifacoum poisoning. From studies of brodifacoum exposure and resulting residues in voles (*Microtus* spp.), Myllymäki *et al.* (1999) estimated that the probability of vole survival started to decrease clearly in association with liver concentrations of 0.20 ppm.

While mortality is the most unwanted outcome for non-target wildlife, use of liver tissue predisposes to generally retrospective monitoring – looking to confirm exposure and its possible contribution to mortality after the fact. Reliance on liver tissue to determine exposure creates

limitations; (*i*) it is generally not acceptable to kill (protected) non-target wildlife species considered at risk of exposure in order to determine whether this has occurred so that (*ii*) residue data for many 'at risk' species that are based largely on post-mortem (carcass) samples have reduced certainty around the contribution of brodifacoum exposure to mortality.

1.3.3 Sublethal effects of brodifacoum exposure

The toxicological implications of sublethal brodifacoum exposure are not well described (e.g. Kaukeinen *et al.* 2000; Lechevin & Vigie 1992) and as noted by Howald (1997), potential remains for adverse effects following repeated or sustained sublethal exposure. Other coumarin anticoagulant compounds such as warfarin have been shown to affect bone mass (e.g. Price 1988) but this aspect has not been well studied in wildlife. Knopper *et al*. (2007) undertook a small investigation of bone density and breaking strength in raptors with sublethal exposure to SGARs including brodifacoum, as indicated by the presence of residual concentrations in liver. Coumarin anticoagulants may also have teratogenic effects (e.g Astedt 1995). The literature on sublethal effects of brodifacoum seems to relate mostly to reproductive or teratogenic effects in mammals, generally suggesting maternal toxicity (haemorrhage) resulting in abortion, rather than direct effects on the fetus.

Brodifacoum given by oral gavage to female rats at daily doses of 0.001, 0.01 or 0.02 mg/kg during days 6-15 of pregnancy produced no apparent effects on fetuses at termination, but daily doses above 0.05 mg/kg caused an anticoagulant effect in the mothers and a high incidence of abortion (Hodge *et al.* 1980 cited by World Health Organisation 1995). Female rabbits dosed daily with 0.005 mg/kg brodifacoum over days 6-18 of pregnancy showed a high incidence of haemorrhage, and resultant mortality. In surviving dams that showed signs of haemorrhage there were no effects on the developing fetuses (Hodge *et al.* 1980 cited by World Health Organisation 1995). Twigg and Kay (1995) cite unpublished data where brodifacoum caused a 50% increase in aborted or still-born lambs when administered to pregnant ewes (*Ovis aries*) 7 weeks after mating, and 22% increase in lamb mortality when pregnant ewes were administered brodifacoum one week before giving birth. An outbreak of abortions and haemorrhages in sheep and goats in Egypt was attributed to accidental exposure to brodifacoum (Feinsod 1986). While Hornfeldt (1996) reported successful treatment of brodifacoum toxicosis in a pregnant dog, a more recent report (Munday and Thompson 2003) of *in utero* brodifacoum toxicity in dogs reported two puppies dying shortly after birth showing haemorrhage and having liver brodifacoum residues.

Because the bitch appeared unaffected, it was suggested that fetuses were more susceptible to brodifacoum toxicity than adults. While no publications about reproductive effects of brodifacoum in birds were found, Stejskal et al (1994) found that spider beetles (*Ptinus tectus*) could reproduce and develop on a brodifacoum bait formulation in field conditions, but there was a high frequency of teratologic adult individuals that was possibly due to brodifacoum exposure of the larval stage.

Besides potential adverse sublethal effects on wildlife, there is also a concern for human exposure through consumption of meat from wild game animals that carry brodifacoum residues (e.g. Clear 2003). Given these information gaps, it is important to continue field monitoring and research to identify which, when, how and to what extent wildlife are being exposed to brodifacoum in their environment and what the outcomes of this exposure might be. Sublethal exposure of wildlife to a range of anticoagulants may be far more widespread in the environment than currently thought, but this is not practicable to investigate using liver tissue. A reliable, non-lethal and minimally invasive sampling procedure to determine exposure in live animals would greatly expand the scope of monitoring, in terms of the species and numbers of wildlife that could be surveyed for sublethal exposure to brodifacoum and other anticoagulants. It may be that single or infrequent sublethal exposures do not significantly compromise survival or reproductive fitness of wildlife individuals or populations, but because the effects of cumulative or long-term exposure are not well described, the ability to characterize field exposure profiles would be of great value in a proactive approach to identifying and minimizing risks to non-target wildlife. This is especially so given the high toxicity and bioaccumulative potential of brodifacoum where sublethal exposure is likely to be repeated or sustained.

1.4 Thesis structure

The general, overarching question of "how can we minimise the environmental risks of using anticoagulant rodenticides?" was addressed by series of studies. Figure 1.3 provides an outline of the rationale and studies (each presented as a separate chapter) undertaken against specific research questions. Chapters Two and Three concern residual concentrations of brodifacoum in tissues following sublethal exposure of birds (chickens) and lethal exposure of mammals (rats) respectively. Chapter Four provides an overview of toxicological information about diphacinone

and the rationale behind its selection as an alternative to brodifacoum. Chapters Five and Six describe studies of diphacinone in mammals (pigs) and invertebrates (weta).

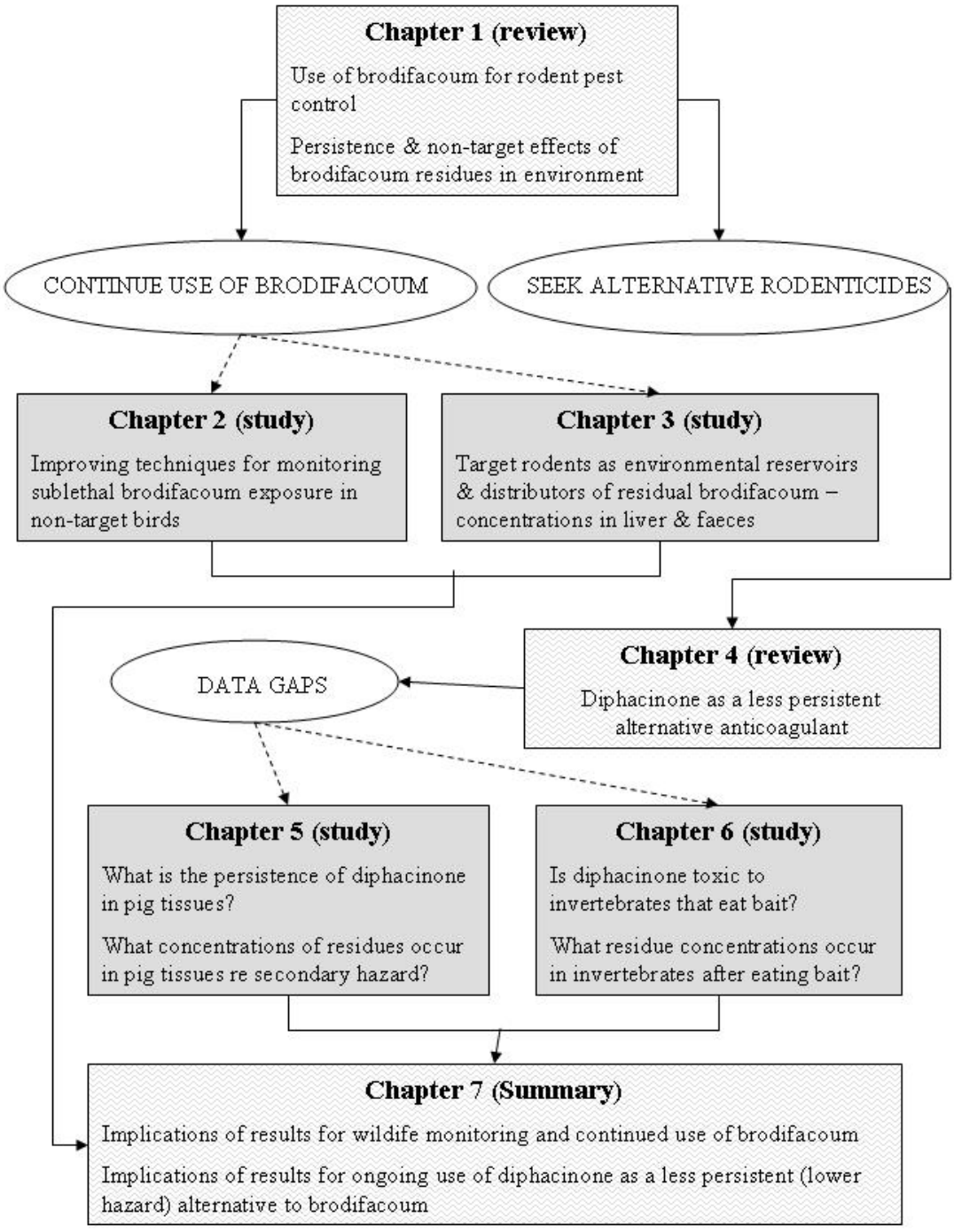


Figure 1.3 Outline of topic areas and specific research questions addressed in this thesis, in the context of New Zealand management options for the use of the anticoagulants brodifacoum and diphacinone for management of introduced vertebrate pests.

Chapter 2: Sublethal brodifacoum exposure in chickens, effects on coagulation time and residual concentrations in tissues, eggs and faeces

2.1 Introduction

Monitoring indicates that environmental exposure to the anticoagulant rodenticide brodifacoum, as indicated by residual concentrations in liver, occurs in a range of bird species (reviewed in section 1.2.2). Mortality or adverse sub-lethal effects resulting from secondary exposure to rodenticides may be an important consideration in the conservation of non-target birds, particularly predators and scavengers (Brakes and Smith 2005). Secondary exposure to brodifacoum may occur when birds of prey (e.g. Barnett *et al.* 2003) take rodents that have ingested bait, or insectivorous birds eat contaminated invertebrates (e.g. Dowding *et al.* 2006). As outlined in section 1.3, current monitoring for brodifacoum exposure relies largely on analysis of liver tissue obtained post-mortem. The requirement for lethal sampling to obtain liver limits practicable monitoring of protected species or large samples of wildlife, and especially does not allow investigation of how sublethal exposure to brodifacoum may affect survival and reproduction in the field. Hence, development of a non-lethal and minimally invasive sampling procedure to determine anticoagulant exposure in live birds would improve the ability to characterise sublethal exposure in field populations. This is especially relevant to NZ, because of its relatively extensive field use of brodifacoum for vertebrate pest control (see section 1.1.2) and the predominance of birds, including many endemic species, in its native biodiversity (e.g. Duffey 2001).

Blood sampling has been used as an indicator of brodifacoum exposure, with plasma samples analysed for residual concentrations, and also for increased coagulation times that may occur in response to exposure (e.g. Howald et al 1997; Poulinquen *et al.* 2006). Prothrombin time (PT) is the most commonly used to indicate an anticoagulant effect and is sensitive to Factors II, VII and X (part of the extrinsic clotting pathway) (e.g. Poller & Hirsch 1996). However, at least 5 mL (ideally 10 mL) of blood is required to derive sufficient plasma for testing, and this may limit the size of birds that can be monitored as it is generally not recommended to remove more than 15% of blood volume in a single sample (Diehl *et al.* 2001). The invasive nature of blood sampling has an additional drawback if birds have been exposed to anticoagulant, as the small injury from sampling may pose a risk of haemorrhage to birds that have elevated coagulation times. Reducing

the volume of blood (plasma) required for valid analysis of brodifacoum concentration would extend the utility of this indicator in field studies, especially for small or conservation-status bird species, where invasive manipulations are undesirable. Trudeau *et al.* (2007) describe application of a small volume dried blood spot (DBS) sampling for monitoring pesticide exposure in birds. Preliminary establishment of an analytical technique for detecting brodifacoum in DBS samples (A. Shlosberg, Kimron Veterinary Institute, pers. comm.) indicated a potential application to monitoring sublethal exposure in birds. Samples that do not require invasive procedures, such as faeces or eggs, were also considered as potential indicators of brodifacoum exposure. Gray *et al.* (1992) estimated that pellets regurgitated by barn owls (*Tyto alba*) contained approximately 29% of a brodifacoum dose the owls ingested via contaminated mice over fifteen days. Collection and analysis of owl pellets has been used to index exposure of owls to brodifacoum and other anticoagulants (Gray et al. 1994; Eadsforth et al. 1996), although there appears to have been no further development of this potentially useful monitoring tool for other bird species.

Using domestic chickens (*Gallus gallus*) as a representative avian species, an investigation of tissue residues and coagulation following sublethal brodifacoum exposure was undertaken. The objectives of this study were (*i*) to measure residual brodifacoum over time in various tissues, faeces and eggs; (*ii*) compare the residues measured in DBS and plasma samples and (*iii*) measure plasma coagulation over time, after a single sublethal exposure.

2.2 Methods

2.2.1 Housing, dosing and tissue sampling of chickens

All procedures carried out using chickens were conducted under Landcare Research Animal Ethics Committee approval (No. 05/03/03 - DBS sampling to detect anticoagulant exposure in birds). Twenty Brown Shaver strain hens (22-24 weeks old, 'point of lay') were obtained from a commercial egg production facility. Each bird was given a numbered leg band, and acclimatised for three weeks to outdoor housing at the Landcare Research animal facility, Lincoln. They were group housed in large grassed pens with sheltered roosts with fresh water and commercial food (Feedworld Premium Gold Poultry pellets) freely available. After weighing they were randomly allocated to either a control (n=5) or brodifacoum treatment (n=15). A dose of 0.50 mg/kg brodifacoum was selected to represent a low sublethal exposure, based on known brodifacoum toxicity in chickens and other birds; Lund (1981) reported that brodifacoum killed four leghorn

hens in 6-12 days after an average intake of 10.5 mg/kg, an LD_{50} estimate of 3.3 (95% CI 2.2-5.2) mg/kg in quail (*Callipepla californica*) (Godfrey 1985) and a report by Bailey et al. (2005) of 100% mortality in ten chickens gavage-dosed with 3 mg/kg brodifacoum. A 0.5 mg/kg exposure was roughly equivalent to a 1.65 kg chicken ingesting 41.25 g of bait containing 0.002% brodifacoum. A dosing solution of 0.4 mg/mL brodifacoum in monopropylene glycol (MPG) (Landcare Research toxicology laboratory Cert No. P05/22, Appendix Two) was administered by stomach tube to hens in the brodifacoum treatment at 1.25 mL solution per kg of bodyweight. Control hens were administered 1.25 mL/kg of MPG alone, with a 2.5 mL maximum dose volume in either treatment. After dosing, the hens were placed back in group housing.

Hens were sampled in groups of 5 (n=4 brodifacoum-dosed and 1 control hen) on days 1, 4, 7 and 14 after dosing. One hen was mistakenly dosed with control treatment, so the Day 14 sample included only three brodifacoum-dosed hens. Dried blood spot (DBS) samples were taken first, where hens were restrained on their side, with the legs and upper wing held and the lower wing extended outwards to expose the brachial vein. The feathers underneath the wing were swabbed with disinfectant and held to one side, while a second person drew blood (maximum 0.2 mL) from the brachial vein with a 25 G needle. Single drops of blood were immediately placed directly from the syringe onto the three marked 'wells' on Schleicher & Schuell No. 903 filter paper cards. The DBS cards were dried in a slide rack at room temperature for approximately 2 h then placed in ziplock plastic bags. Immediately after DBS sampling, the hens were euthanased by decapitation and whole blood samples were collected directly from the blood flow into two 4.5-mL tubes (Vacutainer®Blood Collection Tubes, 3.8% sodium citrate). Samples of whole liver, breast muscle, abdominal fat, eggs/ovaries, kidneys and cloacal faeces were dissected out, whole organs weighed and all samples stored at -20°C.

2.2.2 Testing plasma samples for coagulation time

Within an hour of sampling whole blood was centrifuged at 2500×g for 15 min at 4°C, with plasma divided into two Eppendorf tubes and frozen at −80°C if coagulation testing could not be carried out that day, or stored at 4°C if testing was to be done within 4 hours. Plasma dilutions were prepared with 0.9% saline and tested in duplicate using a commercially-available PT assay kit (Simplastin® Excel) and an automated coagulometer (Amelung KC4Amicro, Sigma Diagnostics). The kit included control plasma reagents, which were also tested in duplicate to provide internal validation standards, where samples that did not clot were allocated a value of

999 seconds. Prolonged prothrombin times result from using mammalian thromboplastin as a reagent in PT testing of avian blood (Howald 1997), possibly due to differences in coagulation factor activities between birds and mammals (e.g. Stopforth 1970) or class specificity of protein reactions in thromboplastin from mammalian or avian sources (e.g. Griminger 1986; Kase et al. 1980). To account for potentially reduced sensitivity in detecting changes in chicken PT through using the rabbit-derived thromboplastin reagent supplied with the Simplastin kit, an avian thromboplastin solution was also prepared. As described by Bailey et al. (2005) and 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 400 mg of powdered brain tissue with 20 mL of 0.9% saline (with stirring) for 15 min at 27°C. The resulting suspension was centrifuged for 10 min at 500×*g* and the supernatant decanted in 20 mL 0.025M $CaCl_2$. This 'in house' avian thromboplastin was used in a set of duplicate PT tests on the chicken plasma samples, where samples that did not clot using the avian thromboplastin were allocated a value of 300 seconds.

2.2.3 Analyses of brodifacoum concentration

Analysis of the various tissues for brodifacoum concentration was carried out at the Landcare Research toxicology laboratory, Lincoln as below. Preparation methods and method limit of detection (MLD) differed for each tissue type. In general, analyses used HPLC with fluorescence detection and a post-column pH switching technique was used to exploit the natural fluorescence of brodifacoum, with difenacoum as an internal standard.

Dried blood spot samples

Preparation and analysis of these samples were based on a calibration carried out by Dr. Alan Shlosberg (Kimron Veterinary Institute, Israel) at the Landcare Research toxicology laboratory (unpublished data). After defrosting the sample cards at room temperature, one of the three 'wells' containing dried blood spots was cut from each card, folded and placed in a 20-mL screw-cap tube to which 3 mL of 0.05M tetra butyl ammonium dihydrogen phosphate (TBAP) in methanol was added, and the blood spot extracted by vortexing and sonicating. The extract was transferred to a clean 10-mL test tube and gently evaporated at 50°C under air. The paper was also dried at 50°C under air, then re-extracted with 3 mL of 0.05M TBAP in methanol, and this

second extract added to the first. The solvent was again gently evaporated at 50°C under air, the sample dissolved in a mobile phase of methanol/water/acetic acid, and passed through a 0.45-µm syringe filter for HPLC analysis. Aliquots were chromatographed on an Alltech Alltima C18 column using methanol/water/acetic acid as the mobile phase. The post-column reagent was ammonia/methanol/water (10/10/80), with a flow rate adjusted until the effluent had a pH of approximately 10.1. The HPLC was run at a flow rate of 1.5 mL/min with degassing, with the gradient program: initial 65%A: 35%B; 8 minutes 84%A: 16%B; 16 minutes 95%A: 5% B; 20 minutes 95%A: 5% B; 23 minutes 65%A: 35%B. The fluorescence detector was at 310-nm excitation and 390-nm emission. Samples spiked with 1, 5 or 10 µL of brodifacoum in methanol were also analysed. The MLD for brodifacoum in dried blood spot samples was 0.04 mg/mL (Landcare Research toxicology laboratory report T2361, Appendix Two).

Plasma samples

Analysis of 1 mL plasma samples for brodifacoum was based on methods described by Primus *et al*. (2001) and Jones (1996). Plasma samples stored at -80°C were thawed in a hot water bath at 37°C and 1 mL extracted with acetonitrile to remove protein. Ethyl ether was added to remove water and the sample evaporated on a vacuum evaporator. The residue taken up in methanol was analysed by HPLC, with MLD 0.005 µg/mL and uncertainty ±8% (Landcare Research toxicology laboratory report T2370, Appendix Two).

Liver, muscle, fat, egg, ovary and faeces samples

Subsamples of 2 g of faeces were prepared and extracted in the same way as tissue samples. A sample of tissue was chopped and a 2 g subsample mixed with anhydrous sodium sulphate, followed by 15 mL of the extraction solvent (chloroform/acetone/ammonia). The mixture was homogenised with a tissue disperser, shaken and centrifuged. The supernatant was decanted and the extraction repeated twice more. The combined extracts were evaporated and taken up in hexane / chloroform / acetone for SPE clean-up on an aminopropyl column. The analyte was eluted from the column using 0.05M TBAP in methanol, which was evaporated off and the sample taken up in a mobile phase of methanol/water/acetic acid for HPLC analysis. MLDs and uncertainties (± 95% C.I.) were; liver 0.005 µg/g,± 8%, muscle and fat 0.001 µg/g ± 8%, egg/ovary 0.001 µg/g ± 8%, and faeces 0.005 µg/g ± 10% (Landcare Research toxicology laboratory report Nos. T2369, T2633, T2648 and T2787 respectively, Appendix Two).

2.2.4 Statistical analyses

Exponential decay models of the form $y=ae^{-bx}$ (where y is the brodifacoum residue and x is time) were fitted to the day 1 to 7 residue data (day 14 results excluded) for liver, plasma, muscle, fat and ovary tissue. Values for the brodifacoum residue were transformed using natural logarithms and regressed against time using the linear regression procedure in the statistical package GenStat (Genstat Committee 2007). Elimination half-lives and their 95% confidence intervals were calculated using the formula $t_{½} = \ln(2)/b \pm (t_{df} * SE)$ (where b is the slope of the regression, t_{df} is the value of Students *t* from tables for the relevant residual degrees of freedom and SE is the standard error of the slope).

2.3 Results

Hens retained normal food intake, with no significant changes in bodyweight in either control or brodifacoum groups over the 14 days after dosing (Figure 2.1). There were no signs of poisoning e.g. anemia, lethargy, bleeding, in daily observation of the hens and no evidence of internal haemorrhage during tissue sampling.

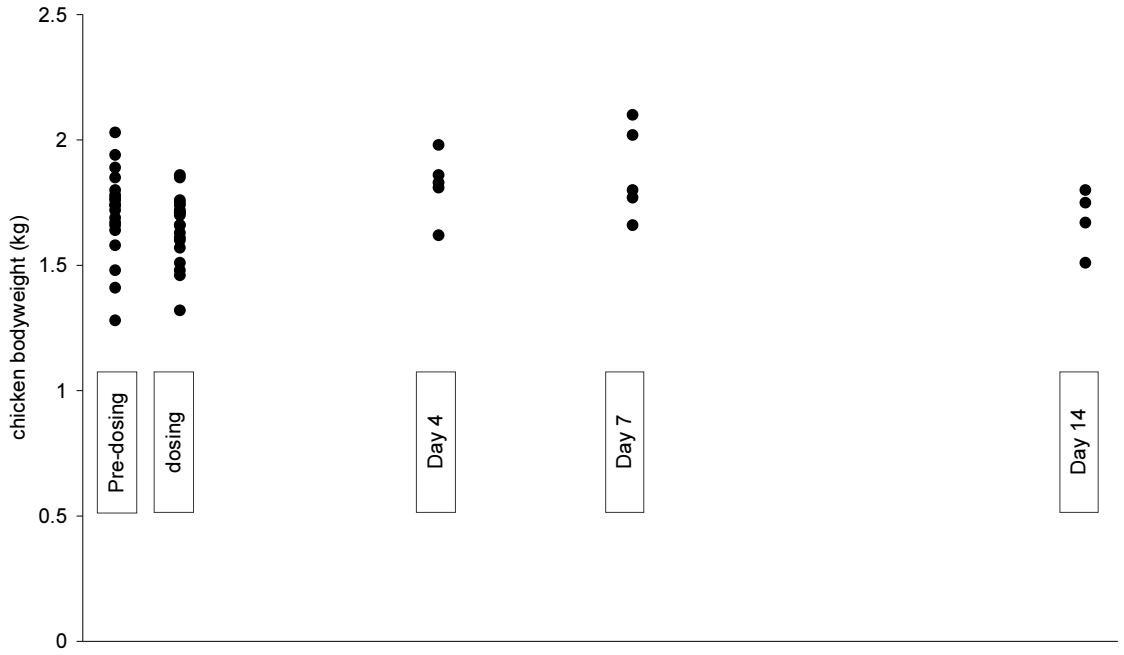


Figure 2.1. Chicken bodyweight before and after dosing with 0.5 mg/kg brodifacoum or MPG (control).

2.3.1 Brodifacoum concentrations in tissues

Residual brodifacoum concentrations in chicken liver remained relatively constant over the 14 days after dosing (Table 2.1), with mean values (μg/g ± SEM) of 0.660 ± 0.058 at Day 1, 0.645 ± 0.069 at Day 4, 0.705 ± 0.107 at Day 7 and 0.617 ± 0.64 at Day 14. These means were not significantly different by ANOVA ($F_{3,11}$=0.21, P=0.89) and a half-life calculation could not be made because there was no decrease in concentrations over the 14 day period. At sampling the mean bodyweight of the hens was 1.78 ± 0.03 kg and mean wet weights of whole livers 45.16 ± 1.88 g, so that liver comprised on average 2.54 % of bodyweight. Assuming that brodifacoum was distributed evenly throughout liver tissue, chickens retained a mean total of 30.91 ± 2.08 μg (range 17.64–52 μg) brodifacoum in liver, which represented on average 3.81 ± 0.26% of the brodifacoum dose administered.

Liver concentrations were consistently much higher (approximately one order of magnitude) than concentrations in muscle and fat (Table 2.1, Fig 2.2). Brodifacoum concentrations in muscle and fat were highest on day one, both with mean concentrations of 0.062 μg/g. In muscle, concentrations declined to means of 0.050 μg/g on day four and 0.028 μg/g on days seven and fourteen. In fat, concentrations declined to means of 0.030 μg/g on day four and 0.015 μg/g on days seven and fourteen (Table 2.1, Fig 2.2). Although plasma concentrations on day one approached those measured in liver (Fig 2.2), plasma concentrations declined to below MDL after day four (see section 2.3.2). Half life estimates with 95% confidence intervals were; 5.3 (3.82-8.66) days for muscle, 2.79 (2.32-3.51) days for fat, 3.17 (1.22- not defined) days for ovary and 1.14 (0.92-1.51) days for plasma.

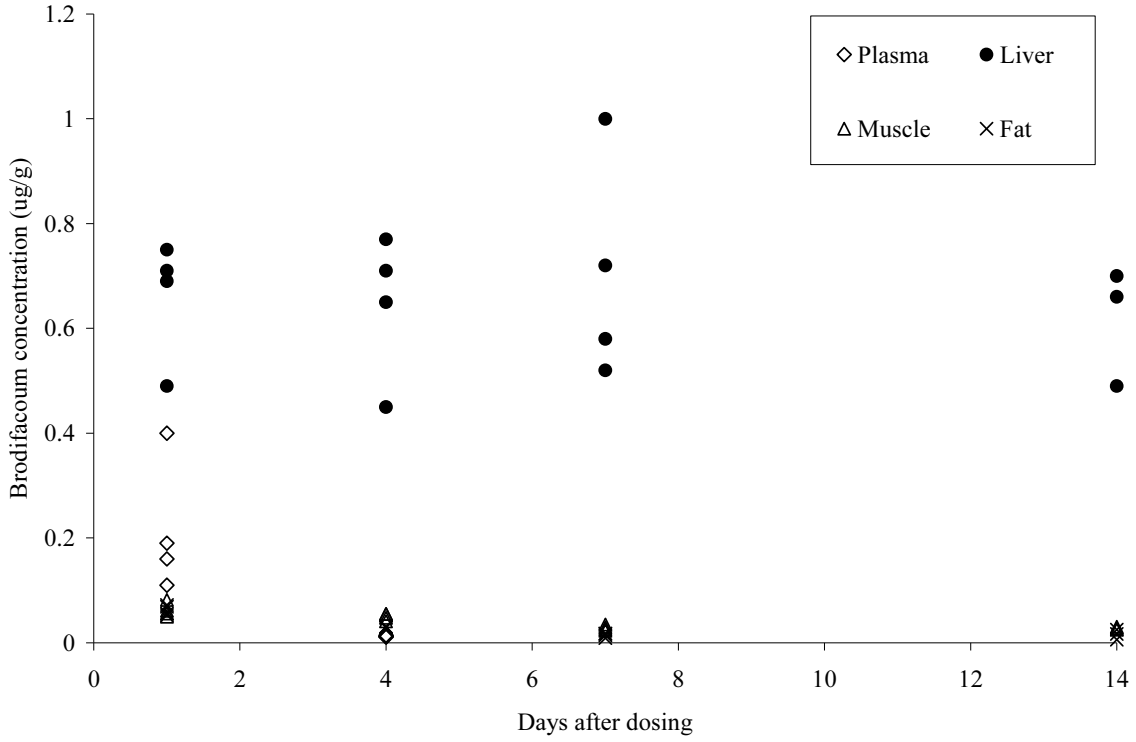


Figure 2.2. Brodifacoum concentrations (μg/g) in chicken liver, plasma, muscle and fat at 1, 4, 7 and 14 days after dosing with 0.5 mg/kg brodifacoum.

Brodifacoum concentrations in ovaries were highest on day one (mean 0.135 μg/g) and declined (day four mean 0.041 μg/g, day seven mean 0.024 μg/g) to just above detectable concentration by day fourteen (*n*=1 treated hen) (Fig 2.3, Table 2.1). Brodifacoum was detected at relatively low concentration in most egg samples from all days, with the highest concentration in a sample measured on day fourteen (0.035 μg/g) (Fig 2.3, Table 2.1). Brodifacoum was detected in faeces on day one (mean 0.17 μg/g) and day four (mean 0.01 μg/g) but had declined to below detectable concentrations by day seven (Table 2.1).

Table 2.1 Concentrations of brodifacoum (μg/g wet weight) in chicken tissues (liver, muscle, fat, egg, ovary, plasma, DBS and faeces) at days 1, 4, 7 and 14 after dosing with 0.5 brodifacoum. Method detection limit shown as [MDL] for each tissue type. Some hens had no egg forming at the time of sampling ('ns' indicates no sample) and ovary samples from two hens on day 14 were not taken due to a technical oversight. Laboratory reports for these analyses are shown in Appendix 2.

		Brodifacoum concentration (μg/g)							
Day	**Chicken ID**	**Liver [MDL 0.005]**	**Muscle [MDL 0.001]**	**Fat [MDL 0.001]**	**Ovary [MDL 0.001]**	**Egg [MDL 0.001]**	**Plasma [MDL 0.005]**	**Dried Blood Spot [MDL 0.04]**	**Faeces [MDL 0.005]**
1	#247	0.75	0.061	0.072	0.33	ns	0.40	0.25	0.22
	#686	0.49	0.056	0.053	0.13	ns	0.19	0.11	0.15
	#666	0.71	0.082	0.056	0.053	0.002	0.16	0.12	0.21
	#683	0.69	0.05	0.069	0.025	0.002	0.11	0.097	0.11
	#229(control)	<MDL	0.003*	<MDL	<MDL	ns	<MDL	<MDL	<MDL
4	#695	0.65	0.048	0.030	0.052	0.028	0.012	<MDL	<MDL
	#690	0.45	0.055	0.029	0.001	ns	0.011	<MDL	0.01
	#681	0.71	0.041	0.031	0.061	0.061	0.014	<MDL	0.028
	#652	0.77	0.055	0.029	0.051	0.010	0.012	<MDL	0.033
	#693(control)	<MDL	0.001*	0.005*	0.001*	0.002*	<MDL	<MDL	<MDL
7	#669	0.52	0.023	0.014	0.023	0.005	<MDL	<MDL	<MDL
	#685	0.72	0.026	0.017	0.025	0.014	<MDL	<MDL	<MDL
	#238	0.58	0.035	0.018	0.020	ns	<MDL	<MDL	<MDL
	#668	1.00	0.029	0.009	0.026	0.014	<MDL	<MDL	<MDL
	#677(control)	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
14	#218	0.49	0.028	0.006	ns	ns	<MDL	<MDL	<MDL
	#653	0.7	0.025	0.017	0.005	<MDL	<MDL	<MDL	<MDL
	#700	0.66	0.031	0.025	ns	0.035	<MDL	<MDL	<MDL
	#205(control)	<MDL	<MDL	0.007*	<MDL	ns	<MDL	<MDL	<MDL

* control hens where analyses showed detectable brodifacoum in tissue – see discussion section 2.4.1.

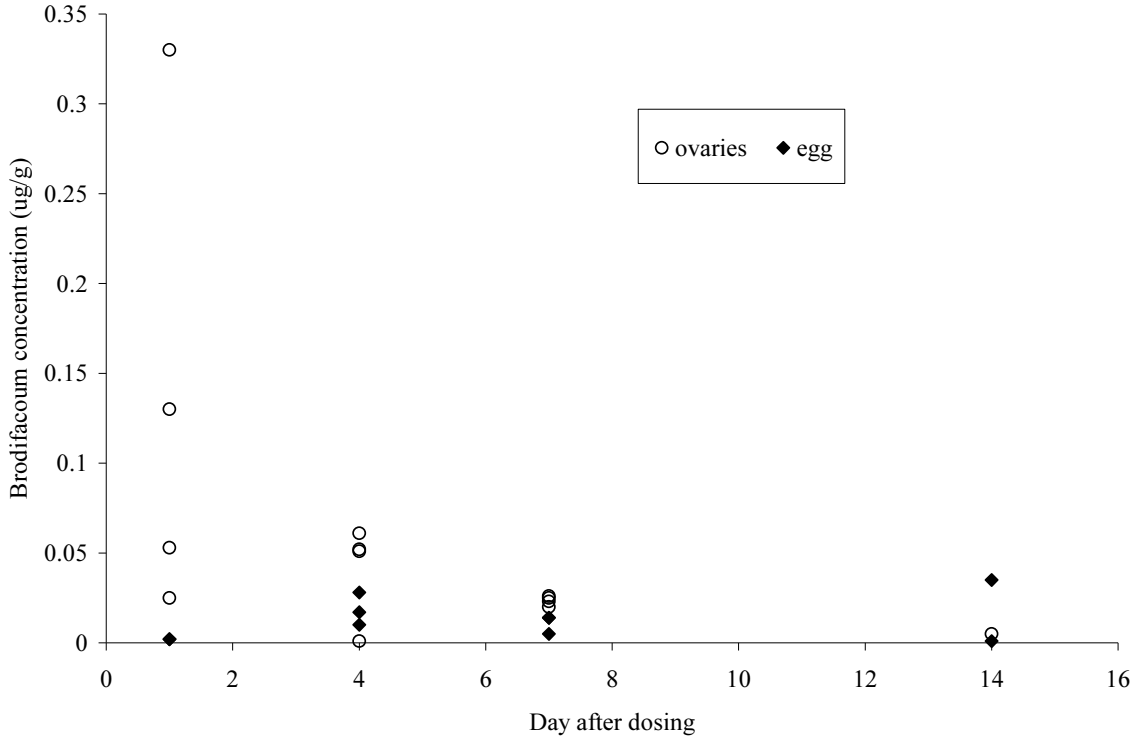


Figure 2.3. Brodifacoum concentrations (µg/g) in chicken ovaries and eggs at 1, 4, 7 and 14 days after dosing with 0.5 mg/kg brodifacoum.

2.3.2 Brodifacoum concentrations in DBS and plasma samples

Spiked DBS samples had an 89-99% recovery and method uncertainty (95% CI) was ±10% and spike recovery in plasma ranged from 88 to 100% with method uncertainty ±8%. Concentrations were below the MDL for plasma and DBS samples from control hens. Day 1 hens had a mean brodifacoum concentration in DBS of 0.144 µg/mL (± SE 0.036) and in plasma 0.215 µg/mL (± SE 0.064). Plasma concentrations had fallen below MDL by Day 7, and in DBS were nominally below MDL from Day 4 onwards (Table 2.1). However, the analyzing laboratory noted that Day 4 DBS samples had detectable concentrations of brodifacoum which ranged from 0.010 to 0.029 µg/g, and while below the calculated MDL (Landcare Research Toxicology laboratory report T2361, Appendix Two) these results were repeatable in duplicate. The Spearman Rank Correlation Coefficient between concentrations in DBS and corresponding plasma samples from Days 1 and 4 was 0.8.

2.3.3 Coagulation times

The 'plasma control' reagents supplied in the kit for internal control and verification of the test were; Verify 1 (normal, coagulation time c. 13.6 seconds), Verify 2 (factors II, VII, IX and X removed) and Verify 3 (factors II, VII, IX and X removed), with the latter two representing 'abnormal' coagulation times of c.27 and 42 seconds respectively. Table 2.2 shows that the avian thromboplastin reagent consistently produced higher PT values than the kit thromboplastin reagent (4 to 7 times longer), but duplicate results were consistent for each.

Table 2.2 Duplicate coagulation time (seconds) from three 'plasma control' verification tests using the kit (Simplastin Excel S) thromboplastin, and the 'in-house' avian thromboplastin.

	Coagulation time (s)	
	Simplastin Excel S	**Avian thromboplastin**
Verify 1 (normal)	15, 15.1	116.3, 113.1
Verify 2 (abnormal)	29.7, 30.6	169, 163.2
Verify 3 (abnormal)	46.1, 48.4	210.6, 205.5

Control PT values were variable, with the Day 1 and 14 samples failing to coagulate with either thromboplastin reagent. The whole blood samples for both were noted to have partially coagulated in the tube before being spun down for plasma, perhaps due to a delay in mixing the blood adequately with citrate reagent during sampling (see section 2.4.3 for discussion). The large residual values presented by these two control results (failure to clot), meant it was not possible to establish confident baseline 'control' PT values, so that statistical interpretation e.g. using general linear modelling procedures, was not possible. Despite this shortcoming, there was a possible response in chicken PT time to sublethal brodifacoum exposure (Fig 2.4) with elevated PT in chickens on Days 1 and 4. Three of the four plasma samples from chickens at Day 4 failed to coagulate with the nominally 'less active' mammalian thromboplastin, but did coagulate with an increased PT with the avian thromboplastin (Table 2.3). Day 7 samples showed a mean reduction in PT with both reagents, and Day 14 PT were similar to those in the two control samples that had measurable times (Fig 2.5). A significant difference in the avian thromboplastin data was present using a Kruskal-Wallis non-parametric analysis of variance by ranks (H=7.65, Genstat 2007), and post-hoc multiple comparison tests (Zar 1999) identified a significant difference between PT values on Day 4 and Day 14 (Q=2.74).

Table 2.3 Duplicate coagulation time (seconds) from PT tests taken 1, 4, 7 and 14 days after chickens were administered 0.5 mg/kg brodifacoum or a control treatment, using mammalian (Simplastin) or avian thromboplastin. (-) indicates that the sample could not be tested because it had already coagulated in the tube.

		Coagulation time (s)	
	Chicken ID	**Simplastin Excel S**	**Avian thromboplastin**
Day 1	#683	539.1, 539.3	55.5, 55.6
	#686	207.6, 202.9	32.7, 32.8
	#247	154.5, 157.9	30.7, 30.6
	#666[b]	999.9, 999.9	58.5, 57.8
Day 4	#690	482.7, 487.3	62.4, 61.6
	#652	999.9, 999.9	85.3, 82.9
	#695	999.9, 999.9	77.8, 76.8
	#681	999.9, 999.9	104.8, 103.0
Day 7	#238[a]	-	-
	#668	245.5, 247.1	43.1, 43.1
	#685	266.7, 272.3	41.7, 40.7
	#669	243.1, 245.0	41.6, 41.7
Day 14	#218 [a]	-	-
	#653	110.5, 111.1	27.6, 27.5
	#700	73.9, 131.4	21.9, 21.0
Controls	#229 (Day 1)[b]	999.9, 999.9	300, 300
	#693 (Day 4)	93.3, 92.8	14.3, 14.1
	#677 (Day 7)	43.8, 44.8	7.3, 8.6
	#205 (Day 14)[b]	999.9, 999.9	300, 300

[a] sample too coagulated to be tested

[b] coagulation visible in tube but available plasma sample tested

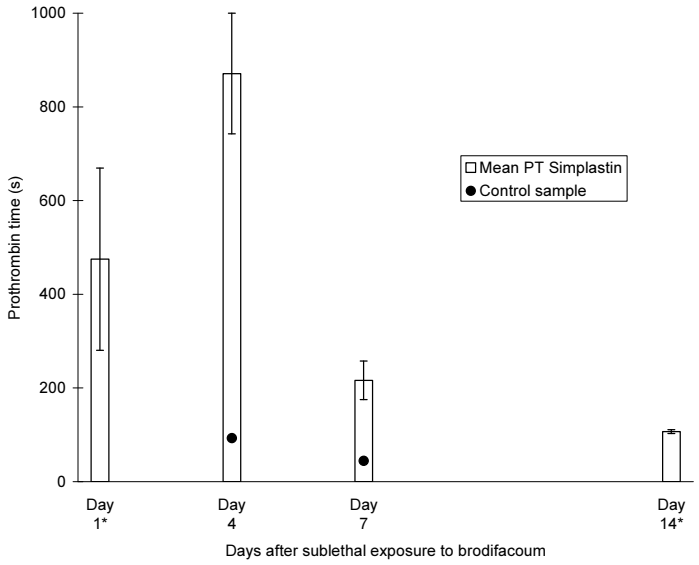


Figure 2.4. Mean (± standard error of mean) prothrombin times (seconds) measured in chicken plasma samples using the 'Simplastin' mammalian thromboplastin reagent, at Days 1, 4, 7 and 14 after sublethal (0.5 mg/kg) brodifacoum. * indicates where the control sample for that day did not coagulate i.e. 999 seconds.

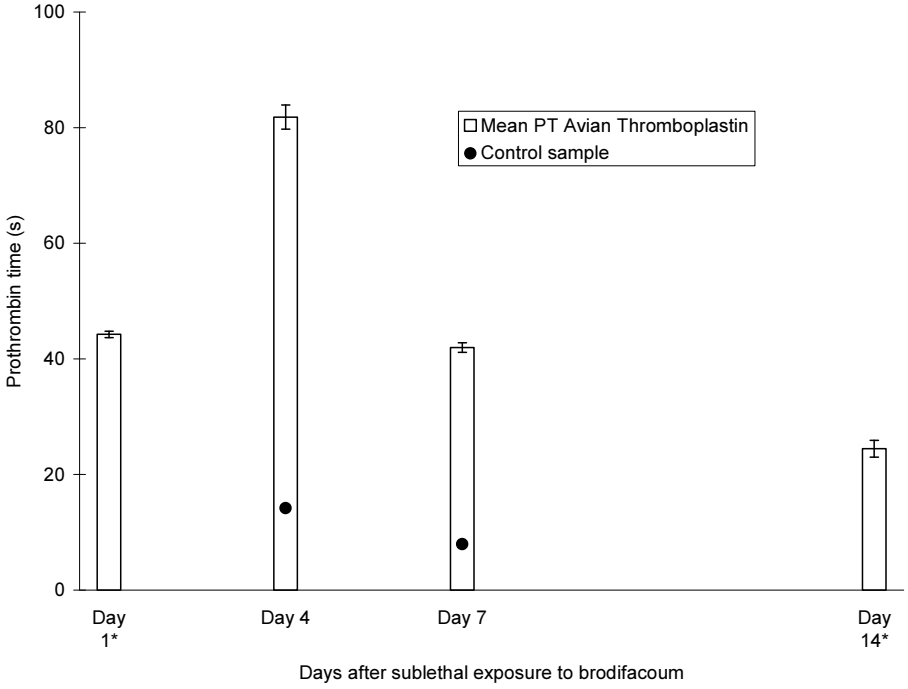


Figure 2.5. Mean (± standard error of mean) prothrombin times measured in chicken plasma samples using the 'in house' avian thromboplastin reagent, at Days 1, 4, 7 and 14 after sublethal (0.5 mg/kg) brodifacoum. * indicates where the control sample for that day did not coagulate i.e. 999 seconds.

2.4 Discussion

2.4.1 Brodifacoum concentrations in tissue after sublethal exposure

Brodifacoum concentrations in chicken livers showed little variation over the 14 days after dosing (mean 0.66, range 0.45-1.00 μg/g). Biphasic (rapid-initial, slower-terminal) elimination rates of anticoagulants have been reported in rats (e.g. Parmar et al. 1987) - this either did not occur in chickens at this exposure, or the rapid-initial phase occurred in the 24 hours before the sampling on day 1. While no hepatic half-life values for brodifacoum in avian species were found in the literature, the results here are consistent with prolonged hepatic persistence of brodifacoum in mammalian species (see summary in Chapter 4.1), and the estimate by Huckle et al. (1989) of >100 day hepatic half-life of the second-generation anticoagulant flocoumafen in Japanese quail.

The highest concentrations in muscle, fat and ovaries all occurred on day 1 with means of 0.6, 0.6 and 0.13 μg/g respectively (Table 2.1). In muscle and fat, this was approximately ten times less than the concentration in corresponding liver samples. Concentrations in these three tissues showed a decline from day 1 (Figs. 2.2 and 2.3) but were still detectable at day 14. Given that plasma concentrations declined more rapidly after day 1, and had declined to below 0.005 μg/g by day 7, the presence of detectable brodifacoum in muscle, fat and ovaries at day 7 suggests that brodifacoum was bound to some extent in these tissues. In terms of potential for secondary exposure, chicken liver is the most hazardous tissue by virtue of retaining relatively high residual concentrations of brodifacoum in an apparently healthy bird. While residues in chicken muscle and fat had lower concentrations and were less persistent, by mass these are the tissues are also likely to constitute secondary exposure to predators, including human consumers. This also raises questions about the hazard posed to wildlife that might eat gut contents of birds contaminated with brodifacoum. While residue concentrations were not measured in the gut of chickens in this study (as the gavage administration used was not considered representative of voluntary ingestion of brodifacoum in food), this may also be an important transfer pathway for brodifacoum in some field situations if predators take birds, or rodents, that have recently fed on brodifacoum bait.

The possibility of brodifacoum contamination of eggs was previously acknowledged by Robertson *et al.* (1999) in a field monitoring study, however they found no detectable brodifacoum in nine kiwi (*Apteryx mantelli*) eggs collected over 18 months after adults were first potentially exposed. The data presented here confirms transfer of brodifacoum to eggs can occur

following sublethal exposure. The pattern of residues in ovary and eggs (Fig 2.3) suggests that if brodifacoum is present in ovary tissue it is partly transferred to any egg being formed at the time, and the mature egg takes with it the residue burden acquired during formation. Whether the presence of residual brodifacoum would adversely affect the development of fertilised eggs or the viability of hatched chicks is unknown, but such investigations are important - perhaps even urgent, given the relative lack of information about reproductive effects of brodifacoum in birds and the possibility of extensive but unmeasured sublethal brodifacoum exposure in avian wildlife. Lund (1981) addressed the question of consumer risk from eggs in a study where four laying hens were fed on brodifacoum bait, and eggs from these birds were fed to a single laboratory rat over a period of 10 days. The four hens died within 6-12 days after an average intake of 10.5 mg/kg brodifacoum, and the rat showed no signs of poisoning after eating 218 g of egg - this result led Lund (1981) to state "..eggs laid during an anticoagulant feeding period contain no toxic residues representing a risk to the consumer". Notwithstanding this conclusion, the concentrations (0.002-0.035 μg/g) of brodifacoum detected in eggs here would be of concern from a human consumer perspective, for example, in NZ the maximum residue limit (MRL) for brodifacoum is 0.001 μg/g (NZ Food Safety Authority 2007). If brodifacoum is used for rodent control at egg production facilities, even caged hens could potentially be exposed to fragments of bait moved from stations by rodents, or to residual brodifacoum in rodent faeces (see Chapter 3.3.2). Survey information about the extent of brodifacoum use in egg production facilities would be useful to scope the probability of sublethal exposure in hens producing eggs for human consumption, and the corresponding potential for contamination of eggs. Whether brodifacoum concentrations would decline in infertile eggs after they were laid also remains to be investigated but would indicate whether a 'withholding period' for consumption of potentially contaminated eggs was appropriate.

The presence of dye coloring in bird faeces can provide strong evidence of primary exposure to brodifacoum baits e.g. ravens on Langara Island (Howald 1997), especially if associated with relatively high residual concentrations in other tissues. The short-lived (four days or less after exposure) presence of residual brodifacoum in faeces found in this study could facilitate the development of a non-invasive means of monitoring recent exposure in field conditions. This approach, using analysis of regurgitated owl pellets has previously been investigated as a monitoring tool for owls in the UK (Gray 1992). Although field monitoring of faeces for residual brodifacoum would probably require collection of fresh material and entail uncertainty in the

identification of the individual or even species of bird that produced the faecal material, it could be especially useful for scoping avian populations considered at high risk of exposure e.g. raptors in the close vicinity of rodent baiting operations.

The most likely explanation for the presence of very low (just above MDL) brodifacoum concentrations in some, but not all tissue samples from two control hens (Table 2.1) was contamination during dissection for tissue sampling either from gloves or instruments. Fresh scalpel blades and detergent-washed instruments were used on each sampling day, but these were only rinsed in water between sampling the five birds on each day and this may not have been adequate to remove all traces of brodifacoum-contaminated tissue. Each tissue type was analysed in separate laboratory runs, so that contamination during analyses was considered unlikely since four tissues (muscle, fat, ovary and eggs) from the control hen on day four all contained low concentrations. It is also possible that this hen may have ingested faeces containing brodifacoum after the hens were returned to group-housing after dosing, but if this was the case the liver sample would also be expected to have low concentrations of brodifacoum. In the context of comparing tissue concentrations, if contamination occurred during tissue sampling on each day it would have contributed only a very small increase to overall tissue concentrations - so that the data from brodifacoum-dosed hens still represent accurate estimates of the brodifacoum concentrations that were present. The design limitations of using only one control hen on each sample day are discussed further in section 2.4.2 below.

2.4.2 Comparison of DBS and plasma brodifacoum concentrations

The reasonably close correlation between brodifacoum concentrations measured in DBS and plasma samples from hens at Day 1 indicates that DBS provided an accurate index of circulating brodifacoum concentrations within this timeframe. Plasma brodifacoum concentrations had fallen below MLD by day 7, but in DBS were below MLD from Day 4 onwards (Table 2.1). Although the plasma analysis was the more sensitive, it is probable that brodifacoum was also present in DBS samples at Day 4, as per the low but repeatable measurements noted by the reporting laboratory. Coumarin anticoagulants bind to plasma albumin proteins (Sutcliffe et al. 1987), so dilution by non-binding fractions such as erythrocytes may have accounted for the generally lower brodifacoum concentrations measured in DBS (whole blood) in comparison to plasma samples (Table 2.1), suggesting that a correction factor accounting for erythrocyte packed cell volume could be applied to DBS data.

At 24 hours after dosing, chicken plasma concentrations in this trial were approximately ten times higher than those reported by Howald (1997) in Japanese quail (*Coturnix japonica*) following sublethal exposure to brodifacoum. In his study groups of six quail were dosed with 0.7, 1.4 or 0.35 mg/kg brodifacoum and plasma concentrations were not significantly different between the three dose groups 24 hours later with a mean of 0.028 mg/L. In quail plasma concentrations declined to a mean of 0.005 mg/L at five days and then to 0.002 mg/L by ten days, showing a consistent rate of decline with the concentrations found here in chickens at four days (0.012-0.014 mg/L) and ten days (below MDL, 0.005 mg/L) after dosing. This presence of detectable concentration of brodifacoum in avian plasma for approximately 7-10 days indicates a relatively rapid elimination of circulating (plasma-bound) brodifacoum, probably due to sequestration of brodifacoum in hepatic binding sites (viz. consistent concentrations in liver here). It is likely that the duration for which residues are detectable in plasma(or DBS)will also vary with the magnitude of the sublethal brodifacoum exposure, with higher exposures probably taking longer to eliminate. Results here support the conclusion of Howald (1997), that detection of brodifacoum in plasma is a more reliable index of brodifacoum exposure in birds than changes in coagulation time. In addition, as an index of recent brodifacoum exposure DBS sampling may also provide an alternative method that is less invasive and requires less labour to prepare than plasma samples.

The field utility of either plasma or DBS for monitoring brodifacom exposure in birds will be limited by the period during which brodifacoum is at measurable concentrations in blood (plasma). From the results here, this 'window of detectability' is estimated as less than seven days for plasma and less than four days for DBS samples. Improvements in the sensitivity of analysis of DBS samples e.g. through LC-MS techniques may extend the 'window' of a sampling method that offers a number of advantages over plasma or liver sampling. The results in this trial, where repeatable but below MDL concentrations were detected in Day 4 DBS samples, suggests that further improvements in the sensitivity of this analysis are feasible. A further reduction in invasiveness of monitoring for anticoagulant exposure may be possible with saliva samples. Sakai *et al*. (1983) found warfarin was excreted in the saliva of rabbits after oral adminstration, the salivary concentration corresponded with that in plasma and was correlated with the effect of warfarin on prothrombin complex activity. It would be useful to investigate whether coumarin anticoagulants are also excreted in the saliva of birds in detectable concentrations, comparable to

those present in plasma and DBS. Application of a low volume saliva sampling method, similar to that used for DBS in this trial, would further reduce the sample invasiveness for monitoring exposure of wild birds and mammals to anticoagulants, even if this retains the limitation of only being able to detect exposure within the last few days.

2.4.3 Effect of sublethal brodifacoum exposure on coagulation time

The consistently higher PT seen with the avian thromboplastin in the verification tests (using control samples of mammalian-derived plasma supplied with the kit) contrast with the reverse situation in the testing of the chicken plasma samples, where use of the avian thromboplastin produced consistently lower PT. This is consistent with previous findings that the shortest PT for a given species is obtained when homologous (brain-derived) thromboplastin is used (Spurling 1981). Bigland & Triantaphyllopoulos (1961) noted greatly increased clotting times of chicken plasma with bovine thrombin and with rabbit thromboplastin, taken as evidence of species specificity for thromboplastin. Particularly with the data from the avian thromboplastin, there was an apparent elevation in the mean PT at Day 4 and a return to 'baseline' times at Day 7 and 14. However, the use of a single control hen for each sampling day was a poor experimental design, which was selected on the basis of budgetary limitations in the number of tissues samples to be analysed for brodifacoum. Failure of the Days 1 and 4 control samples to coagulate (Table 2.3) meant that baseline PT values could not be confirmed for comparison to elevated values, and that the failure of samples from treated hens to coagulate with the mammalian thromboplastin could not confidently be attributed to the brodifacoum exposure and may have been a sampling artefact. The use of decapitation to obtain blood samples, and delays in mixing the blood adequately with citrate reagent during sampling probably contributed to the partial coagulation observed in some whole blood samples before extraction of plasma (Table 2.3). Miletich 1995 (cited in Howald 1997) found that blood collected from decapitation showed a significantly shorter PT than blood collected from the jugular vein and release of tissue thromboplastin (e.g. Stopforth 1970) upon decapitation may have contaminated the blood samples and prompted coagulation. Future studies of this nature should avoid decapitation as a method of whole blood collection to avoid this possibility.

However, the mean avian thromboplastin PT in control hens from Days 4 and 7 (14.2 and 7.9 seconds respectively) may have been valid measurements. They were lower than the 25 seconds reported in chickens by Bailey et al. (2005), and the 38.6 seconds reported by Frost et al. (1999),

but more consistent with other values; mean 9.4 seconds and range 7.8-11.4 seconds (Doerr et al. 1975), 9.0-16.5 seconds (Stopforth 1970) and mean 13.02 seconds (Timms 1977). The use of non-homologous (mammalian) thromboplastin in this trial produced higher mean PT values in the control hens on Days 4 and 7 (93.0 and 44.3 seconds respectively), consistent with the observation of Kase (1978) that plasma tend to be clotted in the shortest time by homologous thromboplastin.

In a pen study Savarie et al. (1979) used the PT 'Quick' test with chick embryo thromboplastin to measure the response of golden eagles (*Aquila chrysaetos*) to secondary diphacinone exposure. Eagles consumed an average of either 0.87 mg/kg diphacinone over five days (four birds) or 1.6 mg/kg over 10 days (three birds) and showed substantial increases in PT at five days after first feeding on meat containing diphacinone. Some eagles also showed haemorrhage from existing wounds and general weakness, but recovered normal PT by 21 days. While these results suggest that PT could be useful to indicate anticoagulant exposure, they were obtained in a controlled trial where the degree and timing of anticoagulant exposure was known. In field monitoring following a brodifacoum baiting operation for rodent eradication on Langara Island, Howald et al. (1999) used a fibrinogen counter with mammalian reagents to evaluate PT times of bald eagles (*Haliaeetus leucocephalus*), alongside analysis of plasma for residual concentrations of brodifacoum. While there was no evidence of anticoagulation in the PT results, some eagles had detectable brodifacoum in plasma. As discussed by Howald (1997), a demonstrable association between tissue residue and an increase in coagulation time would provide the most compelling evidence of an adverse effect on birds that could increase the risk of haemorrhage. While brodifacoum residues were present in all samples from hens on Day 4 of this trial, these could not be definitively linked with an increase in PT. Notwithstanding the equivocal results of this trial, it is suggested that measurement of a coagulation effect as an index of exposure to brodifacoum or other anticoagulants in birds, especially those sourced through 'wildlife incident monitoring' (e.g. Stone et al 2003, Pouliquen et al. 2006), has limitations due to;

i. the short-lived nature of a coagulation response to sublethal exposure
ii. uncertainty around the degree of coagulation compromise that will result in lethal haemorrhage, given environmental variables such as activity and injury
iii. invasive sampling and technical skill required to obtain valid blood samples of suitable volume
iv. differences in coagulation test systems used.

2.5 Conclusion

Measuring residues in avian tissues, particularly liver, is a more reliable indicator of previous brodifacoum exposure than changes in coagulation time. This presents an ongoing dilemma for monitoring of wild birds because tissue analysis is currently practicable as a *post-mortem,* retrospective clue as to how brodifacoum exposure may have contributed to mortality. While samples such as plasma, DBS, faeces and possibly saliva, have the limitation of being only able to detect recent (within 4-7 days) exposure to brodifacoum, refinement of sampling protocols and detection sensitivity would provide an alternative approach to incident-based, *post-mortem* monitoring of avian populations where concern exists about brodifacoum exposure.

Chapter 3: Brodifacoum concentrations in liver and faeces of poisoned rats

3.1 Introduction

The delayed onset of brodifacoum toxicity is a practical advantage for effective vertebrate pest control, as it minimises the likelihood of bait shyness developing (e.g. Kaukeinen & Rampaud 1986). Typical progression of the outward signs of anticoagulant poisoning in rats includes increasing pallor and weakness reflecting blood loss (Pelfrene 2001). A loss of appetite seems to be characteristic of brodifacoum poisoning in mammals at least; in brushtail possums (*Trichosurus vulpecula*), the mean time to reduced feed intake was 13 days and mean time to death was 20.1 days and in Norway rats (*Rattus norvegicus*) the mean time to reduced feed intake was 4 days and mean time to death was 7.2 days (Littin *et al.* 2000). In a radio-tracking study of four wild ship rats (*R. rattus*) before and after brodifacoum poisoning, Hooker & Innes (1995) reported times of death of 4 to 6 days, with no apparent restriction of rat movements during the nights before death. Brodifacoum is one of the most acutely toxic of the anticoagulants with consistently high oral toxicity to rodent species (Table 3.1), such that they only need to ingest a relatively small amount of bait (typically containing 20 or 50 ppm brodifacoum) to receive an effective lethal dose. Approximating bodyweights of 25 g for house mice (*Mus musculus*), 100 g for ship rats and 250 g for Norway rats and assuming a toxicity of 0.77 mg/kg (highest LD_{50} / lowest toxicity shown in Table 3.1) these three species would have to eat approximately 1, 4 and 10 g of 20 ppm brodifacoum bait respectively for an LD_{50} dose. On this basis, lethal quantities of brodifacoum are likely to be consumed in a single feed of palatable bait, a desirable characteristic in an effective rodenticide (e.g. Hadler & Buckle 1992).

In operational pest control using brodifacoum, ready availability of palatable bait to the target species is important to maximise efficacy. However rodents may continue to consume bait after they have ingested a lethal dose, over the period of days until appetite loss occurs and other behavioural signs of poisoning become evident. From a cost perspective, such 'overdosing' is unwanted because more bait than necessary to kill each rodent is used. It is also undesirable because it effectively transfers brodifacoum from a relatively controlled, concentrated primary non-target hazard (in bait) to a more diffuse but mobile secondary non-target hazard in the environment (in the tissues of rodents). While 'pulsed' baiting strategies have been proposed to minimise the occurrence of unnecessarily high residual concentrations of brodifacoum in rodent

liver and gut (Hadler & Buckle 1992, Merson *et al.* 1984, Kaukeinen 1982), Kaukeinen *et al*. (2000) suggest that measuring the liver concentration of brodifacoum cannot determine the magnitude of initial exposure, because liver binding sites have a saturable capacity, past which anticoagulation effects occur. A laboratory study by Fisher *et al.* (2004) simulated three different scenarios of anticoagulant bait intake by Norway rats and measured resulting residual concentrations in rat liver, to underpin an assessment of secondary hazard to non-target species. Data from the brodifacoum treatments in this study were reanalysed to investigate the relationship between brodifacoum exposure and liver concentrations in rats.

Table 3.1 Acute oral toxicity of brodifacoum to rodent species as indicated by published lethal dose (LD_{50}) values. 95% confidence intervals, where reported are shown in [ ].

Species	Brodifacoum LD_{50} (mg/kg)	Reference
Norway rat (*Rattus norvegicus*)		
laboratory strain	0.27	Godfrey (1985)
laboratory strain	0.26	Hadler & Buckle (1992)
laboratory strain (male)	0.41 [0.35-0.50]	United States Environmental Protection Agency (1998)
laboratory strain (female)	0.56 [0.47-0.66]	United States Environmental Protection Agency (1998)
wild-caught	0.17	Booth & Wickstrom (1998)
Ship rat (*R. rattus*)		
wild-caught	0.46	Booth & Wickstrom (1998)
wild-caught	0.77	Marthur & Prakash (1981)
wild-caught (male)	0.9 [0.27-2.9]	Sridhara & Krishnamurthy (1992)
wild-caught (female)	0.7 [0.16-3.0]	Sridhara & Krishnamurthy (1992)
Polynesian rat (*R. exulans*)		
wild-caught	0.32	Booth & Wickstrom (1998)
House mouse (*Mus musculus*)		
laboratory strain	0.40	Godfrey (1985)
wild-caught	0.52	O'Connor & Booth (2001)

A potential environmental transfer pathway of brodifacoum that has received little attention is excretion in faeces - Lavoie (1990) estimated that voles (*Microtus* spp.) either excreted or metabolised 70% of ingested brodifacoum and Laas (1985) reported that sheep (*Ovis aries*) excreted an estimated third of a 2.0 mg/kg brodifacoum dose in faeces over 8 days. If rats excrete

a significant proportion of brodifacoum as unchanged compound between ingestion of a lethal dose and death, brodifacoum will become more widely spread in the environment especially while rats continue to feed on bait.

Two laboratory studies were undertaken, the first having the objective of comparing liver concentrations of brodifacoum in laboratory Norway rats after different bait intakes and the second with the objective of estimating the proportion of an oral brodifacoum exposure excreted in faeces by wild-caught ship and Norway rats, during ingestion of bait up to death through poisoning.

3.2 Methods

3.2.1 Brodifacoum bait intake and liver residue concentrations in laboratory rats

All procedures were conducted under Landcare Research Animal Ethics Committee approval (No. 01/07/03). Young adult female rats (*Rattus norvegicus* Wistar) were housed singly in a controlled-temperature environment (18ºC ± 2ºC) with a 12 hour light/dark cycle and acclimatised with free access to water and cereal feed pellets (Weston Animal Nutrition, Rangiora) for 14 days. They were weighed at the beginning of trials 1-3 (see below), at euthanasia or death, and daily if anticoagulant toxicosis was evident. To account for any changes in bait weight due to environmental conditions, in each trial 'environmental control' baits were weighed into containers (n = 3) and placed in the room housing the rats. These were reweighed each morning, and any change in weight averaged and used as a correction factor for daily estimates of bait consumption by the rats. Trials 1, 2 and 3 used "Pestoff® rodent bait 20R" (Animal Control Products, NZ), a cereal pellet formulation nominally containing 20 ppm brodifacoum. To confirm the brodifacoum concentration in bait, samples of bait were analysed by the Landcare Research toxicology laboratory, as described by Fisher *et al.* (2004). Brodifacoum doses ingested by rats were calculated according to individual's bodyweight and the analysed concentration of brodifacoum.

Liver samples were analysed for brodifacoum concentration by the Landcare Research toxicology laboratory (NZ) using a method based on that described by Hunter (1983). Tissue samples were chopped and mixed with anhydrous sodium sulphate and the extraction solvent (chloroform/acetone). The mixture was homogenised with a tissue disperser, shaken and centrifuged. The supernatant was decanted and the extraction repeated twice more. The combined

extracts were evaporated and taken up in hexane/chloroform/acetone for application to a gel permeation column for clean-up. The eluent from the column was again evaporated and taken up in mobile phase for HPLC determination, which employed post-column pH switching and fluorescence detection. The method detection limit (MDL) was 0.02 μg/g.

Trial 1: Rats offered an effective lethal dose of brodifacoum bait

Procedures involving rats in this study were approved by the Landcare Research Animal Ethics Committee (03/10/02). Toxic bait was offered to twelve rats and as a control treatment three rats were offered similar amounts of non-toxic feed pellets. Approximating an effective lethal dose as twice the LD_{50} estimate of 0.27 mg/kg for brodifacoum in Norway rats (Godfrey 1985), the target minimum brodifacoum intake per rat over three days was 0.54 mg/kg. Rats were weighed on the first day of the trial, before normal food was removed and a weighed amount of bait equivalent to an LD_{50} for each rat was placed in the cage feeder. The following morning (approximately 24 hours later) remaining bait was collected and weighed, and replaced with fresh bait equivalent to the amount required to ingest an LD_{50}. This was repeated for another night (total three nights of bait offered). Rats were weighed and returned to a normal diet when they had ingested the target intake or after three nights of being presented with bait. Signs of anticoagulant poisoning, weight and mortality were recorded daily. Rats that lost greater than 25% of their bodyweight or were deemed to be suffering during this time were euthanased by cervical dislocation whilst under carbon dioxide / oxygen anaesthesia. The three control rats were euthanased within 24 hours of all rats offered toxic bait dying. Samples of liver and muscle were taken post-mortem from all rats for residue analysis.

Trial 2: Rats offered brodifacoum bait ad libitum for 24 hours

As for Trial 1, toxic bait was offered to twelve rats and three control rats were offered similar amounts of non-toxic feed pellets. Rats were weighed before normal food was removed and approximately 40 g of bait was offered in the morning. This amount was expected to be in excess of the amount they would consume over 24 hours, after which uneaten bait was removed and weighed. Rats were returned to normal diet and euthanased as described above after approximately 24 hours. Samples of liver were taken post-mortem for residue analysis.

Trial 3: Rats offered brodifacoum bait and non-toxic food ad libitum until death

As for Trials 1 and 2, toxic bait was offered to twelve rats and three control rats were offered similar amounts of non-toxic feed pellets. Each morning rats were offered 40 g of toxic bait and a 'maintenance diet' quantity (15 g or approximately 5 g food per 100 g of bodyweight) of non-toxic feed. Uneaten bait was removed and weighed after approximately 24 hours, and replaced with fresh baits of the same amount in alternate positions in the feeder each time. This choice was offered until the rats died, or for twelve days after which time the rats were to be euthanased. The three control rats were euthanased within 24 hours of all rats offered toxic bait dying. Samples of liver were taken from all rats post-mortem for residue analysis. Rats were weighed at the beginning and end of the trial but not daily, as handling of poisoned rats may have increased the likelihood of haemorrhage and decreased the time to death.

3.2.2 Brodifacoum concentrations in faeces of rats feeding on bait

A small laboratory study was opportunistically undertaken during an investigation described by Morriss *et al.* (2008) of the effect of weathering on bait acceptance by wild-caught ship and Norway rats. In brief, these rodents were presented with a choice of 'Pestoff® rodent blocks' (20 ppm brodifacoum, Animal Control Products, NZ) and non-toxic cinnamon-lured RS5 pellet bait (Animal Control Products, NZ) to determine relative acceptance over five days. During that trial, an opportunity to investigate brodifacoum concentrations in faeces during bait consumption was taken. Two ship rats and two Norway rats (one male and one female of each species) were randomly selected for collection of faeces one day before (baseline) and throughout the bait acceptance trial until the rat died of poisoning. Each morning, all faecal pellets were collected from the cages of these rats and total wet weight taken. Care was taken not to include any fragments of the green-coloured toxic bait during collection - while only complete faecal pellets were collected some of these had green colouration in them, presumably as the result of bait ingestion. The samples were dried at 40°C overnight and reweighed to determine dry weight. Dried samples of faeces were analysed for brodifacoum concentration using Landcare Research Toxicology Laboratory Method 017 Assay of Brodifacoum Baits and Concentrates by HPLC, a method based on that of Hunter (1983) and ICI method PPSM (1983). Samples were extracted in 25mL instead of 50mL and while no MDL was determined, method uncertainty (95% C.I.) was ±7% (report T2585 in Appendix 3).

3.2.3 Statistical analyses

All mean residue concentrations are reported with the standard error of that mean in brackets. Data from Trials 1, 2 and 3 were pooled for regression analysis of brodifacoum dose ingested against liver residues, using the linear modelling procedure in R (version 2.6.0). Pooling of this data was justified as the three trials used the same strain and sex of laboratory rats, were conducted in the same housing / experimental conditions and used the same brodifacoum bait type.

3.3 Results

3.3.1 Brodifacoum bait intake and liver residue concentrations in laboratory rats

Brodifacoum concentrations measured in 'Pestoff® rodent bait 20R' cereal pellets were 19.7 µg/g for Trial 1 (Landcare Research toxicology laboratory report T1422, Appendix 3) and 18.3 µg/g for Trials 2 and 3 (Landcare Research toxicology laboratory report T1588, Appendix 3). These concentrations were used in calculations of brodifacoum exposure. None of the control rats in Trials 1 to 3 showed any signs of poisoning, and there was no detectable brodifacoum in their liver samples.

In Trial 1 (rats offered an effective lethal dose of brodifacoum bait) all twelve of the rats died, ingesting a mean total of 0.64 ± 0.02 mg/kg brodifacoum over the four days. They all showed some signs typical of anticoagulant poisoning, e.g. anemic and ungroomed appearance, hunched posture, reduced food intake, visible bleeding from nose. Times to death ranged from 24 to 168 hours, with a mean of 102.0 ± 13.25 hours (approximately 4.25 days). Liver brodifacoum concentrations ranged from 1.50 to 2.20 µg/g with a mean of 1.86 ± 0.07 µg/g (Landcare Research toxicology laboratory report T1475, Appendix Three) and muscle concentrations ranged from 0.11 to 0.24 µg/g with a mean of 0.16 ± 0.01 µg/g (Landcare Research toxicology laboratory report T1491, Appendix 3). In Trial 2 (rats offered toxic bait *ad libitum* for 24 hours) the three control rats consumed a mean of 15.92 g of non-toxic food. One of the twelve rats offered toxic bait consumed only 0.20 g, but the other eleven consumed between 10.73 and 18.18 g toxic bait for an overall mean of 12.73 ± 1.30 g bait (equivalent to a mean brodifacoum dose of 1.31 ± 0.13 mg/kg). Liver residues ranged from 0.67 µg/g (in the rat that ate only a small amount of bait) to 11.0 µg/g, for a mean concentration of 5.01 ± 0.82 µg/g (Landcare Research toxicology laboratory report T1705, Appendix 3).

In Trial 3, mean daily intake of non-toxic feed by the three control rats ranged from 17.94 to 24.28 g per day. In the first five days, mean daily intake of toxic bait by the twelve rats ranged between 13.38 and 19.35 g but decreased sharply from day five onwards as signs of poisoning and mortality occurred (Fig 3.1). Rats consistently ate more toxic bait than non-toxic food (Fig 3.1), indicating high palatability of Pestoff® 20R pellets. Rats consumed an estimated five times LD_{50} dose on the first day, and ate a total mean of 93.67 ± 3.27 g bait before death, equivalent to a mean 6.55 ± 0.19 mg/kg intake of brodifacoum. All twelve rats died with times to death ranging from six to thirteen days (Fig 3.1). Brodifacoum concentrations in liver ranged from 6.70 to 17.00 µg/g, with a mean of 10.70 ± 1.1 µg/g (Landcare Research toxicology laboratory report T1751, Appendix 3).

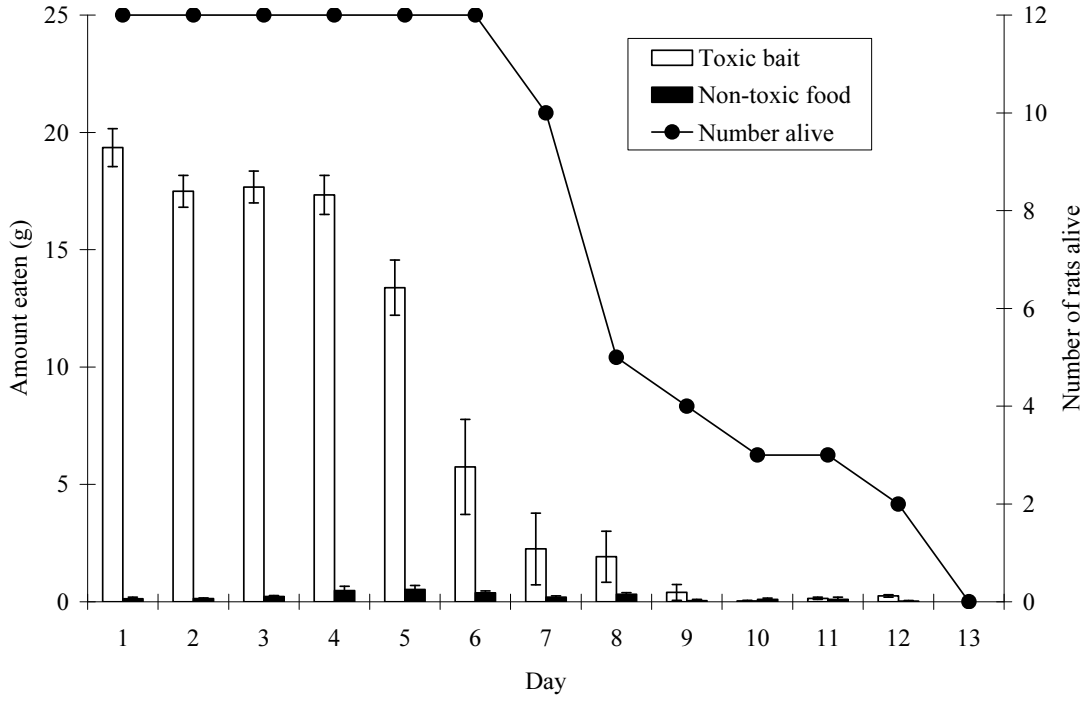


Figure 3.1. Mean amounts (g ± standard error) of brodifacoum bait and non-toxic food eaten by rats in Trial 3, and the mortality of these rats over 13 days. The estimates of mean daily amounts eaten were calculated for each rat on each day, so the means shown account for the decrease in the numbers of rats alive as the trial progressed.

The combined liver residue results are summarised in Fig 3.2. There was a highly significant effect of brodifacoum ingested (dose) on liver residue concentration (slope=1.2961, SDE_{slope}=0.1864, t_{33}=6.95, $p<0.0001$) regardless of whether the rat had died of brodifacoum poisoning (Trials 1 and 3) or had been euthanased for sampling (Trial 2).

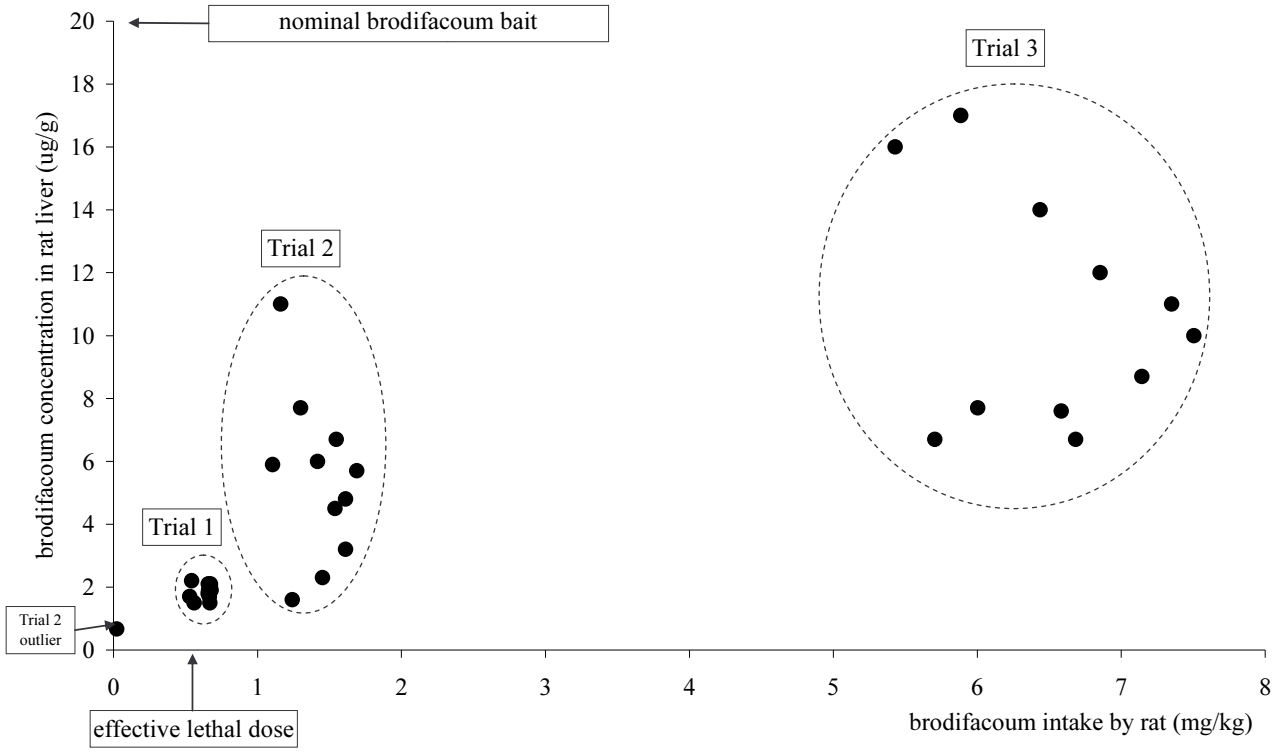


Figure 3.2. Concentrations of brodifacoum in laboratory rat liver after three different bait (20 ppm brodifacoum) intake scenarios; Trial 1 where rats were offered an effective lethal dose (*c*. 0.64 mg/kg) of brodifacoum bait and livers sampled after poisoning mortality, Trial 2 where rats fed *ad libitum* on brodifacoum bait for one night and were euthanased for liver sampling the following day and Trial 3 where rats fed on brodifacoum bait until poisoning mortality.

3.3.2 Brodifacoum concentrations in faeces of rats feeding on bait

Some faecal pellets were observed to have variable shades of green colouration instead of the usual brown, indicating (at least) poor digestion of the dye contained in the brodifacoum bait. The female Norway rat survived, preferring the non-toxic bait and eating only an estimated 0.6 g of toxic bait over ten days (Fig 3.3). The male Norway rat died on day five, ingesting an estimated total of 9.7 g toxic bait (equivalent total 146.2 µg brodifacoum, approximating 0.45 mg/kg dose) (Fig 3.4). The female ship rat died on day ten, ingesting an estimated total of 9 g toxic bait (equivalent total 134.4 µg brodifacoum, approximating 0.95 mg/kg dose) (Fig 3.5). The male ship rat died on day eight, ingesting an estimated total of 16 g toxic bait (equivalent total 240 µg brodifacoum, approximating 1.39 mg/kg dose) (Fig 3.6).

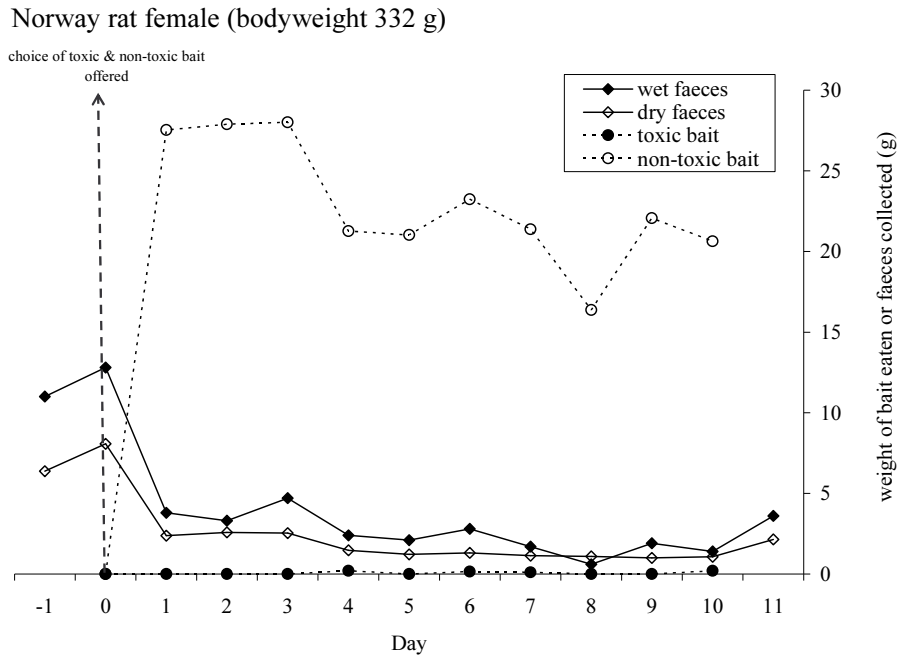


Figure 3.3. Amounts of toxic (Pestoff Rodent Blocks, 20 ppm brodifacoum) and non-toxic (RS5 pellets) bait eaten by a female Norway rat, and the daily weights of faeces collected and dried at 40°C. This rat did not ingest a toxic dose of brodifacoum bait over eleven days.

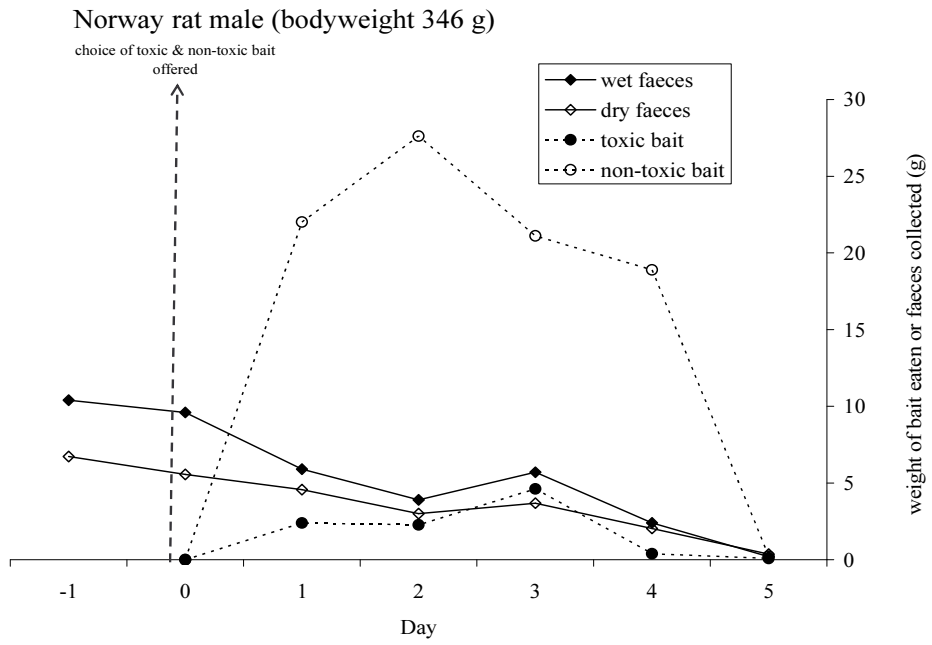


Figure 3.4. Amounts of toxic (Pestoff Rodent Blocks, 20 ppm brodifacoum) and non-toxic (RS5 pellets) bait eaten by a male Norway rat, and the daily weights of faeces collected and dried at 40°C. This rat died on day 5 after ingesting an estimated total of 0.45 mg/kg brodifacoum.

Figure 3.5. Amounts of toxic (Pestoff Rodent Blocks, 20 ppm brodifacoum) and non-toxic (RS5 pellets) bait eaten by a female ship rat, and the daily weights of faeces collected and dried at 40°C. This rat died on day 10 after ingesting an estimated total of 0.95 mg/kg brodifacoum.

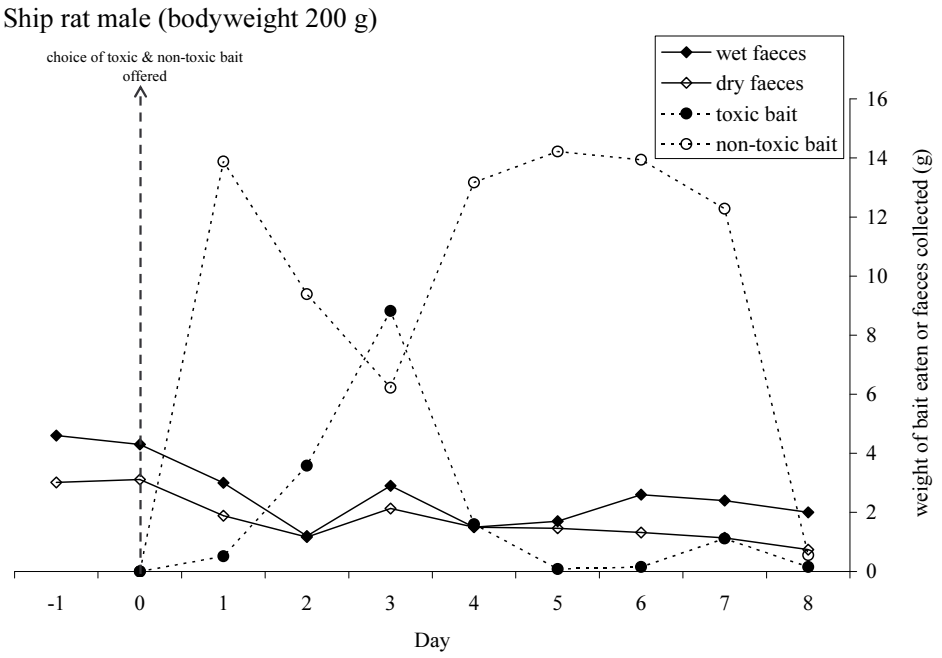


Figure 3.6. Amounts of toxic (Pestoff Rodent Blocks, 20 ppm brodifacoum) and non-toxic (RS5 pellets) bait eaten by a male ship rat, and the daily weights of faeces collected and dried at 40°C. This rat died on day 8 after ingesting an estimated total of 1.39 mg/kg brodifacoum.

Brodifacoum was not detected in any faeces collected from the female Norway rat that did not eat a lethal amount. Using the dry weight of faeces and the measured brodifacoum concentration in each daily sample of faeces, it was estimated that the male Norway rat excreted a total 20.70 µg brodifacoum (*c*. 14% of the total 146.25 µg brodifacoum ingested) (Fig 3.7), the female ship rat excreted a total 29.08 µg (c. 21.6% of the total 134.4 µg brodifacoum ingested) (Fig 3.8) and the male ship rat excreted a total 51.41 µg (c. 21.4% of the total 240.0 µg brodifacoum ingested) (Fig 3.9). The concentrations of brodifacoum measured in daily collections of dried faecal pellets ranged from 0.36 µg/g (female ship rat) to 9.52 µg/g (male Norway rat), with an average concentration (all rats and all days where brodifacoum was detectable in faeces) of 3.56 ± 0.037 µg/g.

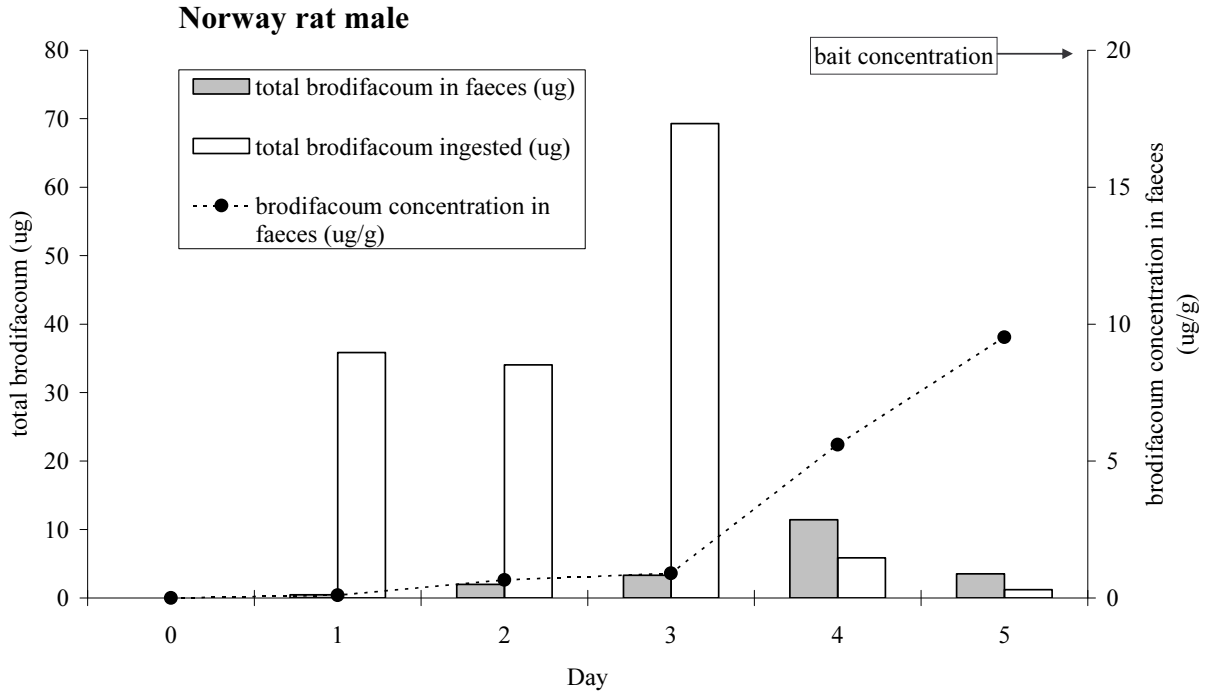


Figure 3.7. Estimated total daily amounts of brodifacoum (µg) ingested in bait and excreted in faeces by a male Norway rat before poisoning mortality. Concentration of brodifacoum measured in dried faeces (µg/g) shown as --●-- with scale relative on the right hand *y* axis relative to brodifacoum concentration in bait.

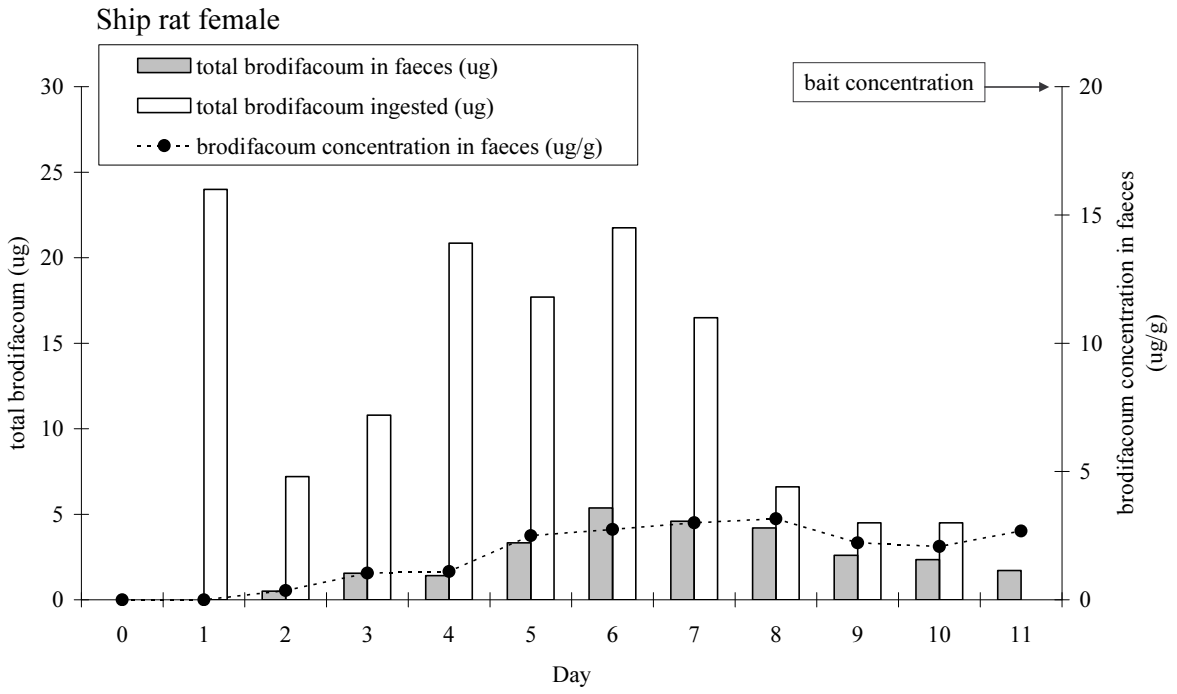


Figure 3.8. Estimated total daily amounts of brodifacoum (µg) ingested in bait and excreted in faeces by a female ship rat before poisoning mortality. Concentration of brodifacoum measured in dried faeces (µg/g) shown as --●-- with scale relative on the right hand *y* axis relative to brodifacoum concentration in bait.

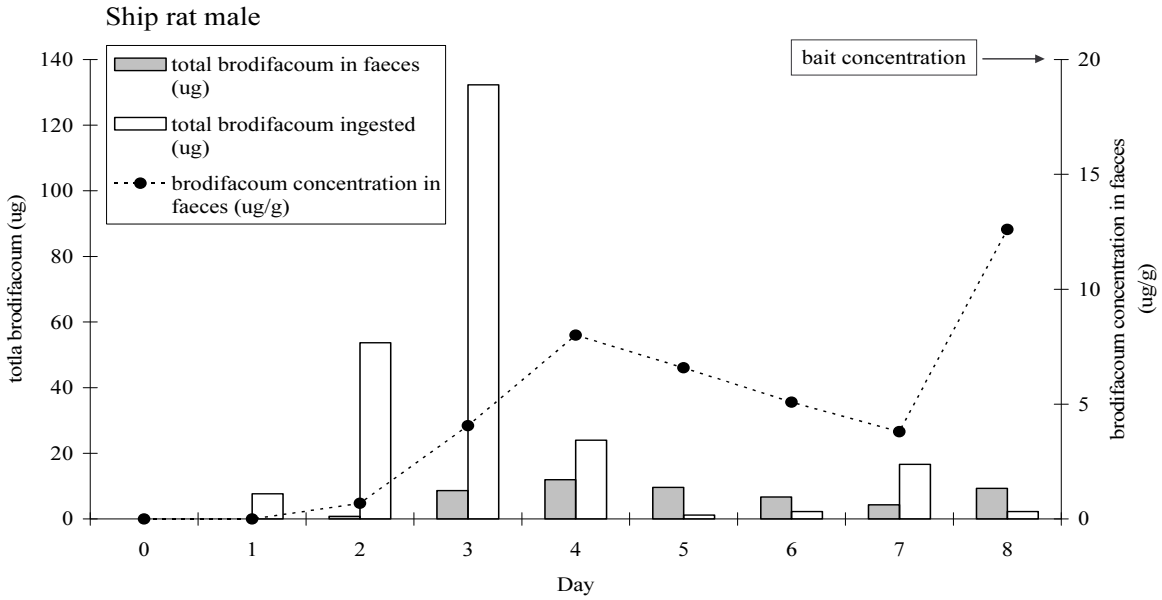


Figure 3.9. Estimated total daily amounts of brodifacoum (µg) ingested in bait and excreted in faeces by a male ship rat before poisoning mortality. Concentration of brodifacoum measured in dried faeces (µg/g) shown as --●-- with scale relative on the right hand *y* axis relative to brodifacoum concentration in bait.

3.4 Discussion

3.4.1 Brodifacoum bait intake and liver residue concentrations in laboratory rats

The positive relationship between amount of brodifacoum ingested and resulting concentrations in rat liver has implications for baiting practices and minimization of secondary hazard. Morin *et al*. (1990) described a similar correlation between the quantity of bromadiolone consumed in bait by coypu (*Myocastor coypus*) and the amounts of bromadiolone quantified in liver. The liver concentrations of brodifacoum measured in Trials 1 to 3 are in the general range reported in other studies with laboratory rats; Bachmann and Sullivan (1983) reported liver concentrations of 2.9-4.9 μg/g in male laboratory rats dosed with 0.2 mg/kg brodifacoum and euthanased 95 hours later. Lower concentrations were measured by Mosterd & Thijssen (1991) following administration of 0.2 mg/kg brodifacoum to laboratory rats and euthanasia of groups at intervals from day 1 to 30 afterwards, with mean brodifacoum liver concentrations of 0.57 to 0.79 μg/g. Ray *et al.* (1989) fed rats 50 ppm brodifacoum bait, and three of these died within 7 days, after ingesting estimated doses of 7.5, 10.0 and 11.25 with corresponding liver concentrations of 8.1, 21.0 and 0.9 μg/g. If the exposure and liver residue values from these three other rat studies were imposed on Fig 3.2, they would fall within the confidence intervals estimated for the slope of the regression.

The highest brodifacoum concentration measured in rat liver here (17 ug/g in Trial 3) approached the 20 ppm used in bait formulations, among the highest residual concentrations previously reported for rat liver. Howald *et al*. (1999) reported liver brodifacoum concentrations of 24.8-35.3 μg/g (wet weight) in Norway rats recovered dead after an eradication baiting operation on Langara Island. Trials 1 and 3, where rats died of poisoning, simulate best and worst case (respectively) for secondary hazard to scavengers of rat carcasses or predators of moribund rats. Taking the average liver concentration from Trial 1 (1.86 μg/g), a theoretical non-target animal weighing 500 g and with the same susceptibility as rats to brodifacoum (LD_{50} 0.27 mg/kg) would have to ingest *c*.72.5 g of rat liver to be at 50% risk of mortality – but using the average liver concentration from Trial 3 (10.67 μg/g) the same theoretical non-target animal would only need to ingest *c*.12.6 g of rat liver to be at risk. Average liver weight in adult laboratory (Norway) rats has been estimated at 9.62 g, approximately 3.2% of the total bodyweight (Landcare Research, unpubl. data). A rat liver of this weight and containing the 'worst case' 17 μg/g residual

brodifacoum would probably constitute a lethal exposure for a susceptible non-target animal of approximately 600 g bodyweight. Overall these results indicate that secondary hazard to non-target wildlife (represented by the residual concentration of brodifacoum in rat liver) can be minimised by limiting brodifacoum bait uptake by the target species to an effective lethal dose only. Dubock (1982) provides a comprehensive rationale for using brodifacoum in pulsed baiting for rodent control in urban and agricultural situations, largely justified in terms of improved cost-efficacy over 'saturation baiting' techniques used with other rodenticides. However he also states "the pulsed baiting technique is designed to ensure that individual rodents ingest little more bait than is absolutely necessary for a lethal dose. The opportunity for heavy toxicant loading of tissues at death should therefore be reduced compared to animals which consume far more than a lethal dose before death...". This observation is well borne out by the positive relationship shown here between brodifacoum ingested and residual concentration in rat liver.

Growing evidence of secondary risk (e.g. Erickson & Urban 2002) has curtailed some applications of brodifacoum, but other use patterns continue (as reviewed in Chapter 1.1.2). In particular, 'over the counter' availability to the public and ongoing bait station (field) applications of brodifacoum in NZ are unlikely to minimise bait intake by the target species to effective lethal doses, creating an increased residue burden in poisoned animals and subsequent secondary hazard to non-target wildlife. Development of broadscale baiting techniques for eradication of rodents has seen brodifacoum become an important conservation tool (e.g. Buckle and Fenn 1992) with many potential island applications (e.g. Howald *et al.* 2007). While Dubock (1982) may not have anticipated that island eradications would be added to the traditional focus of rodent baiting on protecting human health and production values, he describes a scenario for the use of pulsed baiting against populations where all rodents can feed on the first and only bait application, stating "..such a situation occurs where the relationship between the number and toxicity of bait points and number, susceptibility to the toxicant, and behaviour of individual rodents is such that no individual animal is prevented by social interaction or any other factor from ingesting a lethal dose of the first bait application. Consequently all rodents ingest a lethal dose and, on the average, all are dead about 1 week after placing the bait. This situation does not only occur when the rodent population is of a low density; it may also occur where the rodent population density is very high but the density of the bait and its distribution is arranged so that all individuals have the opportunity for lethal ingestion of rodenticide within the same short time". Perhaps not coincidentally, Dubock's statement provides a neat summary of the rationale

underpinning aerial applications of brodifacoum baits on islands, where all target rodents must be put at risk to achieve eradication (e.g. Bomford and O'Brien 1995). Thus, current 'one off' applications of brodifacoum for island eradications of rodents utilise the principles of pulsed baiting and are likely to minimise the amount of brodifacoum entering the environmental reservoir comprised by poisoned rodents. This is an important contrast to sustained or repeated applications, which needs to be considered in risk assessments or any regulatory review of brodifacoum.

Although other tissues are likely to contain lower residual concentrations of brodifacoum than liver, the focus on liver in this study is a limitation in full evaluation of non-target risk. Measurement of whole-body residue burdens provide a more realistic basis for risk assessment as predators and scavengers will consume other tissues, and sometimes whole carcasses of contaminated rats. In particular, gut contents comprising partially digested bait may have relatively high brodifacoum concentrations and present a hazard to predators that eat whole rats. The presence of brodifacoum in the faeces of rats that had been feeding on bait suggests that gut contents at all stages of digestion (not just stomach contents) are a potential non-target hazard.

3.4.2 Brodifacoum concentrations in faeces of rats feeding on bait

Field observations of rodent faeces coloured to different extents by the green or blue dyes commonly used in anticoagulant bait formulations have frequently been made by the author. Similar observations in other species such as common geckoes (*Hoplodactylus maculates)* (Hoare & Hare 2006b) and ravens (*Corvus corax*) (Howald 1997) have supported inferences of bait ingestion. In rats, colouration of faeces probably also indicates the presence of residual (undigested) poison - the results here confirm that an estimated 14-21.6% of ingested brodifacoum is excreted unchanged in rat faeces throughout the period between ingestion of a lethal dose and death. Thus in field baiting situations residual brodifacoum is not just contained in rat tissues, but some is also distributed in the environment through rat faeces. The implications of this for invertebrates that feed on faeces, or for soil contamination with brodifacoum are not known but could be investigated by studies of the rate of degradation of faecal brodifacoum in different environmental conditions. Contamination of stock feed or human food by rodent faeces is of general concern from the perspective of zoonotic disease transmission, however the possibility of brodifacoum contamination through this pathway could be especially important where intensive farms have large feed stockpiles and associated populations of rats subject to

control through sustained brodifacoum baiting. While the potential for faecal brodifacoum to pose a secondary hazard remains unclear, it would be desirable to minimise the amount of brodifacoum entering the environment through this pathway. As for tissue residues, baiting strategies that minimise brodifacoum intake by target pest species to an effective lethal dose would address this.

3.5 Conclusions

The secondary hazard presented to non-target predators or scavengers by contaminated rat liver is likely to be directly related to the amount of brodifacoum ingested by rats. Baiting strategies that maintain high efficacy of brodifacoum against rodents and other vertebrate pest species, also need to find some balance in minimising the amount of brodifacoum available in the environment through rodent tissues, gut contents or faeces. While 'one off' applications of brodifacoum for island eradication of rodents are more likely to meet this criterion simply through discrete delivery of a defined quantity of bait, other current use patterns do not. In particular, ongoing field applications for possum and rat control in NZ are unlikely to minimise bait intake by the target species to effective lethal doses, creating an increased residue burden in poisoned animals and increased secondary hazard to non-target wildlife.

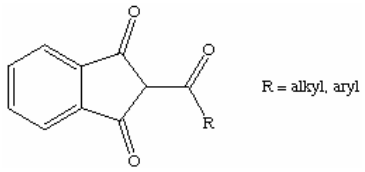
Chapter 4: Diphacinone as an alternative to brodifacoum – a review

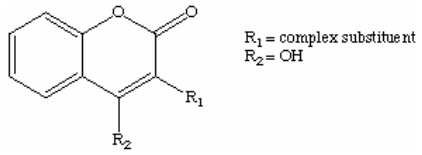
4.1 Overview of anticoagulant toxicity, rodent resistance and persistence

Since the 1950s, anticoagulant compounds have been used worldwide as poisons for the control of commensal rodents, particularly rats (*Rattus* spp.) and house mice (*Mus musculus*). Their common mode of toxicity, largely due to inhibition of normal synthesis of vitamin K-dependent blood clotting factors in the liver, has been well described by other authors (e.g. Thijssen 1995; Parmar & Batten 1987; Sutcliffe *et al.* 1987) and will not be repeated here except to note recent identification of the molecular binding target of hydroxycoumarin anticoagulants at the site of vitamin K epoxide reductase (Gebauer 2007). Anticoagulants can be classified as indandiones or coumarins by chemical structure, and also as first-generation (FGAR) or second-generation (SGAR) according to when they were first available as rodenticides (Table 4.1).

Table 4.1. Date of development and use of first- and second-generation anticoagulant rodenticides, and their grouping by chemical structure (British Crop Protection Council 2001).

First generation	1942: Pindone 1952: Diphacinone c.1962: Chlorophacinone	Indandione
	1944: Warfarin 1962: Coumatetralyl	Coumarin
Second generation	1975: Difethialone 1976: Brodifacoum 1978: Bromadiolone 1984: Flocoumafen 1986: Difenacoum	





Development of physiological resistance in rat populations subjected to intensive use of FGARs (e.g. Jackson *et al.* 1988; MacNicoll 1993) saw succession by the more potent SGARs that were effective against resistant rodents (e.g. Kaukeinen & Rampaud 1986). Resistance to some SGARs, including difenacoum and bromadiolone, has since been documented (e.g. Quy 1998; Misenheimer *et al.* 1994) although the contribution of resistance to the reduced operational efficacy of SGARs has been questioned (Cowan *et al.* 1995). The genetic basis of anticoagulant resistance has been characterized towards DNA-based monitoring of rodent populations (Pelz *et al.* 2005) and this adaptive response of rodent pests remains a challenge for their management

particularly in Europe, the United Kingdom and the United States (table in Pelz *et al.* 2005). No published reports of brodifacoum or flocoumafen resistance in rodents were found in literature, thus these two compounds appear currently the most 'universally' effective anticoagulant rodenticides. While a more comprehensive review of anticoagulant resistance is beyond the scope of this thesis, a lack of formal assessments of the resistance status of NZ rodent populations is noted.

In general, FGARs are most toxic when ingested as multiple, consecutive doses whereas the SGARs, particularly brodifacoum, are considered 'single feed' poisons because of their greater acute toxicity. The operational advantages of single-feed efficacy are outlined in Chapter 1. The generally lower toxicity of the first-generation anticoagulants is attributed to a lower binding affinity to sites in the liver (Parmar *et al.* 1987; Huckle *et al.* 1988), however there can also be considerable inter-sex and inter-strain differences in toxicity of the same anticoagulant to rodents (e.g. Ashton *et al.* 1987). The lethal dose (LD_{50}) is the amount of pesticide required to kill 50% of a population of animals, expressed as milligram of rodenticide per kilogram of animal bodyweight (mg/kg). Table 4.2 shows representative acute oral toxicity values (LD_{50}) for anticoagulants in non-resistant Norway rats (*R. norvegicus*). The persistence and half-life values reported for various anticoagulants in blood (plasma) and liver are summarised in Table 4.3.

Table 4.2 Representative acute oral toxicity values (LD_{50}) for different first-generation (FGAR) and second-generation (SGAR) anticoagulants in non-resistant adult Norway rats (*R. norvegicus*) of both sexes.

Anticoagulant	**Oral LD_{50} (mg/kg) or (mg/kg per day) in *Rattus norvegicus***	**Reference**
Pindone (FGAR)	50	Dubock & Kaukeinen (1978)
Diphacinone (FGAR)	3	Correll *et al.* (1952)
	43.3 (males)	Kusano (1974)
	22.7 (females)	Kusano (1974)
	0.35 for 5 days	Ashton *et al.* (1987)
Chlorophacinone (FGAR)	11	Erickson & Urban (2004)
	20.5	Jackson & Ashton (1992)
	0.19 for 5 days	Jackson & Ashton (1992)
Warfarin (FGAR)	323 (males)	Hagan & Radomski (1953)
	58 (females)	Hagan & Radomski (1953)
	2.5-50	

		Erickson & Urban (2004)
Coumatetralyl (FGAR)	16.5 0.3 for 5 days	Dubock & Kaukeinen (1978) British Crop Protection Council (2000)
Difethialone (SGAR)	0.56	Jackson & Ashton 1992
Difenacoum (SGAR)	1.8 (males) 2.5 (females)	Bull (1976) Bull (1976)
Brodifacoum (SGAR)	0.41 (males) 0.56 (females)	United States EPA (1998) United States EPA (1998)
Bromadiolone (SGAR)	0.56 – 0.84 1.12	Erickson & Urban (2004) Grand (1976)
Flocoumafen (SGAR)	0.25	Huckle *et al.* (1989)

The tendency for anticoagulants to persist in mammalian (rat) liver is influenced by the magnitude of the dose ingested and the relative affinity of the compound for saturable receptors in the liver, which determines the hepatic elimination half-life (t½) and the proportion of the dose retained (Parmar *et al.* 1987). Hepatic elimination of SGAR coumarins is described as biphasic, with a rapid initial phase and more prolonged terminal phase (e.g. Smith *et al.* 1990). The general trend in the coumarin compounds is for hepatic persistence to increase with toxicity (Tables 4.2 and 4.3) so that SGARs are recognised as persistent for prolonged periods in liver (e.g. Erickson & Urban 2004). Bachman & Sullivan (1983) suggested that serum and liver concentrations of brodifacoum were likely to accumulate with repeated daily exposures, based on simulations of the data they obtained following a single 0.2 mg/kg dose to laboratory rats. Bioaccumulation of flocoumafen, another second-generation anticoagulant with relatively high hepatic persistence, has been demonstrated in rats following up to 10 weekly radio-labelled doses of 0.1 mg/kg (Huckle *et al.* 1988). The persistence of the SGAR coumarins is better described than that of the FGARs, particularly the indandiones, for which there is relatively limited data on hepatic persistence (Table 4.3). A recent study by Fisher *et al.* (2003) (Table 4.3) suggests that the general trend seen with the coumarins, of increasing hepatic persistence with increasing toxicity, may not also apply to indandiones.

Table 4.3 Persistence and half-life values reported for first- and second-generation anticoagulants in blood (plasma) and liver following a range of exposure levels and routes of administration. ‡ Liver retention is expressed as the time period for which residues are reported to persist in the liver unless the value is preceded by t½. Plasma is t½ unless otherwise specified. * Not viewed, unpublished report cited by Erickson & Urban 2004.

	Species	**Blood t½ † (hours)**	**Liver retention ‡ (days)**	**Reference**
First generation anticoagulants				
Warfarin	rat (M,F)	18, 28		Pyrola 1968
	rat		t½ 7-10	Thijssen 1995
	rat		t½ 26.2	Fisher *et al.* 2003
	rabbit	6		Breckenridge *et al.* 1985
	possum	12		Eason *et al.* 1999
	human	35-45		Kelly & O'Malley 1979
	human	14.4–57.6		O'Reilly *et al.*1963
Coumatetralyl	rat	-	t½ 55	Parmar *et al.* 1987
Pindone	rat		t½ 2.1	Fisher *et al.* 2003
	dog	120		Fitzek 1978
	sheep		8-16	Nelson & Hickling 1994
Diphacinone	rat		t½ 3	Fisher *et al.* 2003
	cow		>90	Bullard *et al.* 1976
	human	360–480		World Health Organisation 1995
Chlorophacinone	rat	9.6		Belleville 1981*
	human	156–264		Burucoa *et al*. 1989

	Species	Blood t½ † (hours)	Liver retention ‡ (days)	Reference
Second-generation anticoagulants				
Difethialone	rat	55.2	t½ 108	Lechevin & Poche 1988
	rat		½ 126	Belleville 1986*
			½ 74	Belleville 1991*
	dog	52.8–76.8		Robben *et al.* 1998
Difenacoum	rat		t½ 118	Bratt 1987
	rat		t½ 120	Parmar *et al.* 1987
	rabbit	83		Breckenridge *et al.* 1985
Bromadiolone	rat	26–57		Kamil 1987
	rat	25–26	t½ 170	Parmar *et al.* 1987
	rat		t½ 318	Hawkins *et al.* 1991*
	sheep		256	Nelson & Hickling 1994
Flocoumafen	rat		t½ 220	Huckle *et al.* 1988
	sheep		>256	Nelson & Hickling 1994
	quail		t½ >100	Huckle *et al.* 1989
	dog		>300	Veenstra *et al.* 1991

	Species	Blood t½ † (hours)	Liver retention ‡(days)	Reference
Second-generation anticoagulants				
Brodifacoum	rat		t½ 350	Batten & Bratt 1990*
	rat		t½ 128 (terminal)	Batten & Bratt 1990*
	rat		t½ 282	Hawkins *et al.* 1991*
	rat		t½ 150-200	Bratt & Hudson 1979*
	rat		t½ 136	Belleville 1991*
	rat	6.5	>80	Bachmann & Sullivan 1983
	rat		t½ 130	Parmar *et al.* 1987
	rat		t½ 113.5	Fisher *et al*. 2003
	rabbit	60.8 (terminal)		Breckenridge *et al.* 1985
	dog	144		Woody *et al.* 1992
	dog	21.6–112.8		Robben *et al.* 1998
	dog	1.4 (initial) 8.7 (terminal)		Murphy *et al.* 1985
	possum	480–720	>252	Eason *et al.* 1996
	sheep		>250	Laas *et al.* 1985
	horse	28.8		Boermans *et al.* 1991
	human	16–36		Weitzel *et al.* 1990
	human	487		Breckenridge *et al.* 1985
	human	580.8		Hollinger & Pastoor 1993
	human	1488 (terminal)		Stanton *et al.* (1988)
	human	945.6 (terminal)		Lewis-Younger & Horowitz (2001)

4.2 Diphacinone as an alternative to brodifacoum

In selecting appropriate poisons for vertebrate pest control, managers need to balance benefits and cost efficacy against unwanted effects, such as mortality or sublethal effects on non-target wildlife. Increasing evidence of secondary effects and contamination of wildlife by brodifacoum (outlined in Chapter 1) has in some cases prompted re-evaluation of the use patterns of this highly effective rodenticide. For example, the NZ Department of Conservation implemented restrictions on the use of brodifacoum for conservation purposes on the NZ mainland because of documented levels of direct and indirect poisoning of nontarget species (Department of Conservation 2000). Restrictions of the use of brodifacoum for commensal rodent control in the United Kingdom and Europe are in recognition of its potential for unwanted impacts on biodiversity (e.g. Baker *et al.* 2007). The relatively high risk of secondary poisoning posed by brodifacoum to non-target wildlife is mediated by the persistence of brodifacoum in liver and carcasses, and accordingly attention has turned to identifying less persistent but equally effective alternative rodenticides. While all of the anticoagulants have the advantages of delayed onset of poisoning, and the availability of an effective treatment for accidental poisoning, on current data, diphacinone offers an optimal combination of lower hepatic persistence (Table 4.3) but relatively high toxicity to rodents (Table 4.2).

4.2.1 Diphacinone metabolism, elimination and persistence

Yu *et al.* (1982) reported that diphacinone was not extensively metabolised by rats following oral administration of C^{14}-labelled diphacinone at 0.2 or 1.5 mg/kg, with more than 60% of the dose excreted in faeces and 10% in urine over eight days, with a similar elimination pattern observed in mice. Continued elimination in faeces over four to eight days indicated that biliary excretion rather than incomplete absorption was the major route of elimination in both rodent species. At eight days after dosing rat tissues retained *c*.20% of radiocarbon, with highest concentration in the liver, "significant" residues in kidney and lung and lower residues in brain, fat, blood and muscle (Yu *et al.* 1982). Another study using radio-labelled diphacinone in mice (Cahill & Crowder 1979) reported similar results, with radioactivity reaching highest levels in liver and lungs, with maximum liver concentrations at 3.0-7.5 hours after administration. In contrast to findings in rodents, Bullard *et al*. (1976) reported that cows (*Bos taurus*) dosed with 1 mg/kg diphacinone by intraruminal injection had almost constant liver residues of up to 0.15 mg/g from 30 to 90 days after dosing. However, rats fed for 14 days on the livers of the cattle dosed with diphacinone showed no signs of toxicity during the test period or for 14 day afterwards, and liver

samples taken from the rats at the end of that period contained no detectable diphacinone residues (Bullard *et al*. 1976), indicating relatively rapid clearance. On the basis of this small number of studies, significant inter-species differences in diphacinone elimination were a possibility.

4.2.2 Use patterns of diphacinone

Diphacinone has been used in human treatment (e.g. 'Dipaxin'; Katz *et al*. 1954) for prevention of thrombosis. Systemic treatment of livestock with sublethal doses of diphacinone can cause secondary poisoning in vampire bats (*Desmodus rotundus*) that repeatedly feed on the livestock, which has been utilised for control of vampire bats (e.g. Thompson *et al.* 1972; Sald-Fernandes & Flores-Crespo 1991). Diphacinone has been used in a range of countries for commensal rodent control since the 1950s (Pelfrene 2001), and for field control of agricultural pests such as Californian ground squirrels (*Spermophilus beecheyi*) in the United States (Salmon *et al*. 2007). In NZ, diphacinone is currently under consideration by the Department of Conservation as the best potential alternative to replace (now restricted) brodifacoum use for control of field populations of rodents for conservation benefits (Animal Control Products 2003). Results of field efficacy trials of different 0.005% diphacinone bait formulations against rats in mainland sites (Gillies *et al.* 2006) have indicated that registration of diphacinone should proceed for this application. It is uncertain whether diphacinone would also be effective for possum control. Rammell & Fleming (1978) reported that possums survived single oral exposures of 300 mg/kg diphacinone, although multiple-dose toxicity of diphacinone in possums remains to be estimated. Assuming that diphacinone would not have the single-dose efficacy of brodifacoum against both rodents and possums, future effective control of multiple vertebrate pest species in NZ is likely to rely on a combination of control methods (see Chapter 7 for further discussion).

While brodifacoum has an established 'track record' in successful eradication of introduced rodents from islands (Chapter 1.1.3) concerns over secondary risk to non-target predatory or scavenging wildlife, especially island species of high conservation value, have led to consideration of diphacinone as an alternative. Recent successful use of diphacinone in eradication of ship rats (*R. rattus*) on one of the San Jorge Islands, Mexico (Donlan *et al*. 2003) and on Buck Island, Virgin Islands (Witmer *et al.* 2007) represents the beginning of a successful 'track record' for diphacinone in this context. Registration is being sought for a 0.005% diphacinone bait formulation to be applied by aerial broadcast for the control of rodents in native

Hawaiian ecosystems, with the selection of diphacinone over other rodenticides based on demonstrated effectiveness against rats in Hawaii, favourable environmental track record and previous use as a human pharmaceutical (Eisemann & Swift 2006). A proposed rodent eradication on Mokapu Island, Hawaii, will use diphacinone because of the lower non-target risk expected in comparison to brodifacoum (Dunlevy & Lee 2007).

With registrations of diphacinone formulations for broadscale field applications ongoing in both NZ and the United States, existing regulatory toxicity and environmental data has been collated towards hazard assessment (Eisemann & Swift 2006). Information gaps exist around species differences in toxicity and the environmental transfer of diphacinone *viz.* secondary or tertiary hazard to non-target species, including invertebrates and humans. While monitoring during and after field applications of diphacinone baits will be important to validate the predictions made by prior risk and hazard assessments, laboratory studies can selectively address information gaps with data that can be fed into the hazard assessment process. In the NZ context, specific information gaps included the effects of diphacinone exposure on invertebrates i.e. does diphacinone pose a risk of primary poisoning to native species such as weta? Do invertebrates that eat bait retain residual diphacinone concentrations that could pose a secondary risk to insectivores? Accordingly Chapter 5 describes a laboratory study of weta that investigated whether these native NZ invertebrates would ingest diphacinone bait, and described subsequent effects and residual concentrations of diphacinone in the bodies of the weta. Another information gap relevant to NZ ecosystems and resource use concerned the toxicity of diphacinone to pigs and characteristaion of the residual persistence of diphacinone in pigs, with respect to potential hazard to humans hunting and consuming feral pigs. Chapter 6 describes a pen study with domestic pigs designed to address these questions.

Chapter 5: Persistence of diphacinone in pig tissues

5.1 Introduction

As an introduced species to NZ, feral pigs (*Sus scrofa*) can have localized pest status but nationally are also a significant resource for recreational hunters (McIlroy 2001). Similarly, in Hawaii pigs are an introduced species that also have status as a game animal (Wood & Barrett 1979) hunted for human consumption. In NZ, feral pigs may be exposed to anticoagulants through toxic baits laid for possum or rodent control (primary exposure) or when they scavenge carcasses of poisoned animals (secondary exposure, e.g. Morriss *et al.* 2005), creating the potential for additional exposure of humans consuming meat from feral pigs (Engemann & Pank 1984). The NZ Food Safety Authority (NZFSA) specifies guidelines designed to minimise the risk of meat contaminated with vertebrate toxic agents, including anticoagulants, entering human food. These include certification of suppliers of wild game meat and guidelines for recreational hunters of feral pigs (NZFSA 2006). Where game is to be taken from areas where vertebrate pesticides have been used, the NZFSA currently advises withholding periods of 3 years for brodifacoum and other second-generation anticoagulants, and 2 months for pindone, warfarin and other first-generation anticoagulants. Residue testing of game meat for brodifacoum, bromadiolone or flocoumafen is required, against a maximum residue level (MRL) of 0.001 ppm for brodifacoum (Clear 2003), although there is currently no MRL specified for diphacinone.

Toxicity estimates (LD_{50} values) of brodifacoum to pigs range from 0.1 mg/kg (Godfrey 1985), 0.5 mg/kg with 95% confidence intervals of 0.17-1.2 mg/kg (O'Brien & Lukins 1990) and <2.0 mg/kg (Erickson & Urban 2002). Thus, a 50 kg pig consuming approximately 500 g of bait containing 20 ppm brodifacoum would probably receive a lethal dose, a feasible scenario especially where pigs access bait stations. Five feral pigs fitted with mortality transmitters died within 10 days of aerial application of brodifacoum baits in the Maungatautari fenced reserve (NZ) during September 2006 and had brodifacoum liver concentrations (2.08-3.49 μg/g) suggestive of poisoning. Eason *et al*. (2001) reported brodifacoum concentrations of 0.72-1.38 μg/g in liver and 0.02-0.07 μg/g in muscle of captive pigs at five days after ingesting 500-1776 g of bait. A survey of feral pigs taken from areas with a history of brodifacoum use showed that 29/37 (78%) had detectable liver residues, ranging from 0.01-2.4 μg/g (Eason *et al.* 2001). In general, detectable muscle residues (0.1-0.05 μg/g) in these pigs were associated with liver residues greater than 0.5 μg/g. The occurrence of brodifacoum residues in feral pigs in NZ (e.g.

Booth *et al.* 2001) and subsequent potential for human exposure is one reason that diphacinone is being investigated as an effective but less persistent alternative to brodifacoum (see Chapter 4.2).

With registration of diphacinone bait formulations for broad-scale field control of rodents in NZ (Gillies *et al.* 2006) and Hawaii (Eisemann & Swift 2006), potential human exposure to residual diphacinone through consumption of 'wild pork' required assessment. While diphacinone is less persistent than brodifacoum in laboratory rat liver (Fisher *et al.* 2003), it was important to establish whether this was also the case in pigs. The acute oral toxicity of diphacinone in pigs has been reported as LD_{50}>150 mg/kg (Hazelton Laboratories 1957 cited by Pitt *et al.* 2004) although this estimate was based on two pigs that survived doses of 27 or 150 mg/kg. It suggests that pigs are less susceptible to diphacinone than other mammals: Mount & Feldman (1983) reported an LD_{50} of 3 - 7.5 mg/kg in dogs, and Jackson & Ashton (1992) reported 1.93 - 43.3 mg/kg in a laboratory strain of Norway rat. In general, the oral toxicity of first-generation anticoagulants to mammals is increased when these compounds are ingested in multiple, consecutive doses (see Table 4.2). In a recent study in the United States (Fletcher 2002), pigs fed with diphacinone at 0.133 mg/kg/day for 7 days, all survived without any obvious signs of poisoning. However, pigs fed with 0.333 mg/kg/day for 7 days showed some signs of poisoning and showed haemorrhage pathology on necropsy 21 days later. In an earlier study (Keith *et al.* 1990), pigs were offered diphacinone in food at doses of 0.6 mg/pig/day for 2 days and 1.5 mg/pig/day for 5 days, but since no bodyweights were given for the pigs, the doses (mg/kg) they received are unknown. Pigs showed no signs of poisoning and had normal coagulation times 2 and 10 days after dosing. Given that a diphacinone dose of 0.333 mg/kg/day produced obvious signs of poisoning followed by recovery (Fletcher 2002), the multiple-dose LD_{50} for pigs is likely to be higher. However, even the single acute toxicity estimate of >150 mg/kg is very general and does not allow a precise estimation of what might constitute lethal and sublethal intakes of diphacinone in pigs. It was important to refine estimates of oral toxicity in pigs before selecting an exposure level for the residue persistence study.

An initial pen trial with domestic pigs, simulating single and multiple oral diphacinone exposures that could eventuate if (feral) pigs accessed bait stations in the field, was undertaken to evaluate whether such exposures caused toxic effects. Coagulation time of plasma samples were used to indicate toxicity: prothrombin time (PT) is widely used to monitor the extrinsic coagulation pathway (clotting factors II, VII and X), while the activated partial thromboplastin time (APTT)

test can detect abnormalities in the intrinsic coagulation pathway (clotting factors II, V, VII, IX, X, XI and XII). Prolonged coagulation times in either or both test would indicate a toxic effect. Results of this first trial were used to gauge sublethal oral diphacinone exposures for a second trial simulating a worst-case scenario for concentrations of residual diphacinone in pig tissues (liver, muscle, fat and kidney) and measurement of their decline over time. Some data from these trials has been published in Fisher (2006).

5.2 Methods

5.2.1 Pig housing and husbandry

All procedures were approved by the Landcare Research Animal Ethics Committee (Project No. 04/06/03). Domestic weaner pigs (*c*.8 weeks old; equal sex ratio) identified by numbered eartags were housed in groups of 12. Their 10×12 m pen was contained in a large shed, with wood shavings over a concrete floor and a roofed sleeping area constructed of hay bales and filled with straw. Twelve individual feeding bays formed one wall of the pen. Water was freely available through an automatic drinker and pigs were fed twice daily on commercial pellet feed (Weston Animal Nutrition, Rangiora) to approximate a daily intake of 5% bodyweight. The feed pellets contained Vitamin K_3 at a maximum 2.5 g per tonne, which was not expected to compromise the effect of diphacinone on pigs, as Vitamin K_1 is the antidotal form for anticoagulant poisoning. For two weeks pigs were acclimatised to entering a feeding bay and being shut in while consuming their ration. A dough made of flour and sugar was a palatable food later used to deliver the diphacinone exposures, and a *c*.30 g portion of this was also offered to pigs alongside the morning ration as part of the acclimatisation. Pigs were weighed at least every two weeks to monitor growth and general health.

5.2.2 Trial 1: Coagulation time responses to diphacinone exposure

Pigs were randomly allocated to treatment groups (Table 5.1) intended to simulate potential field exposures to bait. These exposures in food were intended to simulate potential high exposures of feral pigs to diphacinone baits in the field, assuming that a bait station held up 2 kg of 50-ppm diphacinone bait, thus a 40-kg pig eating this would ingest 2.5 mg/kg diphacinone. Individual diphacinone doses, according to bodyweight, were prepared by uniformly mixing the appropriate amount of diphacinone powder (supplied Animal Control Products, assayed as 1.98% by the

Landcare Research toxicology laboratory) into *c*.30 g of the palatable sugar dough. Individual 'dough ball' doses were offered before morning feeds on appropriate day(s) and consumption noted. Pigs were then returned to normal diet and observed at least twice daily for signs of poisoning such as haemorrhage, anemia or inappetance, over the following three weeks. At two weeks after last exposure, surviving pigs were euthanased by captive bolt gun and liver samples were taken and analysed for diphacinone concentrations as described in section 5.2.3.

Table 5.1. Oral diphacinone exposures of pigs in Trial 1.

No. pigs (sex)	**Diphacinone dose (mg/kg) or (mg/kg/day)**	**Exposure scenario (per single 40 kg pig)**
3 (2F, 1M)	12.5 mg/kg (single)	Ingests contents of five bait stations within 1 day
3 (1F, 2M)	2.5 mg/kg (single)	Ingests contents of one bait station in 1 day
3 (1F, 2M)	2.5 mg/kg/d (3 days)	Ingests contents of one bait station per day for 3 days
3 (2F, 1M)	0.5 mg/kg/d (5 days)	Ingests c. 400 g of bait per day for 5 days

Baseline blood samples were taken one week before diphacinone exposure and then at days 2, 7 and 14 after first exposure. The pigs were restrained while lying on their backs and blood drawn from the anterior vena cava using a 18G×1 1/2" needle into a 10-mL syringe. Samples were immediately divided between two 4.5-mL tubes (Vacutainer® Blood Collection Tubes, 9NC, 3.8% sodium citrate, Becton Dickinson), stored on ice and centrifuged within 2 h to obtain plasma. Whole blood was centrifuged at 2500*g* for 15 min at 4°C. Plasma from each blood sample was divided into two Eppendorf tubes, with one 'split' retained at −80°C. The other was also kept at −80°C if testing for coagulation time could not be carried out that day, or at −4°C if testing was to be done within 6 h. Plasma from the day 2 and 7 samples were tested for coagulation time within 6 h to ascertain whether toxicity could be occurring and the pigs were observed at least twice daily for signs of poisoning during this time. A licensed bolt-gun operator was on hand to perform euthanasia in the event of evident toxicosis. Where thawing was required before testing, samples were placed in a hot water bath at 37°C. Plasma samples were tested in duplicate using PT and APTT testing kits (respectively Simplastin® Excel S and Platelin® , BioMèrieux Inc., USA) and an automated coagulometer (Amelung KC4Amicro, Sigma

Diagnostics). The control plasma reagents supplied with each kit were tested to provide internal validation standards for the pig plasma samples. The PT times were converted to International Normalised Ratio (INR) values, which provide a uniform scale for comparing PT data obtained using different thromboplastin reagents in different tests. INR values were calculated as the ratio of the mean PT value obtained from duplicate testing of the plasma from a diphacinone-treated pig to the mean baseline PT, then raised to the power of the International Sensitivity Index (ISI) figure which was 1.23 for the Simplastin® Excel S kit.

5.2.3 Trial 2 Persistence of diphacinone in pig tissues

Based on the results of Trial 1, where all exposures tested caused similar temporary elevations in coagulation times, a single 12.5 mg/kg diphacinone exposure was selected to represent a 'high' sublethal dose in Trial 2. Twelve weaner pigs (6 male, 6 female) were group-housed and acclimatised as previously described, and then administered 12.5 mg/kg diphacinone in a dough ball, representing a high sublethal exposure (based on coagulation-time responses from the previous trial). Pigs were randomly allocated to three groups (each $n = 4$) for tissue sampling on day 1, 4 or 10 after exposure. Tissue sampling was carried out immediately after euthanasia by captive bolt gun. Pigs were bled by severing the carotid artery and jugular vein and whole-blood samples (at least 10 mL) were collected from this flow into citrate tubes. Liver and kidneys were removed from the carcasses and weighed. Samples (approximately 50 g) of liver, kidney, muscle (from the rear haunch) and abdominal fat were stored at −20°C until analysis for diphacinone concentration. Blood samples were centrifuged as before and divided into two plasma samples that were stored at −80°C until analysis for diphacinone concentration.

Concentrations of diphacinone in tissues and plasma were determined by the Landcare Research toxicology laboratory, Lincoln. A sample of tissue (1 g or 1 mL plasma) was weighed into a glass tube. Chlorophacinone (100 µL) as an internal standard and anhydrous sodium sulphate (5 g) were added, followed by chloroform/acetone/formic acid (0.25%, 35 mL), and the contents of the tube shaken and centrifuged. The supernatant was decanted and the extraction process repeated twice more. The combined extracts were evaporated and taken up in hexane/chloroform for clean-up on a carbograph SPE column, followed by an aminopropyl column. The analyte was eluted from the aminopropyl column with mobile phase A, which was then evaporated to dryness and taken up in mobile phase for quantification by HPLC analysis, using a C8 10-µm column and

UV detector set at 284 nm. A method detection limit (MDL) of 0.02 µg/g and analysis uncertainty (95% confidence interval) of ±20% was estimated according to TLM067 (IANZ-registered toxicology laboratory, Lincoln).

5.2.4 Statistical analyses

Coagulation time data were analysed using procedure LME in the statistical package R (R Development Core Team 2007). Linear mixed models were fitted to test for significant differences in the response variables (PT, PT-INR, APTT) with respect to diphacinone dose group (12.5 mg/kg, 2.5 mg/kg, 2.5 mg/kg/day×3 or 0.5 mg/kg/day×5) and day after dosing (14, 7, 2 or 0/baseline). Pigs were treated as random effects, while fixed effects were dose group and days after dosing. An autocorrelation function was fitted to the data, as repeated blood samples were taken from the same pigs, but was not needed in the model and was subsequently removed. Data from the pig euthanased on day 6 after exposure (see Results) were omitted from the analysis because its death did not fall on an allocated 'day after dosing'. Statistical analyses of the tissue residue data were carried out using GenStat (Genstat Committee 2007). An exponential decay model was used to derive equations and estimate half-life figures for residual concentrations of diphacinone in liver, muscle, kidney and fat from pigs that had received a 12.5-mg/kg dose. To ensure conservative estimates of the persistence of diphacinone, tissue samples in which the measured concentration was below the MDL were included in the data as having 0.02 µg/g residual diphacinone.

5.3 Results

5.3.1 Trial 1 Coagulation time responses to diphacinone exposure

From an average of 21.6 kg at first weighing during acclimatisation, the pigs gained weight steadily during the acclimatisation and trial period (Fig. 5.1).

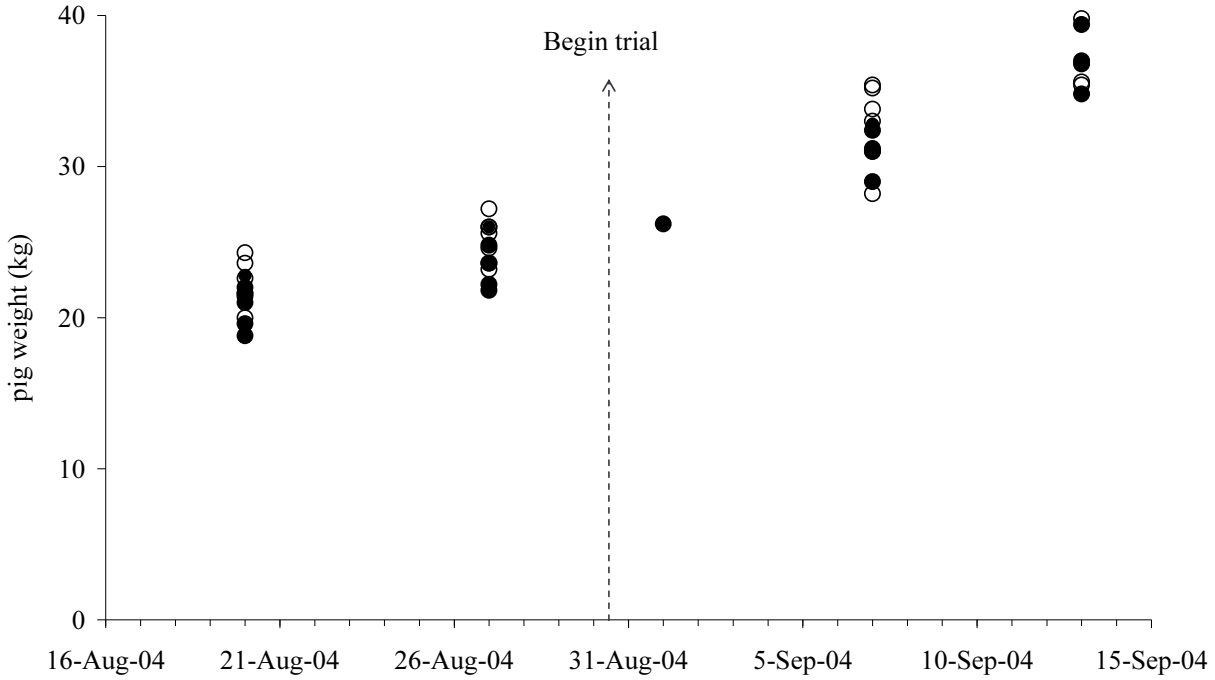


Figure 5.1 Bodyweights of female (●) and male (○) pigs during the acclimatization and Trial 1 period.

Use of an accustomed palatable food to facilitate accurate oral exposure of pigs to diphacinone was generally successful, with all except one eating the entire dose within 15 minutes. One pig was more reluctant than others to consume dough throughout the acclimation period, and this was partly overcome by mixing the uneaten portions with a small amount of normal pellet feed, which resulted in at least 80% of the intended dose (0.5 mg/kg/day × 5 days) being consumed within an hour on each day of dosing. Two pigs in the 0.5 mg/kg/day × 5 days group were euthanased during the trial because of increasingly severe lameness. Elevated PT (64.55 and 311.25 seconds) and APTT (88.3 and 149.85 seconds) were measured in blood samples taken immediately post-mortem from the two euthanased pigs, with INR values of approximately 6 and 49 respectively. Liver diphacinone concentrations were 0.7 μg/g and 0.12 μg/g, respectively. The first pig euthanased, a female, was slightly lame in the right front leg on the day after dosing commenced, and lameness became more pronounced with swelling of the knee joint visible on the following day. Although the pig remained alert, it showed a distinct reluctance to move, prompting the decision to euthanase on day 2. Necropsy revealed haemorrhage spreading from the knee joint upwards along the outer side of the femur, forming an extensive haematoma along the bone,

which presented as visible swelling. The second pig, a male, became slightly lame in the left hind leg four days after dosing commenced, and was euthanased on day 6 due to slight bleeding from around the eartag, relatively more time spent lying down than the other pigs, and increasing severity of lameness accompanied by evidence of pain and/or distress, i.e. squealing when attempting to walk to food. Coagulation times were significantly elevated on day 2 (PT 75.5 seconds, INR 7.37, APTT 117.35 seconds) and even more so on day 6 (PT 311.25 seconds, INR 41.89, APTT 149.85 seconds) just before euthanasia. Necropsy showed most swelling at the top of the right hind leg, with fluid present around the hock joint and a dark haematoma mass where swelling was greatest, mostly in muscle at the rear of the leg.

Mean baseline coagulation times are shown in Table 5.2 (full data Appendix 4). Coagulation times for the euthanased pigs were excluded from the analysis. Neither the interaction nor the effect of dose group was significant in any of the analyses (all $P > 0.05$ with no evidence of a dose-related response), so these were not included in subsequent models. For all coagulation parameters, values on day 2 were significantly different from those on days 0, 7 and 14 (Table 5.2: PT $F = 62.3$, df = 3, 27, $P < 0.001$; INR $F = 51.1$, df = 3, 27, $P < 0.001$; APPT $F = 134.6$, df = 3, 26, $P < 0.001$). Figure 5.2 shows the mean PT times measured in each treatment group, which reflects the overall trends in APPT and INR values during the trial.

Table 5.2. Mean PT and APPT times and INR values (±95% confidence intervals) in pigs dosed with either 12.5 mg/kg, 2.5 mg/kg, 2.5 mg/kg/days × 3 days or 0.5 mg/kg/day × 5 days. Includes data from the one pig euthanased at day 2 due to lameness, but excludes data from a second pig euthanased on day 6.

	Baseline (Day –1)	**Day 2**	**Day 7**	**Day 14**
PT (s)	14.94 (± 0.34)	59.73 (± 1.38)	17.31 (± 0.53)	15.96 (± 0.28)
INR	1.00 (± 0.11)	5.57 (± 0.56)	1.20 (± 0.09)	1.08 (± 0.11)
APTT (s)	33.35 (± 0.60)	99.56 (± 1.76)	40.16 (± 0.69)	40.18 (± 0.95)

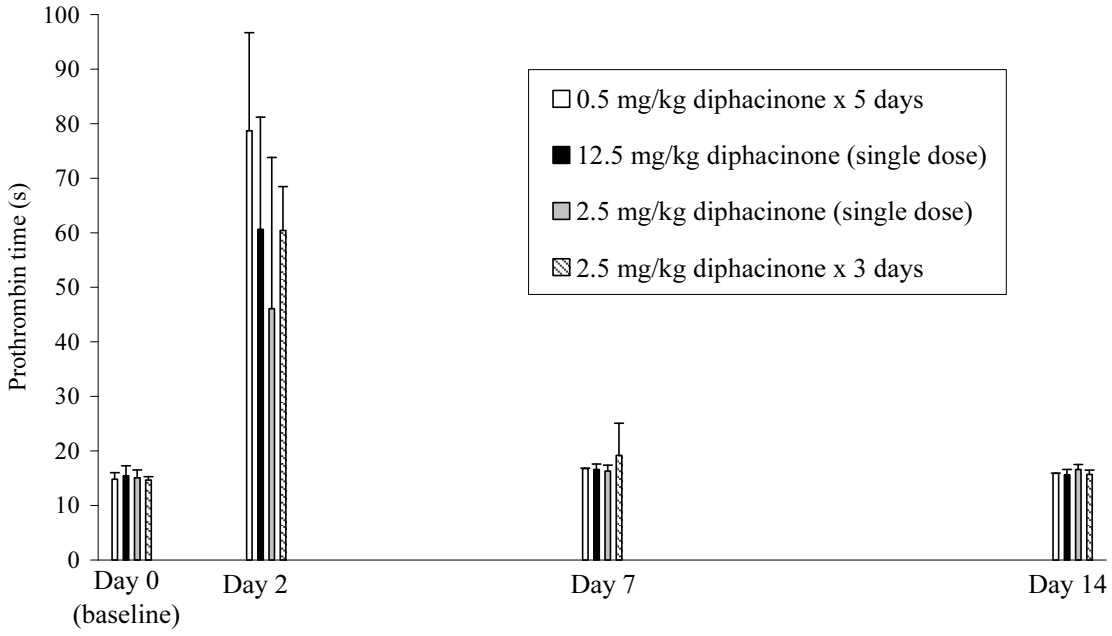


Figure 5.2. Mean prothrombin times (and upper 95% confidence intervals) in pigs dosed with either 12.5 mg/kg, 2.5 mg/kg, 2.5 mg/kg/day for 3 days, or 0.5 mg/kg/day for 5 days. Values on day 2 were significantly different from those on days 0, 7 and 14 (F = 62.3, df = 3, 27, P < 0.001).

Necropsy of the ten pigs euthanased at completion of Trial 1 did not reveal any gross haemorrhage in organs or limbs. In two pigs, however, healing subcutaneous haemorrhages were present on the inner rear legs, which were thought to have occurred during earlier restraint for blood sampling, possibly because of an effect of diphacinone exposure on coagulation. In one pig, the pericardial sac had a little fluid present and some pus was present at a point inside the heart, probably due to a recent infection caused by one of the blood-sampling procedures. There was no significant difference in concentrations of diphacinone in liver between the dose groups (F = 4.25, df = 3, 6, P = 0.06), and the mean residual concentration was 0.44 μg/g (Landcare Research toxicology laboratory report T2262, Appendix Four). No diphacinone was detected in muscle samples from these pigs (Landcare Research toxicology laboratory report T2508, Appendix Four).

5.3.2 Trial 2 Persistence of diphacinone in pig tissues

Method recovery on internal standards was 102% for liver (Landcare Research toxicology laboratory report T2299, Appendix Four), 103% for fat and 87% for muscle (Landcare Research toxicology laboratory report T2313, Appendix Four). Highest diphacinone concentrations were in liver, with a mean of 2.83 µg/g at day 1, 0.69 µg/g at day 4 and 0.62 µg/g at day 10 (Fig 5.3). At Day 1, liver concentrations of diphacinone were on average 9.2 times higher than those in muscle, 6.2 times higher than in fat, 1.2 times higher than in plasma and 1.9 times higher than in kidney. By day 4, diphacinone concentrations in muscle, fat, kidney and plasma were below 0.5 µg/g (Fig 5.3) with plasma samples generally below the MDL of 0.02 µg/g by day 10. Table 5.3 summarizes the elimination half-life (t ½) estimates made from these data for diphacinone in pig liver, fat, muscle, kidney, and plasma.

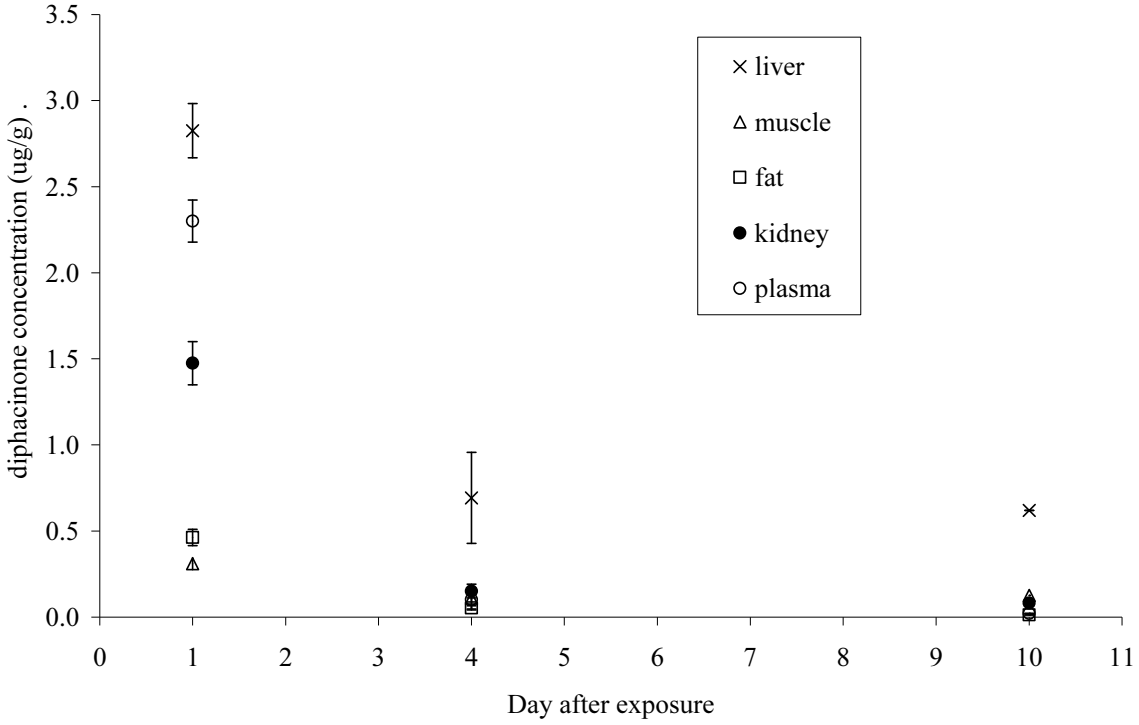


Figure 5.3. Mean concentration of diphacinone (µg/g) in liver, muscle, fat, kidney and liver at days 1, 4 and 10 after exposure of pigs to 12.5 mg/kg diphacinone. Method limit of detection was 0.02 µg/g for all sample types.

From whole organ weights at sampling, liver comprised a mean of 3.41% of total pig bodyweight and kidneys 0.52% of total bodyweight. Using the highest (day 1) diphacinone concentrations measured and the corresponding organ weights of the four pigs sampled on day 1, the estimated burden of diphacinone in liver was 2.64 mg and in kidney 0.20 mg, comprising 0.84% and 0.06% of the total diphacinone exposure respectively.

Table 5.3. Elimination half-lives with 95% confidence intervals for diphacinone in pig tissues, following ingestion of a single 12.5 mg/kg dose.

Tissue	Half-life estimate(days)	95% confidence intervals
Liver	5.43 (overall) 1.30 (initial phase, day 1 to 4) 14.12 (terminal phase, day 4 to 15)	3.55 - 11.52 0.84 - 2.88 5.34 - not defined
Muscle	4.48	3.16-7.68
Fat	2.29	1.66-3.68
Kidney	0.53	0.43-0.70
Plasma	0.33	0.27-0.44

5.4 Discussion

5.4.1 Trial 1 Coagulation time responses to diphacinone exposure

Mean baseline PT and APTT values (14.95 and 33.35 s) measured here were similar to previously reported values for pigs (e.g. Hahn *et al*. 1996, McGlasson *et al.* 1998, Drescher *et al*. 2002), although lower normal APTT values have also been reported (Hahn *et al*. 1996, Munster *et al*. 2000). The significant elevations of both PT and APTT at day 2 indicated an anticoagulant effect of all diphacinone exposures in this trial. In human medicine, the risk of bleeding increases dramatically when the INR exceeds 4.0-6.0 (Hanslik & Prinseau 2004). Assuming the same applies to pigs, the diphacinone doses ingested by pigs in this study all increased the risk of haemorrhage at day 2, with a return to baseline values in most cases by day 7. While Keith *et al*. (1990) found that oral diphacinone doses to captive wild pigs of up to 1.5 mg/pig/day for 5 days were followed by normal coagulation times at 2 and 10 days afterwards, with no signs of toxicity, other studies of anticoagulants in pigs have documented similar effects on coagulation times to those described here. Fletcher (2002) fed four pigs diphacinone at 0.333 mg/kg/day for 7 days and they showed some signs of poisoning, followed by recovery at 8 days after dosing, with hemorrhage-related pathology when they were euthanased 21 days later. McGlasson *et al.* (1998)

dosed pigs daily for 14 days with 2–3 mg of coumadin (equivalent to c. 0.07–0.08 mg/kg/d of warfarin) and measured PT and APTT values at 0, 7 and 14 d, using two different test systems. Pigs responded to the coumadin doses with increased APTT times at 7 d (28.0 s) and 14 d (53.0 s) and INR values above 5 at 14 d (4.3 and 12.8 depending on the test system used), although McGlasson *et al.* (1998) did not document any visible signs of anticoagulation.

Pigs in this trial appeared more susceptible to diphacinone than indicated by Hazelton (1957; cited in Pitt *et al.* 2004) and Fletcher (2002), suggesting that non-target hazard to feral pigs during field diphacinone baiting is higher than previously thought. If feral pigs have sufficient access to bait they will be poisoned, as evidenced by the mortality of thirteen feral pigs following an experimental field application of diphacinone bait in Hawaii, 2003 (Pitt *et al.* 2004; Tummons 2004). The two pigs that developed severe leg joint hemorrhage in Trial 1 had ingested smaller and less prolonged doses of diphacinone than those described by Fletcher (2002). Pigs in the latter study were kept individually in 4' × 8' pens and thus were probably relatively restricted in their movement compared to the group-housed pigs in Trial 1, which displayed frequent play, mounting and running behaviour. Movements such as these may create minor joint injuries or strains that can become starting points for haemorrhage when coagulation is compromised. These results emphasise the unpredictable incidence of haemorrhage during anticoagulant toxicosis, where variables such as movement and injury probably influence the occurrence of severe haemorrhage. Lameness appears to be a typical early sign of anticoagulant toxicosis in pigs. Of eight pigs fed with coumatetralyl, Dobson (1973) noted lameness in one at 3 days and in four others by 6 days, also noting that pigs often showed "difficulty in rising" before death in 8 days. Mean time to death in pigs poisoned with warfarin ranged from 5.4 to 10.0 days with necropsy of 78 pigs finding haemorrhage in fore- and hind limbs of 61.5–74.4% (Hone & Kleba 1984). O'Brien & Lukins (1990) also reported lameness, depression and lethargy as first signs of poisoning approximately 3 d after pigs had ingested a lethal dose of warfarin or brodifacoum, with affected pigs reluctant to move and spending relatively more time lying down. Brakes & Smith (2005) noted limb joint haemorrhage as a likely explanation for staggering gait and decreased mobility of poisoned rodents. Although animal welfare is not the focus of this thesis, the behaviours in the two haemorrhage-affected pigs in Trial 1 suggested that the lameness was painful. While haemorrhages are probably not inherently painful if they occur in a relatively open body cavity, they may lead to pain if they cause tissue swelling or pressure within an enclosed area such as a joint cavity (MacLain & Weinstein 1999). In particular, intra-articular

haemorrhage has been noted as painful in humans, probably owing to stimulation of intra-articular nociceptors through pressure exerted by accumulating blood (MacLain & Weinstein 1999). Given the frequency with which lameness is reported to occur in pigs poisoned with anticoagulants, some welfare compromise of feral pigs poisoned with diphacinone seems certain, especially if times to death are in the order of days in field situations.

5.4.2 Trial 2 Persistence of diphacinone in pig tissues

The hepatic half-life estimate in pigs contrast with the findings of Bullard *et al.* (1976) in cattle (Table 4.2). While no comparable half-life estimates for other anticoagulants in pigs are available, the results here are consistent with the findings of Fisher *et al.* (2003) in laboratory rats, indicating that diphacinone persistence in mammalian liver is relatively short in comparison to second-generation anticoagulants.

In feral pigs exposed to a trial application of diphacinone bait in Hawaii, the highest diphacinone concentrations detected in liver and muscle were 3.07 and 0.12 µg/g respectively and were both present in one pig (Pitt *et al.* 2004). The highest residual diphacinone concentration in pig liver reported by Keith *et al.* (1990) was 0.83 µg/g. Thus Trial 2 produced some of the highest residual concentrations of diphacinone in pig liver and muscle (3.22 and 0.37 µg/g respectively) reported to date, occurring in the same pig at one day after dosing with 12.5 mg/kg. Using the half-life estimates for diphacinone in muscle and fat, it would take 19 and 10.5 days respectively for the highest residue measured in these tissues (0.37 and 0.43 µg/g) to decline to below detectable concentrations. Using the terminal half-life estimate of 14.12 days for diphacinone in liver, it would take 104 days for the highest residue measured in liver (3.22 ug/g) to decline to below detectable concentrations (≤ 0.02 ug/g). This is a conservative measure that does not take account of the overall elimination rate from the liver, including the more rapid initial phase. To define a with-holding period for feral pigs hunted for human consumption in areas where diphacinone has been applied, allowance should be made for the possibility of higher residue concentrations than those measured here. Incorporating a very conservative 'safety factor', by adding half again to the estimated number of days for liver residues to become undetectable, a with-holding period of 156 days is suggested. This is longer than the 2 months currently recommended for first-generation anticoagulants by NZFSA.

5.5 Conclusions

Primary poisoning risks to feral pigs resulting from single and multiple exposures to diphacinone baits in the field remain to be evaluated. However feral pigs are capable of consuming sufficient diphacinone bait to cause mortality if access is allowed. Diphacinone is less persistent than brodifacoum in pig tissues, thus presents a lower duration of hazard to consumers of pigs. Human toxicity data for diphacinone are scarce, but assuming an LD_{50} value of 1.93 mg/kg as reported for laboratory rats (Jackson & Ashton 1992), and using the highest residue concentration from Trial 2, a 70 kg human would need to eat an estimated 41.9 kg of contaminated liver or 365 kg of contaminated muscle to ingest a lethal dose. While these scenarios are unlikely, the potential for repeated, smaller exposures to diphacinone in meat and sublethal effects on coagulation, as discussed by Eason *et al.* (2001) for brodifacoum, remain to be considered. It is suggested that currently available analytical method limits of detection for diphacinone in tissue are refined and validated in order to review a practicable maximum residue level. As well as confirming the validity of the withholding period recommended for diphacinone above, residue surveys of feral pigs from areas where diphacinone has been applied for rodent control would also help to determine the frequency and extent to which an MRL might be exceeded.

Chapter 6: Bait consumption and residual concentrations of diphacinone in weta (*Hemideina crassidens*)

6.1 Introduction

As outlined in Chapter 1, application of brodifacoum baits to control introduced pests in NZ (e.g. Innes *et al*. 1995) has resulted in primary and secondary exposure of non-target birds (e.g. Eason and Spurr 1995) and mammals (e.g. Spurr *et al*. 2005) to brodifacoum. Exposure of non-target invertebrates has also been studied - a range of terrestrial insect species feed on cereal baits used for vertebrate pest control (e.g. Ogilvie *et al*. 1997; Spurr & Drew 1999; Craddock 2003) hence undergo primary exposure to the active ingredient of the bait. Secondary exposure of invertebrates can also occur, as demonstrated by Howald (1997) who measured brodifacoum residues in carrion-feeding invertebrates collected from the carcasses of poisoned rats. Investigation of diphacinone as an effective but less persistent alternative to brodifacoum (summarised in Chapter 4.2) raises questions about possible effects on invertebrates, especially native NZ species. It has been suggested that anticoagulants lack insecticidal properties because insects do not have the same blood-clotting systems as vertebrates (Shirer 1992), and a number of laboratory and field studies suggest this is the case for brodifacoum at least. Captive studies (summarised in Booth *et al*. 2001) indicate low toxicity of brodifacoum to large-headed tree weta (*Hemideina crassidens*) and Ascension Island land crabs (*Gecarcinus lagostoma*). Craddock (2003) found that captive locusts (*Locusta migratoria*) fed readily on cereal-based brodifacoum baits with no significant increase in mortality. In captive cave weta (*Pleioplectron simplex*) and ground weta (*Hemiandrus* sp.) weight loss and mortality was not significantly higher in weta exposed to brodifacoum bait over 60 days (Bowie & Ross 2006).

No similar studies or published acute toxicity data was found for diphacinone in terrestrial arthropods. No mortality was observed in captive Hawaiian snails (*Oxychilus* spp.) and slugs (*Limax maximus* and *Deroceras laeve*) fed 0.005% diphacinone bait over seven days (Johnston *et al.* 2005) indicating that acute toxicity to these species was low. However, there are earlier reports of compounds structurally related to diphacinone (2-(diphenylacetyl)-1,3-indandione) having toxic effects on invertebrates. For example, isomeric valeryl-1,3-indandiones exhibited strong insecticidal properties against houseflies (*Musca domestica*) (Kilgore *et al*. 1942), 2-pivalyl-1,3-indandione (pivalyl) showed toxic effects against body lice (*Pediculus humanus corporis*) (Eddy & Bushland 1948), and 0.025% pivalyl cereal baits applied in field trials for

rodent control also had insecticidal properties (Crabtree & Robinson 1953). Diphacinone is considered "moderately toxic" to the freshwater invertebrate *Daphnia magna* (US EPA 1998). Thus it was important to assess the toxicity of diphacinone in a representative terrestrial invertebrate to confirm whether diphacinone would present low hazard to NZ invertebrates that feed on bait.

Field monitoring following brodifacoum baiting in NZ has detected residues in some invertebrate species, particularly those that eat baits (summarised by Booth *et al.* 2001). Thus invertebrates can also transfer anticoagulant residues in the environment, presenting a secondary hazard to insectivores (as outlined in Chapter 1.2.1). Godfrey (1985) reports that several birds in a zoo aviary died, apparently as the result of eating ants and cockroaches that had eaten brodifacoum baits. More recently, sandhoppers (*Talorchestia* spp.) that fed on brodifacoum bait were identified as the likely source of lethal secondary exposure in New Zealand dotterels (*Charadrius obscurus aquilonius*) following a baiting operation at Tawharanui, NZ (Dowding *et al.* 2006). In general, field-based assessments of secondary risk to non-target insectivores are scarce probably because of the relative scale and complexity of the studies required. Most of the relevant information pertains to brodifacoum residues – the only 'field monitoring' data for diphacinone in invertebrates sourced was by Johnston *et al.* (2005), where residue concentrations were measured in Hawaiian snails and slugs collected within 1 m of diphacinone baits during a baiting operation. These authors then undertook a probabilistic secondary risk assessment for diphacinone based on residue concentrations measured in gastropods. To obtain similar data applicable to secondary risk assessment in the context of non-target NZ insectivores, a laboratory trial was undertaken to determine whether the Wellington tree weta (*Hemideina crassidens*), a regionally common, large native NZ orthopteran would feed on diphacinone bait, whether such exposure was associated with toxicity or mortality and to measure the concentrations of residual diphacinone in weta that fed on baits.

6.2 Methods

6.2.1 Capture and housing of weta

Tree weta were captured in December 2004 from podocarp–broadleaf coastal forest near Harihari, South Island, NZ (2311045E, 5781505N). Adult and late-instar juvenile weta were identified by size and appearance, each placed in a ventilated plastic container with leaf litter

from the point of capture and transported to a laboratory at Landcare Research, Lincoln. Housing and husbandry procedures were derived from those described by Barrett (1991). Weta were housed individually in cylindrical plastic containers (200-mm diameter × 300 mm high) with close-fitting lids and three 2-cm diameter ventilation holes covered with fine metal mesh in the sides. The base of each 'unit' was covered with a 4-cm layer of sand and leaf litter. Hollow flax-flower stalks (150 mm long), split in half and then held together with rubber bands were placed in each container as daytime shelter. The housing units were kept under natural photoperiod on a benchtop out of direct sunlight, with ambient room temperature ranging from 10 to 25°C, and humidity of 50–75%. Two plastic tubes (9.5-cm long, 1.7-cm diameter) taped to the inner sides of each unit were filled with water so that they could hold sprigs of native NZ plants that weta feed on; five-finger (*Pseudopanax arboreus*), mahoe (*Melicytus ramiflorus*), and broadleaf (*Griselinia littoralis*) were used. Evidence of feeding was noted during the twice-weekly checks and plant food replaced fresh at least weekly, or more frequently if substantially eaten or wilting. Weta were weighed every 7–10 days, although any that were moulting were not handled. When humidity in the room fell below 60% as indicated by a digital meter, or if the leaf litter appeared dry, the insides of the units were misted thoroughly with tap water, and the plant tubes were topped up. During trials, faecal pellets were only removed from the housing units if they were visibly mouldy but were cleaned completely out of the housing units between trials. During three weeks' acclimatisation to these conditions, weta were checked twice weekly and confirmed to be alive by removing them from the shelter.

6.2.2 Removal of non-toxic cereal bait by weta

A preliminary trial was undertaken to determine whether bait uptake by weta was affected by the presence of natural plant food. Twenty two adult weta were weighed and allocated by descending bodyweight to two treatment groups (each $n = 11$). One group was presented with a non-toxic, cinnamon-lured, 'RS5' cereal pellet bait (Animal Control Products Ltd., Wanganui, NZ) with normal plant food present, and the other group was presented with the same bait type without normal plant food present. Pellet baits were placed on a glass petri dish in each housing unit - as 'environmental controls', a pellet bait was placed in each of four housing units that did not contain weta – two with plant food in place, and two without. These pellets were weighed daily and replaced. Pellets in weta containers were left *in situ* but visually assessed for evidence of

consumption by weta. Visual estimates were based on intervals of either: less than 5% eaten (recorded as 2.5%), between 5 and 15% eaten (recorded as 10%), between 15 and 25% eaten (recorded as 20%), between 25 and 35% eaten (recorded as 30%) and so on. Daily estimates of the percentage of original pellet remaining intact and general observations were recorded e.g. swelling, presence of mould, 'scrape' marks in bait, presence of bait fragments. On the fourth day, weta were weighed again and the dishes and remaining bait including any sizable fragments were removed from all housing units. Each glass dish and remaining bait and fragments were weighed together, then the dishes emptied, dry-wiped clean and weighed alone. In some cases it was impossible to remove small quantities of sand from bait fragments in the dish or to collect all bait fragments moved from the dish by weta, so these were a potential source of error in weighed estimates of bait eaten. The daily weights of the 'with plant food' and 'without plant food' environmental control pellets were calculated as percentage change from original weight, and averaged for each treatment. These figures were used to correct the calculation of the weight of bait removed by weta in the respective treatment groups. The total corrected amount of bait eaten or removed by each weta was expressed as a percentage of that weta's starting bodyweight.

6.2.3 Consumption of diphacinone bait: pilot trial

In a preliminary assessment of whether weta would feed on diphacinone bait and if this would cause mortality, four late-instar weta (2 females, 2 males) were presented with a Ditrac® bait (Pest Management Services, Paraparaumu, NZ) placed on a glass dish in the housing unit with normal plant food present. The baits were waxed cereal blocks weighing *c*. 30 g, dyed a pale green colour, nominally containing 0.005% diphacinone. A sample of the fresh bait was analysed for diphacinone concentration by the Landcare Research toxicology laboratory, Lincoln, using an HPLC method based on that of Hunter (1984). Weta and baits were weighed at the start of the trial and then regularly over the following forty days, with regular observations of the appearance of the bait and health of the weta.

6.2.4 Consumption of diphacinone bait and residues in weta

Twenty-seven adult or late-instar weta (18 female, 9 male) were presented with a Ditrac® bait as in the pilot trial. Weta and baits were weighed before baits were placed and for the first week, baits were observed daily for fresh feeding marks, presence of crumbs on the bait dish and presence of mould. After this, to minimise disturbance of weta and possible effects on feeding

behaviour, weekly checks of bait condition and the weta were made during routine replacement of plant food. After placement of bait, a sample of four weta (initially 2 male, 2 female) was randomly selected on days 1, 4, 8, 16, 31, and 64. The weta were weighed, placed in labelled specimen containers and left overnight in a –20°C freezer to kill them prior to analysis. Any frass that had been excreted while they were in the freezer were included with the weta sample for analysis as described below. Remaining baits and fragments for each sample group were collected and weighed as described in 6.2.2. Two environmental-control housing units (with bait but no weta) were established and the baits in these were observed and weighed at each sampling interval.

Analyses of diphacinone concentrations in weta were carried out by the Landcare Research toxicology laboratory, using a HPLC method where the method limit of detection (MDL) was 0.2 µg/g. Whole, frozen weta were dissected and mixed with anhydrous sodium sulphate and subsequently extracted with solvent (chloroform/acetone/formic acid). The mixture was homogenised with a tissue disperser, shaken and centrifuged. The supernatant was decanted and the extraction repeated twice more. The combined extracts were evaporated and taken up in hexane / chloroform / acetone for application to a solid phase extraction column for clean-up. The eluent from the column was again evaporated and taken up in mobile phase for HPLC determination, which employed ion-paired chromatography and UV detection at 284 nm. Each batch of samples analysed included a spiked sample, where 50 µL of 10 µg/mL diphacinone was added to a suitable 'blank' matrix in order to determine recovery.

6.2.5 Statistical analyses

All estimates of bait consumption/removal in the trials (either visual estimates of bait removed or weighed estimates from remaining bait) were analysed by weighted regression using generalised linear models in GenStat (Genstat Committee 2007), with weighing being the reciprocals of the variance of the daily measurements. In the diphacinone trial (described in 6.2.4 above) bait consumption by weta was estimated by correcting the start weight of the bait using the corresponding mean change in the environmental control baits at each sampling interval, and then subtracting the weight of the bait at sampling. From this figure, diphacinone intake by weta was estimated at each sampling interval using the analysed concentration of diphacinone in samples

of bait, adjusted according to the weight of the individual weta at sampling (i.e. intake as µg diphacinone / g of weta bodyweight).

6.3 Results

Three of the 38 weta brought into the laboratory died within the first week. One adult female was injured, probably during capture and the other two, an adult male and a late-instar female started moulting soon after being placed in housing units and were found dead two and three days later. At the end of the first week in the housing units, mean weight of 11 adult females (± SE) was 3.40 ± 0.34 g and of 9 adult males was 2.82 ± 0.23 g. Overall, the weta maintained or gained weight during the following month, with the mean weight of the same 11 adult females being 3.98 ± 0.27 g, and 9 adult males 2.97 ± 0.20 g, after one month's acclimatisation.

6.3.1 Removal of non-toxic cereal bait by weta

There was no variation in day 1 percentage estimate of bait removal, and these data were dropped from analysis.

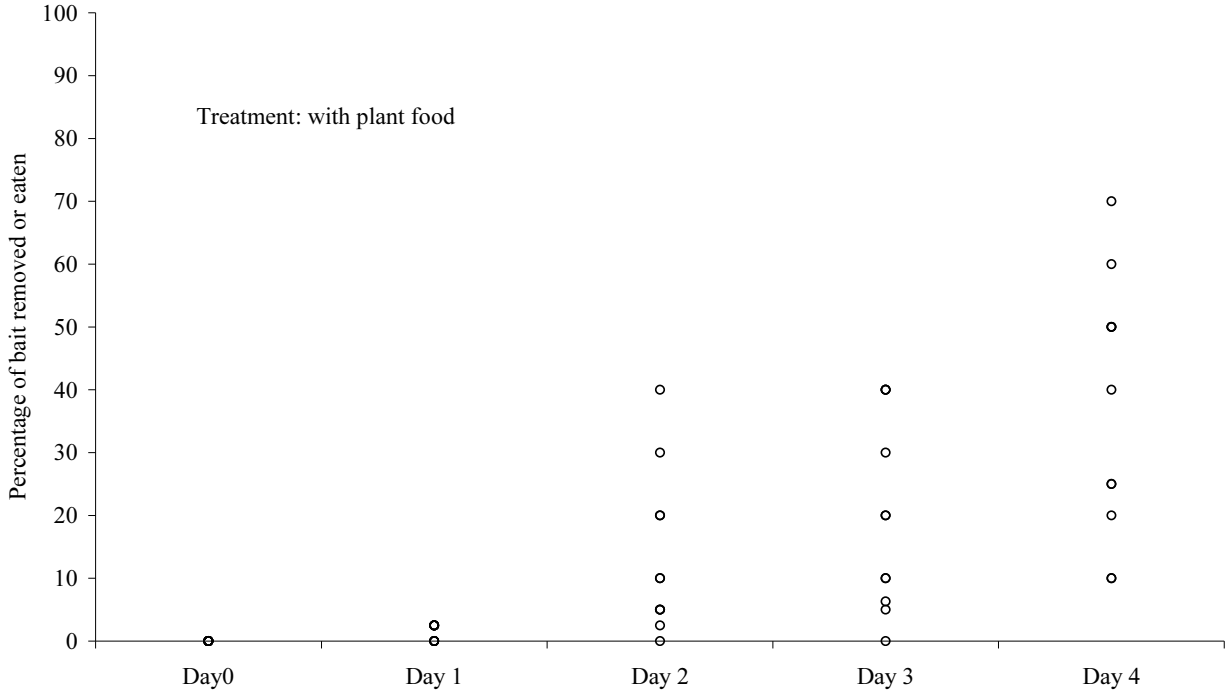


Figure 6.1. Percentage of a non-toxic RS5 cereal pellet bait eaten or removed by weta over 4 days, while normal plant food was also available.

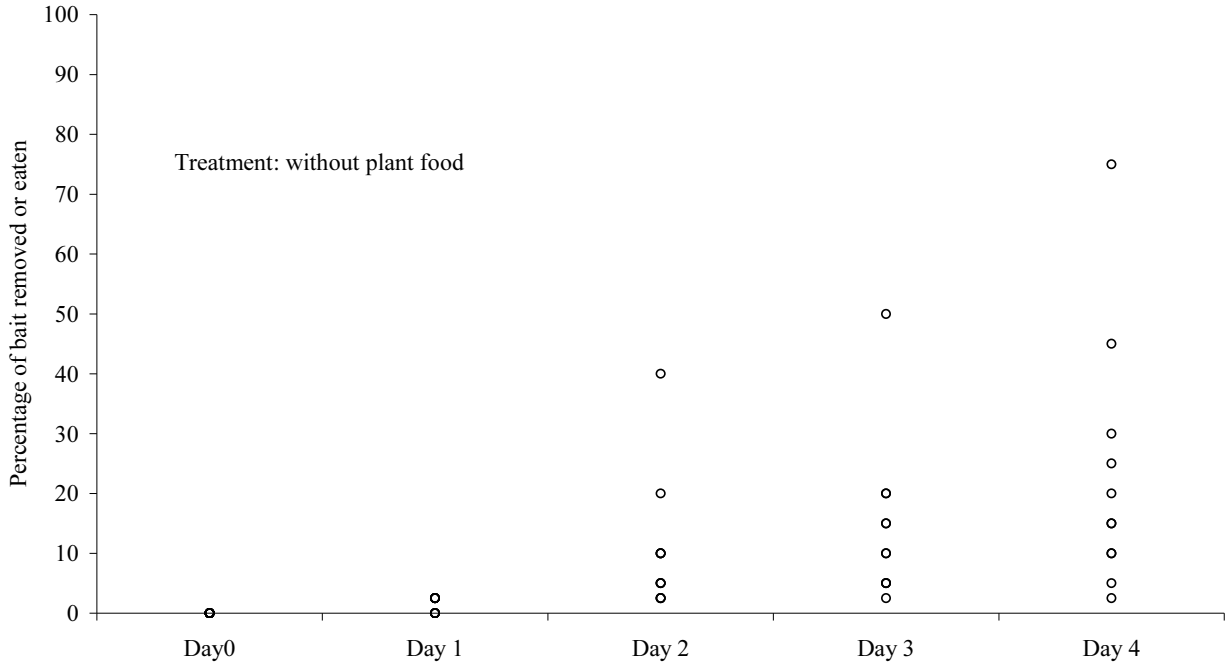


Figure 6.2. Percentage of a non-toxic RS5 cereal pellet bait eaten or removed by weta over 4 days, while normal plant food was not available.

There was no significant difference of slopes between 'with plant food' and 'without plant food' treatments (difference in slopes = 3.77, SE_{diff} = 1.96, t_{84} = 1.93, P = 0.06) and overall removal of the non-toxic pellets over 4 days (assessed by visual estimates) appeared similar for both treatments (Figs 6.1 and 6.2). Most weta nibbled the pellets, but did not eat or remove more than 5% on the first day. Weta in both treatments had eaten or at least fragmented 5–50% of the pellet by the second and third days. Some weta did not eat more than 10% of pellet throughout the trial, whereas others had eaten or fragmented an estimated 70–80% by the fourth day. Weta in the 'with plant food' treatment consumed a mean 0.61 ± 0.05 g of pellet over the 4 days, representing 17.2% (range 9.8–26.5%) of their starting body weights. Weta in the 'without plant food' treatment consumed a mean 0.64 ± 0.07 g of pellet over the 4 days, representing 19.7% (range 6.8–33.2%) of starting body weights. Amounts of pellet eaten/removed in each treatment were not significantly different (t_{20} = 0.31, P = 0.76). Weta in both treatments gained weight over the 4 day trial, but the amount of weight gain as a proportion of initial weight was not significantly different between groups (t_{20} = 1.14, P = 0.27). The four 'environmental control' pellets all gained weight over the 4 days of the trial, presumably through absorption of moisture, and had lost some of their shape but remained intact. The mean gain from the starting weight of the two

pellets with plant food present was 18.2 % of original weight, and that of the two pellets without plant food was 15.3%.

6.3.2 Consumption of diphacinone bait: pilot trial

All four weta appeared healthy throughout the trial and responded normally to disturbance. Three gained weight (+30-70% of starting weight) over the 44 days that they had access to Ditrac® bait, but one female lost approximately 60% of starting bodyweight over this time. All weta had nibbled the Ditrac® bait by the fourth day. Bait consumption, or at least removal, continued steadily until about Day 15–20, after which it appeared to level off (Fig. 6.3). Mould was first observed on the baits after approximately 1 month and covered the bait surface more extensively after this. One of the weta continued to eat the mouldy bait although the other three did not appear to have fed on bait once the mould was present. The bait blocks lost a mean of 1.6 ± 0.6 g over the trial. Laboratory analysis showed that the Ditrac blocks contained 52.5 µg/g diphacinone (Landcare Research toxicology laboratory Report T2301, Appendix Five), slightly higher than the nominal 0.005% by weight. The analysed concentration of diphacinone in bait was used to estimate a mean intake of 113.04 ± 21.79 µg diphacinone eaten or at least removed by each weta over the four days, without accounting for changes in bait weight due to environmental moisture.

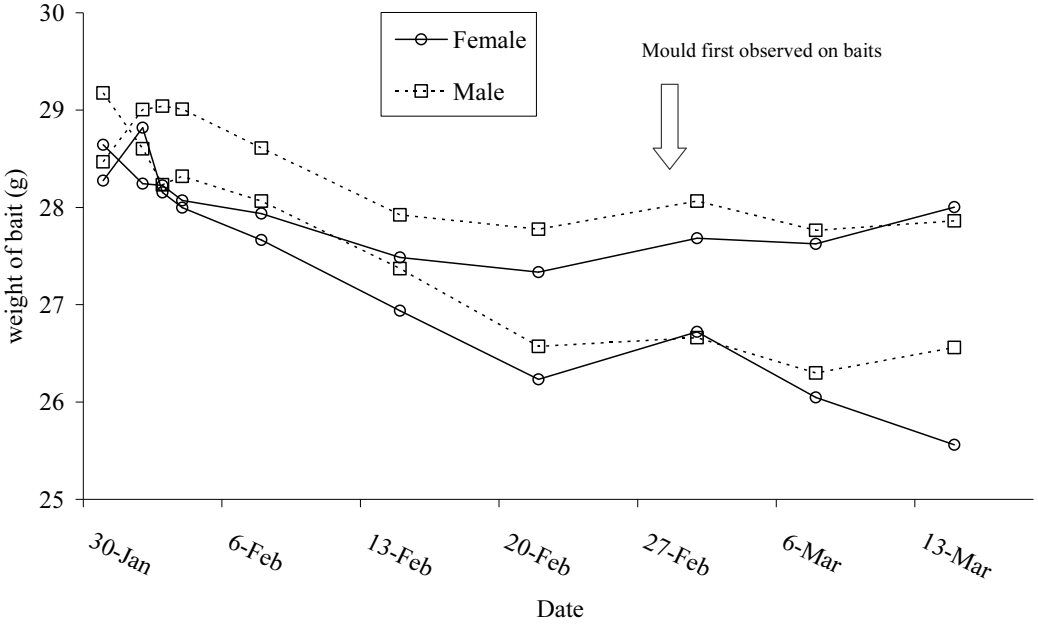


Figure 6.3. Changes in the weights of Ditrac® block baits accessible to two female (○) and two male (□) weta, in the presence of natural plant food, over 44 days.

6.3.3 Consumption of diphacinone bait and residues in weta

The environmental control baits showed a mean increase of 2.68% by the end of the trial. By day 8, all weta left evidence of consumption or removal of the diphacinone bait blocks. Frass produced by weta feeding on bait was light-coloured and often had a striped appearance, in comparison to the uniform dark brown of frass from weta feeding on normal plant food. Exposure to diphacinone baits did not appear to adversely affect weta bodyweight: weight gain was greater during this trial than during acclimatisation to the housing units (Fig. 6.4; difference in slopes = 0.01938, SE_{diff} = 0.00857, t_{100} = 2.26, P = 0.026) although relative growth rates (g/g/day) showed less difference between the acclimatisation and trial phases (difference in slopes = 0.0001955, SE_{diff} = 0.0000392, t_{183}=1.82, P = 0.072).

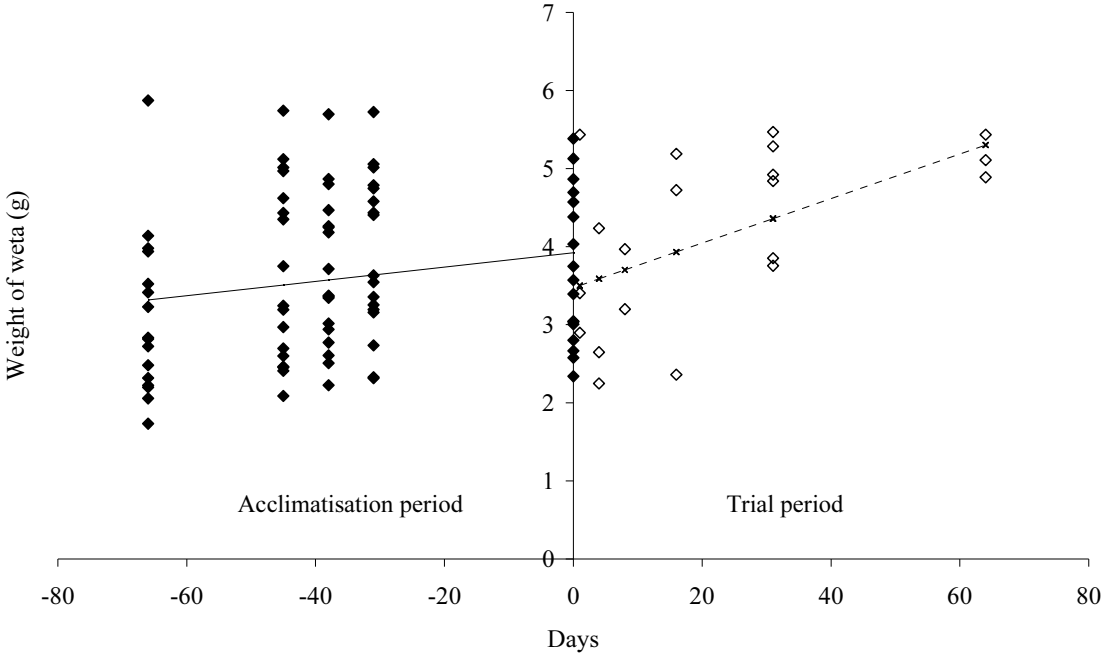


Figure 6.4. Bodyweights of weta during an acclimatisation period before being presented with diphacinone bait and sampled in groups of four during the trial period.

Spiked samples analysed alongside the whole weta yielded estimates of 86, 82 and 76% recovery of diphacinone. The method detection limit for diphacinone in invertebrate tissue was 0.2 µg/g, with an uncertainty (95% c.i.) of ± 20%. The amount of bait consumed or removed over time by weta increased (weighted regression slope = 0.376, se = 0.114, $t_{24} = 3.29$, $P = 0.003$) (Fig. 6.5) while there was a small, but significant decrease in the amount of residual diphacinone measured in the weta over time (weighted regression slope = −0.0441, SE = 0.0123, $t_{24} = 3.59$, $P = 0.002$). The mean (± s.e.m.) concentrations of diphacinone in weta showed a slight increase from weta sampled on day 1 (3.63 ± 1.59 µg/g) to those sampled on day 4 (4.85 ± 0.73 µg/g), but thereafter declined gradually to reach 0.99 ± 0.51 µg/g by day 64 (Fig. 6.5). Cumulative bait intake i.e the amount of bait removed or eaten by each sample group of weta by the day of sampling, was expressed as µg diphacinone / g weta bodyweight (Fig 6.5) to facilitate a comparison between the quantity of diphacinone ingested by weta and the residual concentrations subsequently measured in their bodies.

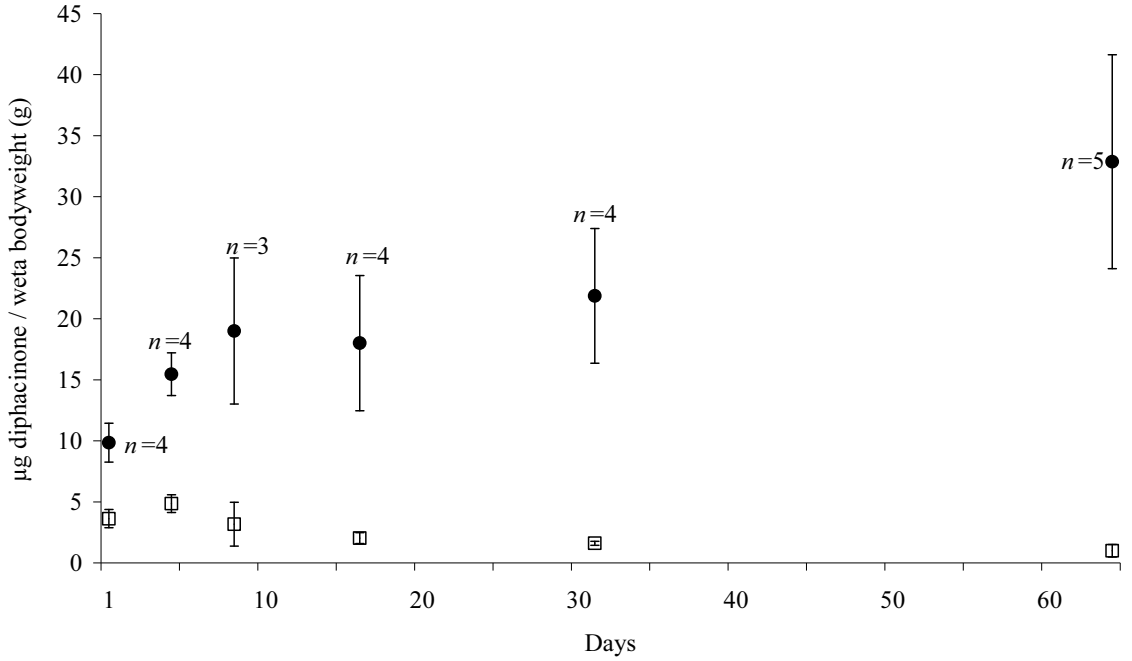


Figure 6.5 Diphacinone consumption by weta (µg diphacinone/g weta weight, mean ± SE). (●) estimated cumulative intake of diphacinone from consumption of Ditrac® block baits, and (□) diphacinone residues measured in the whole body of each weta.

Using the bodyweight of each weta at sampling to estimate the total amount of residual diphacinone contained in a weta gave mean (± s.e.m.) figures of 11.44 ± 0.88 µg diphacinone (day 1), 14.42 ± 4.36 µg (day 4), 9.91 ± 5.06 µg (day 8), 8.06 ± 11.68 µg (day 16) (Landcare Research toxicology laboratory report T2318, Appendix Five), 6.27 ± 0.92 µg (day 31) and 3.64 ± 1.74 µg (day 64)(Landcare Research toxicology laboratory report T2351, Appendix Five). The most residual diphacinone calculated to be present in a single weta was 24.99 µg, in a 4.23 g female sampled on day 4.

Three male weta died during the trial period – two were found dead on day 8 of sampling, with the other unresponsive on day 8 and dead on day 9. Weights of the dead weta were nearly half those recorded at the beginning of the trial. All three weta had consumed bait during the trial period, equivalent to 31.0, 29.5 and 8.09 µg diphacinone /g bodyweight. Respective residual diphacinone concentrations in these weta were 7.9, 3.6 and 2.2 µg/g. These weta were excluded from the overall statistical analyses as the cause of their death was not certain. The amounts of bait these weta had consumed or removed before they died were similar to those estimated for other weta were apparently healthy throughout the trial. Although the residual concentration in

one of the dead weta was slightly higher (7.9 µg/g) than the next highest residual diphacinone concentration measured in the trial (6.2 µg/g in a female sampled on Day 8), the residual concentrations of the other two dead weta were well within the range measured in 'surviving' weta. Whole-body residue loads were not calculated for the three dead weta because of the significant weight loss they underwent over a relatively short period of time and uncertainty about the actual date of death for two of them.

6.4 Discussion

A wide range of NZ invertebrate species, including weta, have been reported to eat cereal-based baits in field conditions (Sherley *et al*. 1999; Lloyd & McQueen 2000; Spurr & Berben 2004). Recent fieldwork in the North Island showed that weta and cockroaches were attracted to cereal-based brodifacoum bait in bait stations, and spent considerable time in contact with the baits (Craddock 2003). The results shown here confirm that cereal-based bait formulations used for field control of vertebrate pests represent a palatable food source to opportunistically foraging weta. Some captive weta in this study ate, or at least removed, up to 80% of an RS5 pellet within 4 days – in field conditions, substantial feeding on baits by weta and other invertebrates may contribute to a decrease in the availability or acceptability of baits to target pest animals.

It was assumed that mortality in the three weta (out of the 24) was due to causes other than toxic effects of diphacinone, possibly because the housing units did not provide suitable conditions for moulting. No adverse effects in twenty four weta following consumption of Ditrac® wax block baits for up to 64 days were observed, suggesting a low oral toxicity of diphacinone in this species. Although acute toxicity or mortality appears unlikely in weta feeding on diphacinone bait, the absorption, distribution, metabolism and excretion of anticoagulant rodenticides in invertebrates remain poorly described. The vitamin-K-dependent carboxylation reactions that produce blood coagulation factors in mammalian liver, which are affected by anticoagulant toxicity, are probably not present in invertebrates. However, vitamin-K-dependent metabolic processes also occur in other tissues (Vermeer *et al*. 1992) and carboxylase enzyme systems are generally distributed in invertebrate systems (Walker *et al*. 2001). While the effects of anticoagulant compounds on these aspects of invertebrate metabolism remain unknown, caution should be used in extrapolating a general lack of acute effects of anticoagulants in arthropod species to nil effect in other invertebrates, or to nil effect on the long-term survival fitness of

arthropods exposed to baits. Stejskal *et al.* (1994) suggested that brodifacoum caused anomalies, particularly duplication of ovipositors and possibly also observed deformations of the elytra, in adult *Ptinus tectus* (spider beetles) that fed on brodifacoum bait larvae. There is limited evidence for mortality in molluscs (e.g. Gerlach & Florens 2000; Primus *et al.* 2005) and earthworms (Booth *et al*. 2003) following relatively high environmental exposures to brodifacoum.

Fig 6.5 shows that the rate of bait removal and consumption was higher in the groups of weta sampled from day 1 through to day 7, and then the rate appeared reduced in the groups of weta sampled later through to day 64. This may be indicative of a novelty effect where consumption of a new, palatable food is higher when first encountered but then reduces as the food is recognised as familiar and readily available. Weta in the day 1 sample group consumed or removed diphacinone bait in quantities estimated from 12 to 27% of their bodyweight within 24 hours, so over this period it was likely that the gut became filled with bait material in various stages of digestion. Although the extent to which weta absorb and metabolise diphacinone is not known, most of the residual diphacinone detected in them was probably in the gut contents. Weta did not appear to accumulate diphacinone, i.e. whole-body concentrations did not increase with the amount of diphacinone eaten which could indicate a 'saturation' body burden, where bait material retained in the gut represented the majority of residue and any diphacinone absorbed was metabolised and/or excreted relatively quickly. Frass from weta that were eating diphacinone bait underwent a substantial change in colour and, by inference, composition. If weta excrete bait material relatively rapidly and without extensive metabolism, they could distribute residual diphacinone in the leaf litter and soil surface via frass. Measuring the relative proportion of ingested diphacinone excreted in weta frass would indicate how significant this pathway of environmental transfer might be.

Persistence of residues in invertebrates is also a determinant of the likelihood of secondary exposure. Limited data is available regarding anticoagulants in invertebrates but generally indicates lower persistence than in mammals. Following sublethal doses, brodifacoum residues were not detectable after 4 days in captive weta (Booth *et al*. 2001) and after 1 month in land crabs (Pain *et al*. 2000). Captive locusts appeared to excrete brodifacoum rapidly, indicating that long-term bioaccumulation was unlikely (Craddock 2003). However, a recent field-based study showed that brodifacoum residues in various invertebrate species took more than 4 weeks to return to background levels with trace concentrations of brodifacoum still detectable up to 10

weeks after the bait had been removed (Craddock 2003). Brodifacoum residues were found in both the gut and foot tissue of common garden snails (*Helix aspera*) 14 days after they were exposed to soil containing ground bait at 2 mg brodifacoum / kg of soil (Booth *et al*. 2003). Similarly in captive snails and slugs fed on diphacinone bait over seven days, Johnston *et al.* (2005) found that residue concentrations did not decline over the seven days after exposure to baits ended, suggesting that doses acquired over a number of days were excreted "relatively slowly". While diphacinone concentrations in weta had a similar range to those measured by Johnston *et al.* (2005) in slugs and snails (<0.02-5.01 μg/g), future consideration of differences between the gastropods and arthropods (rather than simply 'invertebrates') seems appropriate. The study reported here did not set out to assess persistence of residual diphacinone in weta after exposure to bait ceased. However, given the 'gut saturation' mechanism proposed for whole-body concentrations in weta it is considered likely that they would excrete the majority of residual diphacinone in frass within a period of days, rather than retain residual diphacinone bound to certain tissues. Investigation of the gut passage time of bait material in weta, and the retention time of residual diphacinone in their tissues and frass, as separate samples, would help to further quantify the timeframe over which contaminated weta might pose a secondary hazard to insectivores. Definitive measures of anticoagulant persistence e.g. exponential decay curves, in gut and other tissues of gastropods and arthropods could specifically test this assertion and investigate differences between invertebrates as reservoirs and vectors of anticoagulant residues in the environment.

For estimating overall secondary non-target risks, the distribution of residues in weta tissues is of less importance to characterise as predators or scavengers are likely to eat the whole insect, or at least the abdomen. Birds appear to be less susceptible than mammals to single doses of diphacinone; the lowest LD_{50} value (i.e. the single oral dose of diphacinone expected to cause death in 50% of a population) reported for a bird species is > 400 mg/kg in northern bobwhite quail (*Colinus virginianus*), and the lowest single-exposure LC_{50} (i.e. the concentration of diphacinone in food that can be expected to cause the death of 50% of a population) is 906 μg/g (95% confidence interval 187–35 107 μg/g) for mallards (*Anas platyrhynchos*) (US EPA, 1998). The highest weta residue concentration detected in this trial (7.9 μg/g) was approximately six times less toxic than the diphacinone concentration in the Ditrac® bait blocks, and 23 times less than the lower 95% confidence interval for the mallard dietary toxicity value (LC_{50}). On paper, such concentrations of diphacinone in weta represent a very low secondary hazard to birds – a 20

g bird would need to consume over 10 kg of contaminated weta in a single feed to ingest 400 mg/kg diphacinone (as a conservative LD_{50} estimate for birds). While acute secondary diphacinone toxicity in birds that feed on weta seems highly unlikely on the basis of this simplistic 'risk of mortality' calculation, the toxicity of diphacinone in multiple rather than single intakes, and the possibility of adverse sublethal effects on birds requires consideration. As for other first-generation anticoagulants, the toxicity of diphacinone to mammals is enhanced by multiple, consecutive oral doses in comparison to single oral doses (see Chapter 4.1), and this could also be the case for birds. These aspects of the secondary risk assessment for diphacinone remain unquantified, and investigation in terms of lethal or sublethal outcomes in birds feeding regularly on diphacinone bait or contaminated invertebrates is warranted.

6.5 Conclusion

Weta found Ditrac® wax block baits palatable in the presence of natural plant food, showing steady consumption of bait over time. No mortality or weight loss was attributable to the intake of Ditrac® bait indicating very low toxicity of this anticoagulant to this species. All weta that ate bait had detectable diphacinone in their bodies, but did not accumulate diphacinone; i.e. whole-body concentrations did not increase with the amount of diphacinone bait eaten over time. This was possibly because the majority of the residues were present in the gut contents and excreted in frass. A simple deterministic risk assessment suggests that weta containing the residual diphacinone concentrations measured here would present a low, single-exposure risk of acute toxicity non-target birds that ate them. However, multiple secondary exposures and the possibility of sublethal effects in non-target wildlife feeding on contaminated invertebrates await further investigation. Definitive measures of anticoagulant persistence in gut and other tissues of gastropods and arthropods would be useful to investigate differences between invertebrate classes as reservoirs and vectors of anticoagulant residues in the environment.

Chapter 7: Summary and implications

7.1 Continued use of brodifacoum as a rodenticide

The relatively high potential of brodifacoum to cause secondary mortality is established, while the unknown long term effects of sublethal exposure on wildlife may represent a significant and as yet unrecognized cost. Subsequently a proactive approach to defining use patterns of brodifacoum that optimise both benefit (rodent control) and acceptable cost (adverse effects on non-target wildlife) is warranted. Chapters 2 and 3 describe new information relevant to monitoring and assessing pathways of environmental transfer of brodifacoum, summarized in Fig 7.1. This can contribute to future assessment of environmental contamination and non-target risks associated with different uses of brodifacoum as a rodenticide.

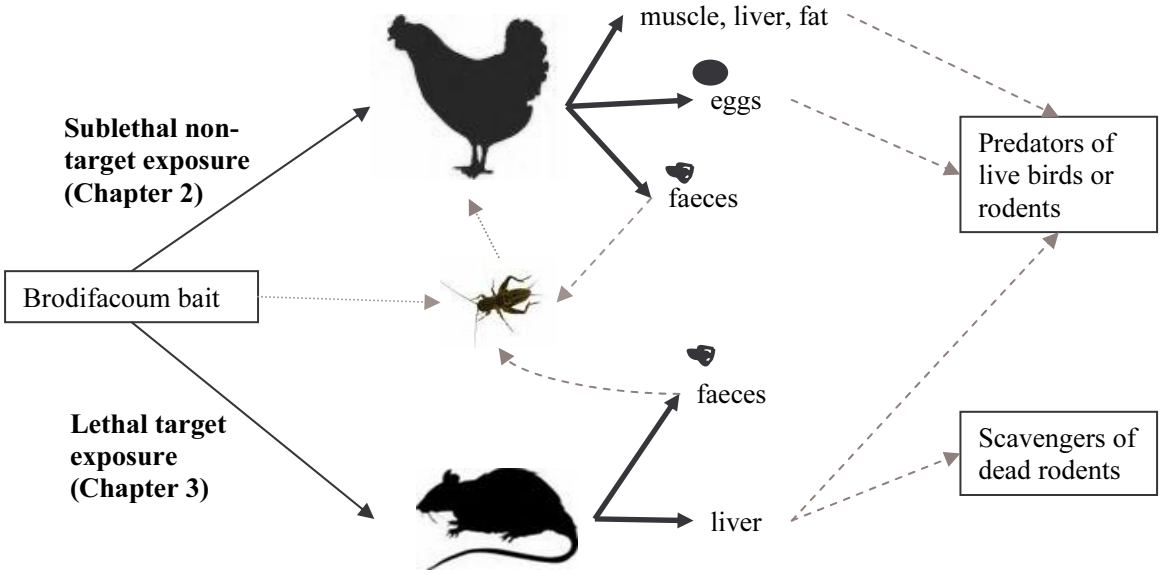


Figure 7.1 Environmental pathways of brodifacoum investigated in this thesis. Black lines represent primary exposure pathways (sublethal exposure in birds, Chapter 2 and lethal exposure in Norway rats, Chapter 3) and tissues in which residual brodifacoum was measured in trials. Relative hazard of residual concentration magnitude is not indicated. Grey dashed lines indicate potential pathways of secondary exposure to brodifacoum.

As a little-investigated environmental pathway of brodifacoum, the occurrence of residual concentrations in eggs following sublethal exposure of hens raises questions around potential adverse effects on the development of fertilised eggs and chicks. Investigation of this seems especially warranted with respect to birds predisposed to brodifacoum exposure through diet e.g.

raptors, where breeding success is important for species conservation. The pathways in Figure 7.1 do not show a relationship between the amount of brodifacoum ingested by rats and the magnitude of secondary hazard presented by rat liver, or the potential for gut contents of rodents to represent a significant secondary hazard, but these are all important factors when considering baiting practices that minimise secondary hazards while maintaining efficacy against target rodent pests. Baiting practices that utilize pulsed availability of toxic bait to rodents, or present a ratio of non-toxic to toxic bait (e.g. one in ten baits contains a lethal dose) to rodents are possible techniques to investigate in this context.

7.2 Diphacinone as an alternative to brodifacoum

The two environmental pathways of diphacinone investigated are shown in Fig 7.2. Use of diphacinone as an alternative field rodenticide to brodifacoum is likely to pose less secondary hazard to humans consuming meat from feral pigs. Residual concentrations of diphacinone would be eliminated from pig liver and probably other tissues more quickly than would brodifacoum, and diphacinone is also less toxic than brodifacoum so that relatively more contaminated tissue would need to be consumed to result in harmful effects. Primary poisoning in feral pigs that access diphacinone bait stations appears higher than previously estimated, with potentially lethal haemorrhages observed in pigs at oral exposures of c. 0.5 mg/kg/day for up to 5 days.

Weta will readily feed on cereal-based bait formulations, but as for brodifacoum, diphacinone seems to have acute low toxicity to insects. Use of diphacinone instead of brodifacoum is likely to reduce the secondary hazard to mammalian or avian predators of insects because of the lower acute toxicity of diphacinone. However, the increased toxicity of diphacinone in multiple, consecutive exposure needs further consideration in estimating secondary hazard in field situations.

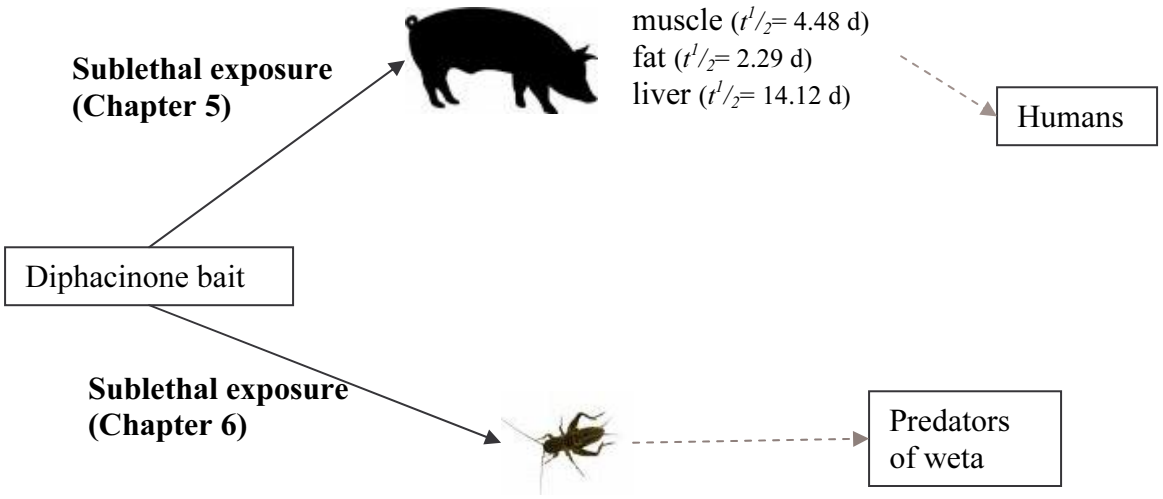


Figure 7.2. Environmental pathways of diphacinone investigated in this thesis. Black lines represent primary exposure pathways (sublethal exposure in pigs, Chapter 6 and in weta, Chapter 6) and tissues in which residual brodifacoum was measured in trials. Grey dashed lines indicate potential pathways of secondary exposure to diphacinone.

Continued development of diphacinone as a field-use rodenticide is warranted, with existing data and new data here confirming lower persistence in mammalian liver than brodifacoum, and also indicating low toxicity to invertebrates. The toxicity profile of diphacinone to mammals and birds – having lower single-exposure oral toxicity than brodifacoum but also enhanced toxicity in multiple consecutive exposures – will however be an important consideration in engineering bait formulations and bait application strategies that provide effective reduction of rodent populations, especially in the context of eradication of rodents from islands. While diphacinone appears to present a low risk of secondary poisoning to birds, theoretical assessments to date have generally been based on single exposures and do not take take full account of the potential for increased risk where non-target birds or mammals might continually feed on prey containing residual diphacinone. This aspect should be further investigated at least in captive trials with representative bird and mammal species before extensive and sustained field applications of diphacinone are undertaken.

7.3 Implications for pest managers - how can we minimize the environmental risks of using anticoagulants?

Brodifacoum and diphacinone can occupy the same environmental reservoirs, in the liver and other tissues of mammals and birds and in the gut and possibly other tissues of invertebrates. Hence, residual concentrations are likely to follow the same pathways of secondary transfer within the environment to non-target predators and scavengers. However the reduced persistence of diphacinone in liver and other tissues, means that the duration (longevity) of the secondary hazard in this reservoir is far reduced in comparison to brodifacoum. Where a persistent secondary hazard in mammalian or bird liver is unacceptable, the findings reported here should encourage the use of diphacinone as an alternative to brodifacoum for rodent control – for example in areas where there is concern for contamination of feral pigs that are hunted and eaten by humans. This thesis has focused on measuring and assessing environmental cost aspects of using anticoagulants for pest control, particularly in the context of field baiting operations aimed at protecting conservation values from rodent pests. It is acknowledged there are many other practical considerations facing pest managers who need to work within budgetary and logistical contraints to achieve maximum efficacy against the targeted pest whilst minimising unwanted effects on non-target wildlife and the wider environment. Thus a range of factors will influence the outcome of decisions about which anticoagulants to apply and when, where and how best to apply them.

The efficacy of brodifacoum against rodents, and brushtail possums in NZ, is well established and backed by a firm track record of operational use. Currently, diphacinone is only established as a rodenticide, and existing formulations are unlikely to be as effective against possums as brodifacoum. This may change with new formulation developments in the future, but currently diphacinone is not an appropriate alternative toxin for possum control. For NZ rodent pest managers considering replacing brodifacoum with diphacinone, there may be uncertainty about whether suitably high mortality and reduction of rodent impacts will be achieved. This can be addressed in a trial-and-error approach to diphacinone applications but this may not be acceptable in all instances, for example, where successful reduction of rodent populations is critical to assure the protection of critically endangered populations of fauna or flora. In particular, brodifacoum has played a key role in eradications of rodents from offshore islands, as an effective ‘big gun’ with concomitant high potential for non-target impacts - these are recognised and often

considered justified against the conservation gains of removing rodents completely from an island. Where 'one-off' applications of brodifacoum are successful in achieving rodent eradication from an island (and subsequent rodent-free status is maintained) this removes the need for further application of rodenticide to the environment – this is an important distinction between island eradications and sustained field applications of brodifacoum for maintenance rodent control.

The sustained field applications of brodifacoum in bait stations for possum and rodent control in NZ remain exceptional in contrast to the restrictions on its use in other countries. Continuation of this use pattern by some regional management agencies and private land managers in NZ seems largely driven by favourable cost-efficacy in comparison to other control tools, due to the high efficacy of brodifacoum, availability of baits to non-licensed users and the relatively low cost of baits and labour required to maintain bait stations. Despite earlier research and monitoring in NZ that identified the potential for environmental transfer of brodifacoum residues and non-target mortality, and growing international evidence that even more restricted uses of anticoagulants can result in residue burdens in wildlife, there has been no ongoing evaluation or monitoring of the longer term environmental impacts of ongoing NZ field applications of brodifacoum. It seems a major oversight that the potential environmental costs of this use are not being considered in optimising the balance of benefits and costs of pest control towards sustainable practices. Accordingly it seems timely that such sustained field use of brodifacoum in NZ is formally reviewed from a regulatory risk assessment and a product stewardship perspective.

Where increased and sustained field use of diphacinone for rodent control is being undertaken in NZ, managers should recognize the potential for the development of physiological resistance traits in rodent populations. To date, there appears to have been no formal assessment of the 'resistance status' of NZ rodents and this would be useful information at least as a baseline against which to compare in future. Use of diphacinone as a replacement for brodifacoum also should be accompanied by monitoring of environmental residues to validate the non-target risk predictions that are currently based on laboratory data. The lower toxicity of diphacinone, especially to birds, indicates an overall lower secondary hazard to non-target wildlife than equivalent (weight for weight) residual concentrations of brodifacoum in tissue. It is important to note that there is relatively much more toxicological data available for brodifacoum than diphacinone – in particular there are very few field-based data pertaining to diphacinone residues.

On this basis, nearly all data underpinning current risk assessments for diphacinone (e.g. Eisemann & Swift 2006) are from laboratory-based studies, rather than monitoring of field applications. As field applications of diphacinone increase, it is critical that risk predictions made on the basis of laboratory data are validated by residue testing from field samples of mammals, birds and invertebrates. This approach is currently being taken in Hawai'i where recent applications of diphacinone baits on the small islets of Mokapu and Lehua in are being followed by monitoring for both rodent kill (efficacy) and environmental residues of diphacinone (Peter Dunlevy, pers. comm.). To date, NZ field applications of diphacinone appear not to have been as formally monitored for residues, partly because of the cost of doing so but perhaps also because of the assumption that the risk predictions from laboratory data can be extrapolated to field conditions. Pest managers should be mindful that the extent of brodifacoum contamination in NZ wildlife only became evident after formal research based on field monitoring was undertaken, and extend a precautionary field monitoring approach to ensure that the field use of diphacinone as an alternative does not have unforeseen, unwanted consequences for native wildlife. As if to emphasise this point, within the month of finalising this thesis diphacinone was implicated in (but not confirmed as the cause of) mortality in endangered New Zealand bats (Harper 2009).

While the persistence of anticoagulant residues in invertebrate bodies is probably shorter than that in mammals and birds, environmental transfer through invertebrates deserves further attention. The persistence of diphacinone and brodifacoum in invertebrate bodies may be quite similar if neither is bound to specific invertebrate tissue in the way they bind to mammalian liver and the majority of the residual concentration resides in invertebrate gut contents. If this was the case, secondary hazard to insectivores would be highest in invertebrates that had very recently fed on anticoagulant bait – an important next step would be to describe the elimination rate of anticoagulants from invertebrates in this scenario. Prevention of invertebrates from feeding on anticoagulant baits e.g. by incorporating a repellent into the formulation, is another area for investigation towards minimisation of environmental risk.

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This thesis was completed part-time during full-time employment in pest management research at Landcare Research. As worker and student in this research environment, my appreciation of NZ's unique landscapes, biota and ecologies has grown alongside an understanding of the threats posed to them by introduced species. Combining this with a fascination for all things toxic still seems ironic to the naive undergrad who opted for zoology through a love of animals. Using poisons to kill animals designated 'pest', while avoiding harm to everything else, makes for a long wish list towards improvement.

My foremost acknowledgement is of the opportunity, resources and support to combine a great job and study - many people at Landcare Research contributed patient science advice, specialised technical expertise, humour and friendship. Thanks to Cheryl O'Connor (for teaching me the ropes, you are missed as friend and boss), Grant Morriss (critter expert, skulls and fellow 'killer'), Lynn and Phil Booth (toxicology experience and motorbike therapy), Les Brown and Kat Trought (lab skills plus some of the funniest/most bizarre tea-room conversations ever), Kathy O'Halloran (more toxicology and an Aussie too), Andrea Airey (technican extraordinaire) and Geoff Wright, Dave Morgan, Eric Spurr, Bruce Warburton and Phil Cowan (from across the ditch I read their publications with admiration, never anticipating that one day I would be working alongside them).

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Appendix 1b. Tables A, B & C summarizing brodifacoum concentrations measured in liver of non-target wildlife

Previous reviews by Eason & Murphy (2001) and Eason *et al.* (2001; 2002) of brodifacoum residues measured in bird and mammal in association with New Zealand brodifacoum operations for the field control of possums or rats are acknowledged. To avoid duplication, data from these review papers are not shown in the tables below, although data from some individual studies also referenced by Eason & Murphy (2001) and Eason *et al.* (2002) are included. Similarly, studies included in the review by Hoare & Hare (2006a) are included in table below because the latter authors noted the presence of detectable brodifacoum residues, but not the concentration. All references cited in the tables are listed in full in the 'References' section of this thesis.

Table A. Liver concentrations of brodifacoum measured in the carcasses of non-target animals and birds known or inferred as killed by brodifacoum poisoning. Note analytical methods and limits of detection differed between studies.

Species (*n*)	**Brodifacoum concentration in liver (µg/g)**	**Context**	**Reference**
Mammals			
Stoat (*n*=9) (*Mustela erminea*)	0.20-0.91	Radio-tagged, recovered (presumed secondary poisoning) after a field baiting operation, NZ	Alterio & Moller (2000)
Stoat (*n*=3)	0.94-1.72	Radio-tagged, recovered (presumed secondary poisoning) after a field baiting operation, NZ	Alterio (1996)
Ferret (*n*=5) (*Mustela furo*)	1.47-1.97	Radio-tagged, recovered (presumed secondary poisoning) after a field baiting operation, NZ	Alterio (1996)
Ferret (*n*=1)	1.59	Found after reinvading an area subject to field baiting, NZ	Alterio (1996)
Dog (*n*=3) (*Canis familiarus*)	1.2-12.7	Presented as veterinary cases showing signs of anticoagulant poisoning	DuVall *et al*. (1989)
Dog (neonate) (*n*=2)	063, 0.23	Died soon after birth, suspected placental transfer of brodifacoum	Munday & Thompson (2003)
Dog (*n*=3)	0.08, 0.4, 0.3	Died 7-11 days after administration of brodifacoum by gavage (laboratory study)	Ray *et al.* (1989)
Cat (*n*=2) (*Felis catus*)	2.71, 3.73	Radio-tagged, recovered (presume secondary poisoning) after a field baiting operation, NZ	Alterio 1996
Cat (*n*=2)	0.2, 0.7	Found after a field baiting operation, NZ	Bell *et al*. 1983
Cat (*n*=3)	0.98, 1.38, 0.91	Found after an island eradication baiting operation, NZ	Dowding *et al*. 1999
Cat (*n*=1)	1.4	Found dead after baiting operatiom, NZ	Murphy *et al.* (1998)
Cat (*n*=1)	0.71	Found 4-28 days after baiting operation, NZ	Rammell *et al*. (1984)
Mountain lion (*n*=2) (*Puma concolor*)	0.57, 0.31	Radio-collared animals living near urban areas recovered and necropsied with signs of anticoagulant poisoning, USA	Riley *et al.* (2007)
Bobcat (*n*=1)	0.05	Radio-collared animal living near urban areas determined to	Riley *et al.* (2007)

(*Lynx rufus*)		have died of anticoagulant poisoning, USA	
Rabbit (*n*=35) (*Oryctolagus cuniculus*)	0.03-11.37	Found after a field baiting operation, NZ	Bell *et al*. (1983)
Rabbit (*n*=5)	0.54-2.01	Found after an island eradication baiting operation, NZ	Dowding *et al*. (1999)
Rabbit (*n*=43)	<0.05-0.79	Found 4-28 days after baiting operation, NZ	Rammell *et al*. (1984)
Rabbit (*n*=44)	mean = 4.4	Carcasses collected as target species of baiting operation, NZ	Williams *et al*. (1986)
Hare (*n*=1) (*Lepus capensis*)	0.77	Found 4-28 days after baiting operation, NZ	Rammell *et al*. (1984)
Pig (*n*=4) (*Sus scrofa*)	0.21 (*n*=1) mean (*n*=3) 1.7	Found after baiting operation, NZ	Murphy *et al.* (1998)
Sheep (*n*=4) (*Ovis aries*)	0.48-3.7	Found 4-28 days after baiting operation, NZ	Rammell *et al*. (1984)
Llama (*n*=1) (*Lama glama*)	1.1	Submitted for veterinary analysis after suspected poisoning, USA	Ray *et al.* (1989)
Norway rat (*n*=11) (*Rattus norvegicus*)	24.8-35.3	Found after brodifacoum baiting operation, Langara Island	Howald *et al.* (1999)
Polynesian rat (*n*=3) (*R. exulans*)	0.6-11.0	Found after baiting operation, NZ	Morgan *et al.* (1996)
Meadow vole (*n*=62) (*Microtus pennsylvanicus*)	2.07 ± 0.17	Found within 7 days after 0.005% brodifacoum baiting at 10.5 kg/ha	Merson *et al*. (1984)
Meadow vole (*n*=74)	4.07 ± 0.20	Found within 7 days after 0.005% brodifacoum baiting at 45.9 kg/ha	Merson *et al*. (1984)
Meadow vole (*n*=43)	0.35 ± 0.03	Found within 7 days after 0.001% brodifacoum baiting at 22.9 kg/ha	Merson *et al*. (1984)
Vole (*n*=9) (*Microtus* spp.)	mean 0.20-0.38	Found after baiting operation, Finland	Myllimaki *et al*. (1999)
Brushtail possum (*n*=21) (*Trichosurus vulepcula*)	mean 0.53 range 0.17 -1.04	Died after ingesting a lethal dose 0.50-1.07 mg/kg brodifacoum in bait, laboratory study	Littin *et al*. (2002)
Brushtail possum (*n*=23)	0.25-2.9	Found after a field baiting operation, NZ	Alterio & Moller (2000)
Human (*n*=1) (*Homo sapiens*)	1.37	Died after deliberate ingestion of brodifacoum bait	Helmuth *et al.* (1989)
Human (*n*=1)	0.05	Died after deliberate ingestion of brodifacoum bait	Palmer *et al.* (1998)
Human (*n*=1)	0.056	Died after deliberate ingestion of brodifacoum bait	Routh *et al.* (1991)
Birds			
Australasian harrier (*n*=2) (*Circus approximans*)	0.12, 0.34	Found 4-28 days after baiting operation, NZ	Rammell *et al*. (1984)
Australasian harrier (*n*=2)	0.61, 0.66	Found after an island eradication operation	Dowding *et al*. (1999)
Black-backed gull (*n*=2)	1.3, 1.5	Found after a field baiting operation	Bell *et al*. (1983)

(*Larus dominicanus*)			
Black-backed gull (*n*=1)	0.58	Found after an island eradication operation	Dowding *et al*. (1999)
Chaffinch (*n*=1) (*Fringilla coelebs*)	8.1	Found after a field baiting operation	Bell *et al*. (1983)
Chaffinch (*n*=3)	0.12-2.31	Found after an island eradication operation	Dowding *et al*. (1999)
Paradise shelduck (*n*=4) (*Tadorna variegatus*)	0.24-0.80	Found after an island eradication operation, NZ	Dowding *et al*. (1999)
Paradise duck (*n*=1)	4.0	Found 4 to 28 days after baiting operation, NZ	Rammell *et al*. (1984)
Mallard (*n*=2) (*Anas platyrynchos*)	0.90, 1.23	Found after an island eradication operation	Dowding *et al*. (1999)
Grey duck (*n*=1) (*Anas superciliosa*)	0.91	Found after an island eradication operation	Dowding *et al*. (1999)
Pukeko (*n*=9) (*Porphyrio porphyrio*)	0.52-1.35	Found after an island eradication operation	Dowding *et al*. (1999)
Blackbird (*n*=2) (*Turdus merula*)	0.56, 0.78	Found after an island eradication operation	Dowding *et al*. (1999)
Blackbird (*n*=4) (*Turdus merula*)	0.6-11.0	Found after baiting operation, NZ	Morgan *et al.* (1996)
Common myna (*n*=3) (*Acridotheres tristis*)	0.54-1.27	Found after an island eradication operation	Dowding *et al*. (1999)
Australian magpie (*n*=2) (*Gymnorhina tibicen*)	0.40, 0.99	Found after an island eradication operation	Dowding *et al*. (1999)
Australian Magpie (*n*=1)	0.48	Found 4 to 28 days after baiting operation	Rammell *et al*. (1984)
New Zealand dotterel (*n*=1) (*Charadrius obcurus aquilonius*)	0.77	Found after baiting operation, NZ. Suspected secondary poisoning through invertebrates	Dowding *et al*. (2006)
Kaka (*n*=3) (*Nestor meridionalis*)	1.2, 3.3, 4.1	Found after a brodifacoum bait operation, NZ	Empson & Miskelly (1999)
Eastern screech-owl (*n*=7) (*Otus asio*)	0.4-0.8	Radio-tagged and recovered after baiting operation, USA	Hegdal & Colvin (1988)
Common raven (*n*=13) (*Corvus corax*)	0.98-2.52	Found after brodifacoum baiting operation, Langara Island	Howald *et al.* (1999)
Barn owl (*n*=1) (*Tito alba*)	1.67	Died 15 days first feeding on contaminated mice, captive study	Gray *et al.* (1992)
Morepork (*n*=1) (*Ninox novaeseelandiae*)	3.4	Found 23 days after baiting operation, NZ	Ogilvie *et al.* (1997)
Morepork (*n*=1)	0.97	Found 22 days after baiting operation, NZ	Stephenson *et al.* (1999)
Kakariki (*n*=1)	0.03	Found 33 days after baiting operation, NZ	Ogilvie *et al.* (1997)

(*Cyanoramphos novezelandiae*)			
Seagull (*n*=2) (not specified)	1.3, 1.5	Found 4 to 28 days after baiting operation	Rammell *et al*. (1984)
Little spotted kiwi (*n*=1) (*Apteryx owenii*)	1.2	Found 25 days after baiting operation, NZ	Robertson & Colbourne (2001)
Saddleback (*n*=1) (*Philesturnus carunculatus*)	0.6	Found after baiting operation NZ	Towns *et al.* (1994)

Table B. Liver concentrations of brodifacoum measured in non-target mammal and birds found dead from unknown causes e.g. road killed and collected as part of wildlife monitoring schemes. Only detectable brodifacoum concentrations are included, noting that analytical methods and limits of detection differed between studies. Exposure to brodifacoum was inferred from the presence of liver residues, but not confirmed as cause or contributory to mortality.

Species (*n*)	**Brodifacoum liver concentration (µg/g)**	**Context**	**Reference**
Mammals			
Red fox (*n*=2) (*Vulpes vulpes*)	1.32, 4.01	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Red fox (*n*=2)	0.04, 0.05	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Gray fox (*n*=1) (*Urocyon cinereoargenteus*)	0.03	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
San Joaquin kit fox (*n*=3) (*Vulpes macroitis mutica*)	0.07-0.47	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Coyote (*n*=12) (*Canis latrans*)	<0.01-0.5	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Bobcat (*n*=3) (*Lynx rufus*)	0.018-0.07	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Bobcat (*n*=31)	up to 0.56	Radio-collared animals living near urban areas that died before and after a mange epizootic, USA	Riley *et al.* (2007)
Mountain lion (*n*=1) (*Felis concolor*)	0.52	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Ferret (polecat) (*n*=2) (*Mustela putorius*)	0.070, 0.052	sampled from road-killed animals, UK	Shore *et al.* (2003)
Ferret (polecat) (*n*=1)	0.008	sampled from road-killed animals, UK	Shore *et al*. (1999)
Gray squirrel (*n*=4) (*Sciurus carolinensis*)	0.7 -4.1	from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at	Stone *et al.* (1999)

		necropsy, USA	
Eastern chipmunk (*n*=1) (*Tamias* spp.)	3.8	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Raccoon (*n*=5) (*Procyon lotor*)	0.32 – 5.3	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Raccoon (*n*=2)	0.08, 0.41	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
White-tailed deer (*n*=5) (*Odocoileus virginianus*)	0.12 – 0.41	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Opossum (*n*=1) (*Didelphis virginiana*)	0.18	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Birds			
Great horned owl (*n*=13) (*Bubo virginianus*)	0.01 – 0.73	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Great horned owl (*n*=42)	0.007-0.97	sampled from carcasses submitted 1998	Stone *et al.* (2003)
Great horned owl (*n*=3)	0.015-0.35	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Screech owl (*n*=2) (*Otus asio*)	0.34, 0.80	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Screech owl (*n*=8)	0.007-0.47	sampled from carcasses submitted 1998-2001 by public, USA	Stone *et al.* (2003)
Barn owl (*n*=3) (*Tyto alba*)	0.07-0.35	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Golden eagle (*n*=8) (*Aquila chrysaetos*)	<0.01-0.13	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Golden eagle (*n*=1)	0.03	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone *et al.* (1999)
Sharp-shinned hawk (*n*=12) (*A. cooperii*)	0.008-0.22	sampled from carcasses submitted 1998-2001 by public, USA	Stone *et al.* (2003)
Red-tailed hawk (*n*=42) (*Buteo jamaicensis*)	0.006-1.28	sampled from carcasses submitted 1998-2001 by public, USA	Stone *et al.* (2003)
Red-tailed hawk (*n*=7)	0.16 –1.6	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy	Stone *et al.* (1999)

		USA	
Red-tailed hawk (*n*=1)	0.01	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Red-shouldered hawk (*n*=2) (*Buteo lineatus*)	0.01, 0.05	sampled from carcasses returned by government agencies and wildlife rehabilitation groups	Hosea (2000)
Raven (n=1) (*Corvus* spp.)	1.04	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone et al. (1999)
Common crow (n=1) (*Corvus brachyrhynchos*)	1.34	sampled from carcasses submitted 1971-1997 by public or wildlife rehabilitation centers and associated with haemorrhage at necropsy USA	Stone et al. (1999)

Table C. Liver concentrations of brodifacoum measured in non-target mammal and birds sampled live e.g. shot or trapped and then killed for sampling at various intervals during or after baiting operations, or administered known doses of brodifacoum in captive studies. Only reports of detectable brodifacoum concentrations are included, noting that analytical methods and limits of detection differed between studies. Sublethal exposure to brodifacoum was inferred from the presence of liver residues.

Species (*n*)	**Brodifacoum liver concentration (µg/g)**	**Context**	**Reference**
Mammals			
Stoat (*n*=3) (*Mustela erminea*)	<0.05,0.08, 0.36	Radio-tagged & trapped 4,5 and 227 days after baiting operation NZ	Gillies & Pierce (1999)
Stoat (*n*=5 male)	mean 0.28 ± 0.1	Trapped after field baiting operation NZ	Gillies & Pierce (1999)
Stoat (*n*=31of 40 tested)	mean 0.37	Trapped during or after baiting operations, NZ	Murphy *et al.* (1998)
Stoat (*n*=55) (*Mustela furo*)	mean 0.15 range 0.0-0.74	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
Ferret (*n*=4)	<0.2-0.34	Trapped 12-14 weeks after baiting operation, NZ	Alterio (1996)
Ferret (*n*=9 of 16 tested)	mean 1.01	Trapped during or after baiting operations, NZ	Murphy *et al.* (1998)
Ferret (*n*=6)	mean 0.82 range 0.0-2.43	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
Weasel (*n*=4 male) (*Mustela nivalis*)	mean 0.6 ± 0.1	Trapped after field baiting operation NZ	Gillies & Pierce (1999)
Weasel (*n*=1 female)	1.0	Trapped after field baiting operation NZ	Gillies & Pierce (1999)
Weasel (*n*=10 of 14 tested)	mean 1.26	Trapped during or after baiting operations, NZ	Murphy *et al.* (1998)
Weasel (*n*=18)	mean 0.33 range 0.0-0.93	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
Cat (*n*=1) (*Felis catus*)	2.70	Radio-tagged & trapped 89 days after a field baiting operation, NZ	Alterio (1996)
Cat (*n*=9)	2.20 - 5.05	Trapped or shot after an island eradication operation, NZ	Dowding *et al*. 1999
Cat (*n*=2)	0.13, 0.25	Radio-tagged & trapped 91 and 212 days after baiting operation	Gillies & Pierce (1999)

		NZ	
Cat (*n*=13 male)	mean 0.40 ± 0.17	Trapped after field baiting operation NZ	Gillies & Pierce (1999)
Cat (*n*=2 female)	mean 0.88 ± 0.83	Trapped after field baiting operation NZ	Gillies and Pierce (1999)
Cat (*n*=1)	0.39	Trapped after baiting operation, NZ	Murphy *et al.* (1998)
Cat (*n*=10)	mean 0.41 range 0.0-1.25	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
Rabbit (*n*=4) (*Oryctolagus cuniculus*)	0.03-0.42	Shot two weeks after an island eradication operation, NZ	Dowding *et al*. (1999)
Pig (*n*=37) (*Sus scrofa*)	0.007-1.9	Shot in areas where brodifacoum bait being used, NZ	Eason *et al*. (2001)
Pig (*n*=19)	0.01-2.4	Shot in areas where brodifacoum baiting had ceased at least 6 months before, NZ	Eason *et al*. (2001)
Pig (*n*=5) + (*n*=1)	0.01-0.03	Five released, radio tagged pigs and one resident pig shot after baiting operation, NZ	Morriss *et al.* (2005)
Pig (*n*=3)	0.007, 0.009, 1.6	Trapped or shot after baiting operation, NZ	Murphy *et al.* (1998)
Sheep (*n*=11) (*Ovis aries*)	0.35-6.50	Shot for sampling 2-128 days after dosing with 0.2 mg/kg brodifacoum (captive study)	Laas *et al*. (1985)
Goat (*n*=2) (*Capra hircus*)	0.01, 0.01	Shot in areas where brodifacoum bait being used, NZ	Eason *et al*. (2001)
Deer (*n*=36) (*Cervus elaphus*)	0.03-0.04	Shot in areas where brodifacoum bait being used, NZ	Eason *et al*. (2001)
Black rat (*n*=9) (*Rattus rattus*)	mean 0.87	Trapped during a baiting operation, NZ	Murphy *et al.* (1998)
Black rat (*n*=8)	mean 0.17	Trapped within three months of a baiting operation, NZ	Murphy *et al.* (1998)
Black rat (*n*=31)	mean 0.76 range 0.0-5.65	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
House mouse (*n*=20) (*Mus musculus*)	mean 0.49 range 0.0-4.57	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
Vole (*n*=7) (*Microtus* spp.)	mean 0.23-0.33	Trapped 2-4 days after baiting operation, Finland	Myllimaki *et al*. (1999)
Vole (*n*=5)	mean 0.01-0.03	Trapped two weeks after baiting operation, Finland	Myllimaki *et al*. (1999)
Hedgehog (*Erinaceus europaeus*)	mean 0.20 range 0.0-1.31	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
Possum (*n*=7) (*Trichosurus vulpecula*)	mean 1.13 range 0.0-2.14	Trapped during baiting operation, NZ	Spurr *et al.* (2005)
Birds			
Eastern screech-owl (*n*=2) (*Otus asio*)	<0.3-0.3	Captured after baiting operation, USA	Hegdal and Colvin (1988)
Northwestern crow (*n*=3)	0.019	Captured after brodifacoum baiting operation, Langara Island	Howald *et al.* (1999)

(*Corvus caurinus*)			
Blackbird (*n*=6)	0.004-0.2	Shot within one week of baiting operation, NZ	Morgan *et al.* (1996)
Barn owl (*n*=3) (*Tyto alba*)	0.55, 0.67, 0.69	Killed for sampling 15 days after last feeding on contaminated mice (captive study)	Gray *et al*. (1992)
Morepork (*n*=1) (*Ninox novaeseelandiae*)	0.61	Shot during or after baiting operations, NZ	Murphy *et al.* (1998)
Magpie (*n=2* of 8 tested) (*Gymnorhinus tibicens*)	0.08, 0.41	Shot during or after baiting operations, NZ	Murphy *et al.* (1998)
Magpie (*n*=2) (*Pica pica*)	1.21, 2.04	Killed for sampling three weeks after last exposure to contaminated voles (captive study)	Myllymaki (1996)

Appendix 2. Laboratory reports for chicken brodifacoum trial

Dosing solution 0.4 mg/mL brodifacoum in monopropylene glycol (MPG)
Landcare Research toxicology laboratory Cert No. P05/22

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CERTIFICATE OF PREPARATION

Product Identification **Cert. No.: P05/22**

Description: Brodifacoum, 0.4mg/mL in MPG

Active ingredient: Brodifacoum

Required dosage: 0.5 mg/kg body weight Dose volume: 1.25 mL/kg Animal: Chicken

Active concentration: 0.4 mg/mL (also known as 0.04 % w/v).

Preparation

General precautions: Wear gloves when handling; avoid contact with skin and eyes.

Weight of active: 0.908g Active strength *(if known)*: 2.23%, 4273 (ref T1590)

Final volume, mL: 50

Remarks on solution preparation: 0.908g of 2.23% brodifacoum solution was added to MPG in a 50mL volumetric flask and made up to volume with MPG.

The concentration of the sample was determined by:

Documented value of concentration: 0.4 mg/mL.
(95% confidence level)

Signature for Preparation
Date: May 5, 2005

Check Signature
for: Pest Control and Wildlife Toxicology
Lincoln Region
5 May 2005

Brodifacoum concentrations detected in dried blood spot samples, MDL 0.04 mg/mL
Landcare Research toxicology laboratory report T2361

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Toxicology Laboratory Analysis Report

Centre for Environmental Toxicology

Report No: T2361

CLIENT: Penny Fisher, Landcare Research, Lincoln, ..

CLIENT REFERENCE No.: 444009 0307 **Telephone No:** 325 6700

SAMPLES: Ten blood spot samples

REQUIREMENT: Examine for brodifacoum

RECEIVED: 10, 13 May 2005

121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 10

Day 4 samples show quite repeatable results that are below the current statistical limit of detection for the method.

LabNo.	Description	Brodifacoum, µg/mL
8400	Dried blood spot, chicken #229, day 1, 10/5/05	<MDL, <MDL
8401	Dried blood spot, chicken #247, day 1, 10/5/05	0.25, 0.25
8402	Dried blood spot, chicken #668, day 1, 10/5/05	0.12, 0.12
8403	Dried blood spot, chicken #683, day 1, 10/5/05	0.098, 0.093
8404	Dried blood spot, chicken #686, day 1, 10/5/05	0.11, 0.11
8457	Dried blood spot, chicken #682, day 4, 13/5/05	<MDL (0.029, 0.020)
8458	Dried blood spot, chicken #681, day 4, 13/5/05	<MDL (0.016, 0.015)
8459	Dried blood spot, chicken #690, day 4, 13/5/05	<MDL (0.012, 0.011)
8460	Dried blood spot, chicken #698, day 4, 13/5/05	<MDL (0.009, 0.002)
8461	Dried blood spot, chicken #686, day 4, 13/5/05	<MDL (0.010, 0.011)

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM/075, the determination of brodifacoum in dried blood spots (DBS) by HPLC. NOT AN ACCREDITED TEST. The method limit of detection is 0.04mg/mL and the uncertainty (95% C.I.) is ± 10%.

TESTED BY: leb **WORKBOOK REF** 40/9

TEST PERIOD: 18-19/5/05

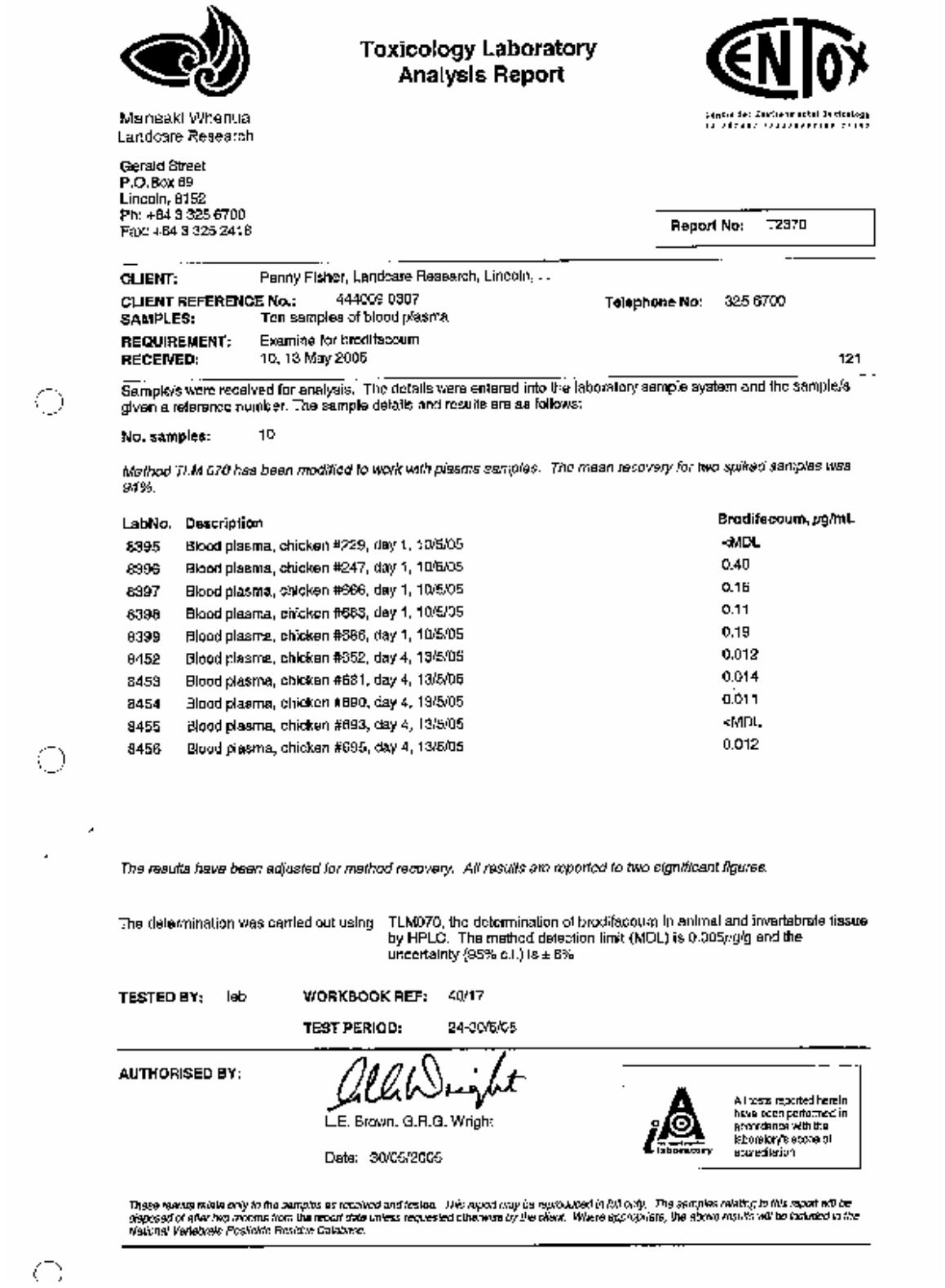
AUTHORISED BY:

L.E. Brown, G.R.G. Wright

Date: 23/05/2005

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Brodifacoum concentrations detected in plasma samples, MDL 0.005 µg/mL
Landcare Research toxicology laboratory report T2370



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Toxicology Laboratory Analysis Report

Report No: T2370

CLIENT: Penny Fisher, Landcare Research, Lincoln, - -
CLIENT REFERENCE No.: 444006 0307 **Telephone No:** 325 6700
SAMPLES: Ten samples of blood plasma
REQUIREMENT: Examine for brodifacoum
RECEIVED: 10, 18 May 2005 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 10

Method TLM 070 has been modified to work with plasma samples. The mean recovery for two spiked samples was 94%.

LabNo.	Description	Brodifacoum, µg/mL
8395	Blood plasma, chicken #229, day 1, 10/5/05	<MDL
8396	Blood plasma, chicken #247, day 1, 10/5/05	0.40
8397	Blood plasma, chicken #666, day 1, 10/5/05	0.16
8398	Blood plasma, chicken #663, day 1, 10/5/05	0.11
8399	Blood plasma, chicken #366, day 1, 10/5/05	0.19
8452	Blood plasma, chicken #352, day 4, 13/5/05	0.012
8453	Blood plasma, chicken #681, day 4, 13/5/05	0.014
8454	Blood plasma, chicken #880, day 4, 13/5/05	0.011
8455	Blood plasma, chicken #893, day 4, 13/5/05	<MDL
8456	Blood plasma, chicken #895, day 4, 13/5/05	0.012

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM070, the determination of brodifacoum in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.005µg/g and the uncertainty (95% c.l.) is ± 8%

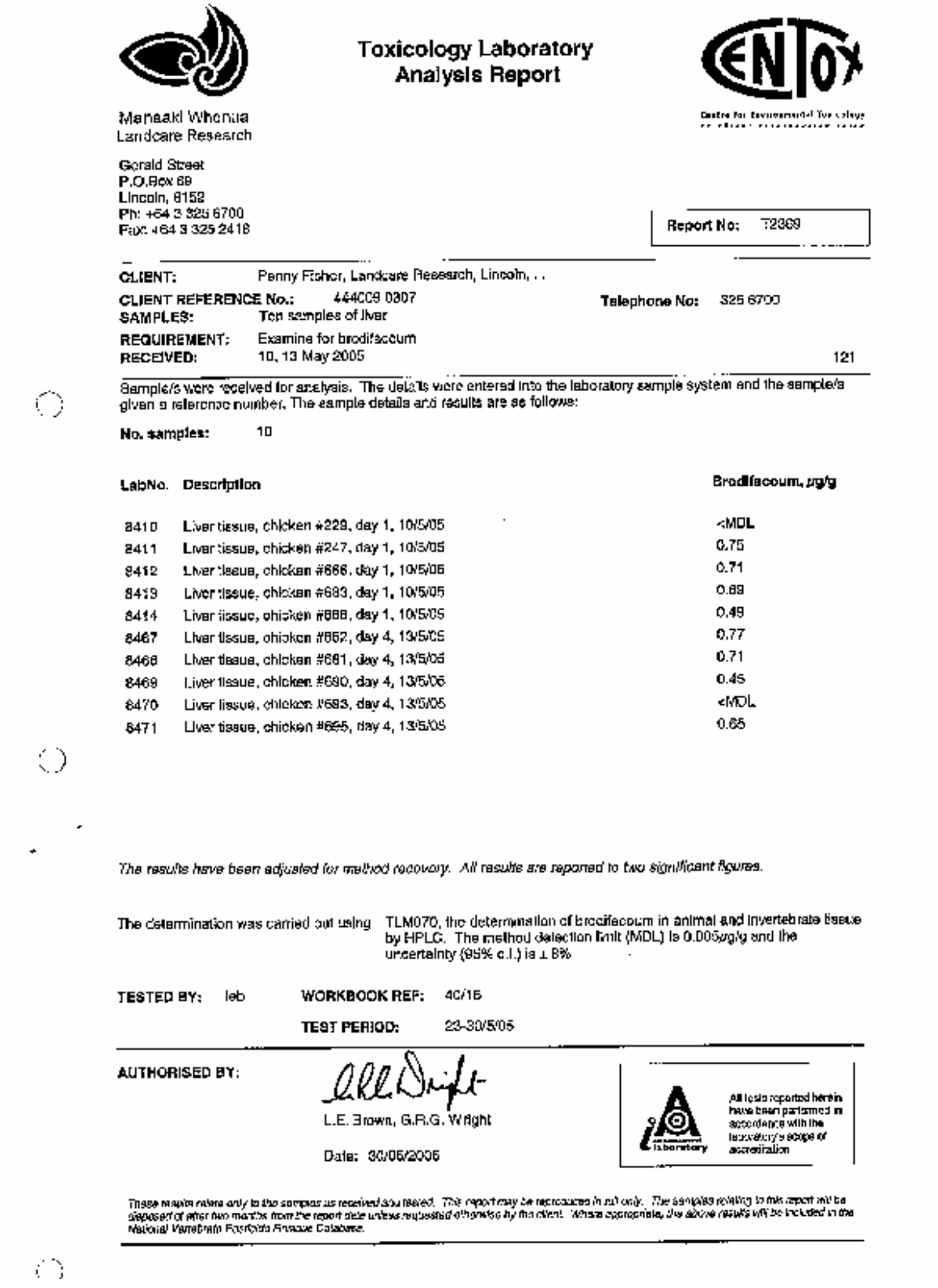
TESTED BY: lab **WORKBOOK REF:** 40/17
TEST PERIOD: 24-30/5/05

AUTHORISED BY:
L.E. Brown, G.R.G. Wright
Date: 30/05/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be calculated to the National Vertebrate Pesticide Database.

Brodifacoum concentrations detected in liver samples, MDL 0.005 µg/g, Landcare Research toxicology laboratory report No. T2369



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Toxicology Laboratory Analysis Report

Report No: T2369

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: 444009 0307 **Telephone No:** 325 6700
SAMPLES: Ten samples of liver
REQUIREMENT: Examine for brodifacoum
RECEIVED: 10, 13 May 2005 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 10

LabNo.	Description	Brodifacoum, µg/g
8410	Liver tissue, chicken #229, day 1, 10/5/05	<MDL
8411	Liver tissue, chicken #247, day 1, 10/5/05	0.75
8412	Liver tissue, chicken #666, day 1, 10/5/05	0.71
8413	Liver tissue, chicken #683, day 1, 10/5/05	0.69
8414	Liver tissue, chicken #688, day 1, 10/5/05	0.49
8467	Liver tissue, chicken #662, day 4, 13/5/05	0.77
8468	Liver tissue, chicken #681, day 4, 13/5/05	0.71
8469	Liver tissue, chicken #690, day 4, 13/5/05	0.45
8470	Liver tissue, chicken #693, day 4, 13/5/05	<MDL
8471	Liver tissue, chicken #695, day 4, 13/5/05	0.65

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM070, the determination of brodifacoum in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.005ug/g and the uncertainty (95% c.l.) is ± 8%

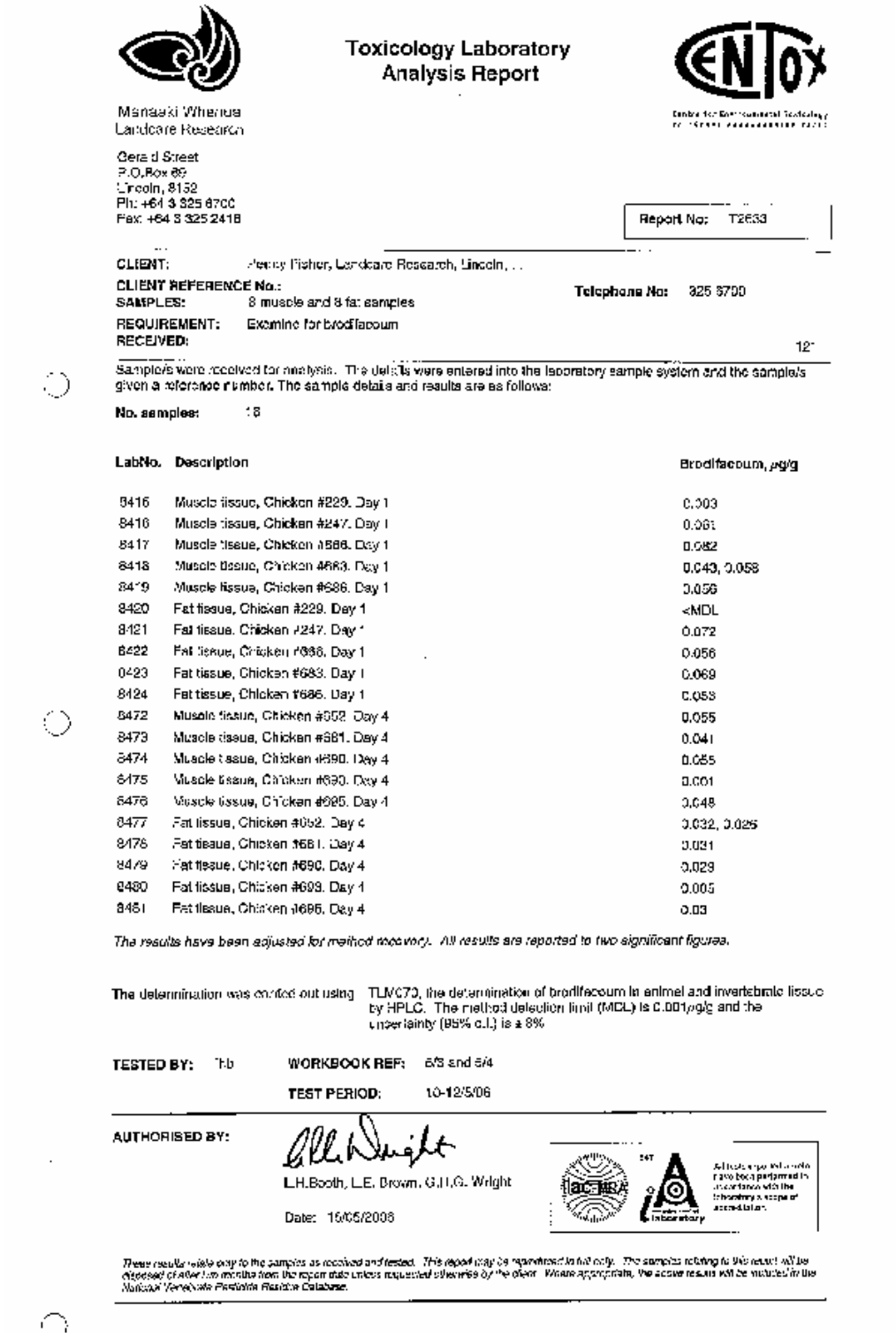
TESTED BY: leb **WORKBOOK REF:** 40/15
TEST PERIOD: 23-30/5/05

AUTHORISED BY:
L.E. Brown, G.R.G. Wright
Date: 30/05/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Brodifacoum concentrations detected in muscle and fat samples, MDL 0.001 µg/g
Landcare Research toxicology laboratory report No. T2633



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Toxicology Laboratory Analysis Report

Report No: T2633

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.:
SAMPLES: 8 muscle and 8 fat samples
Telephone No: 325 6700
REQUIREMENT: Examine for brodifacoum
RECEIVED: 12

Samples were received for analysis. The details were entered into the laboratory sample system and the samples given a reference number. The sample details and results are as follows:

No. samples: 16

LabNo.	Description	Brodifacoum, µg/g
8416	Muscle tissue, Chicken #229. Day 1	0.303
8416	Muscle tissue, Chicken #247. Day 1	0.061
8417	Muscle tissue, Chicken #686. Day 1	0.052
8418	Muscle tissue, Chicken #683. Day 1	0.043, 0.058
8419	Muscle tissue, Chicken #686. Day 1	0.056
8420	Fat tissue, Chicken #229. Day 1	<MDL
8421	Fat tissue, Chicken #247. Day 1	0.072
8422	Fat tissue, Chicken #686. Day 1	0.056
8423	Fat tissue, Chicken #683. Day 1	0.069
8424	Fat tissue, Chicken #686. Day 1	0.058
8472	Muscle tissue, Chicken #662. Day 4	0.055
8473	Muscle tissue, Chicken #681. Day 4	0.041
8474	Muscle tissue, Chicken #690. Day 4	0.055
8475	Muscle tissue, Chicken #690. Day 4	0.001
8476	Muscle tissue, Chicken #695. Day 4	0.048
8477	Fat tissue, Chicken #662. Day 4	0.032, 0.025
8478	Fat tissue, Chicken #681. Day 4	0.031
8479	Fat tissue, Chicken #690. Day 4	0.029
8480	Fat tissue, Chicken #690. Day 4	0.005
8481	Fat tissue, Chicken #695. Day 4	0.03

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLWC70, the determination of brodifacoum in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.001µg/g and the uncertainty (95% c.l.) is ± 8%

TESTED BY: TLb **WORKBOOK REF:** 5/3 and 5/4
TEST PERIOD: 10-12/5/06

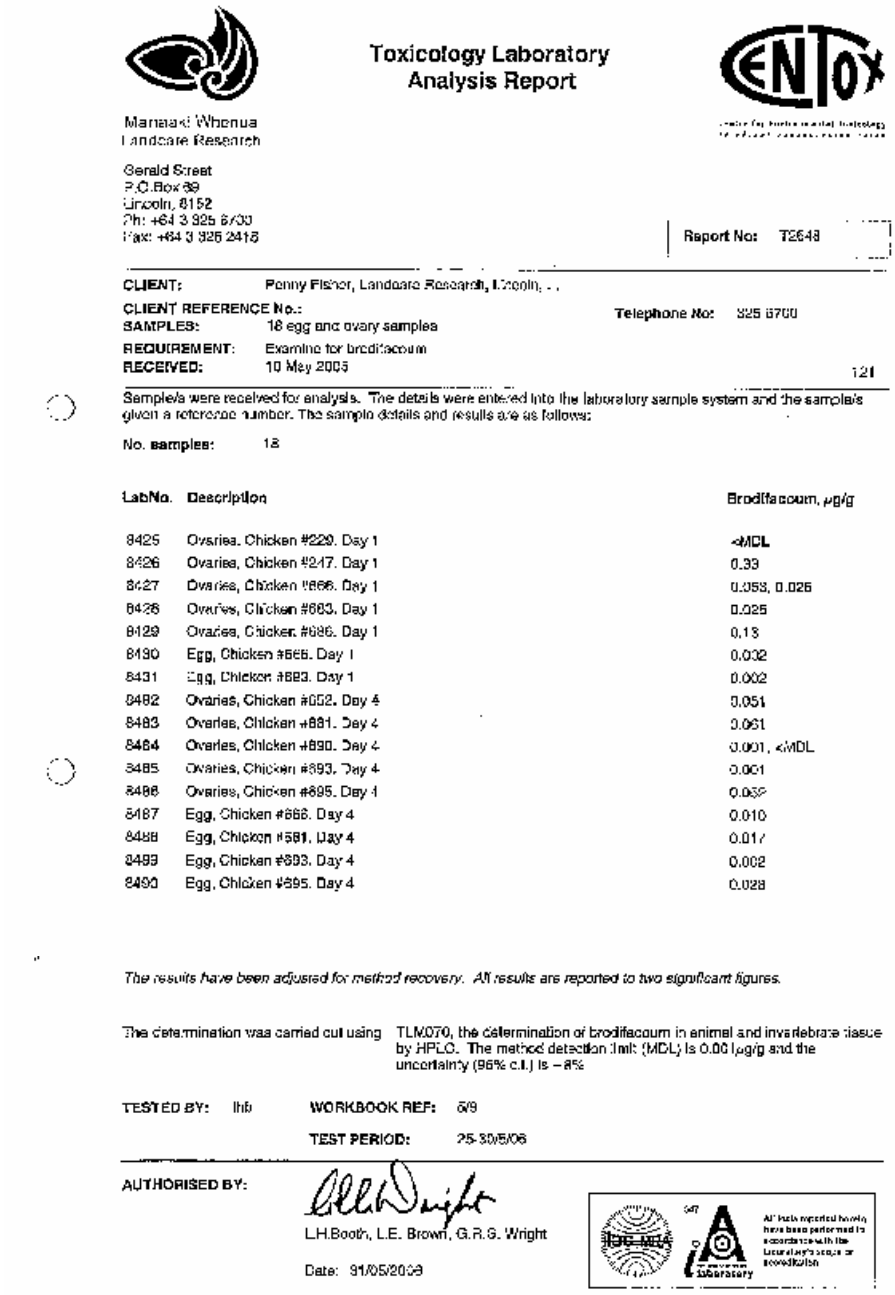
AUTHORISED BY: L.H.Booth, L.E. Brown, G.J.G. Wright

Date: 15/05/2006

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation.

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after 1 month from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Brodifacoum concentrations detected in egg and ovary samples, MDL 0.001 µg/g
Landcare Research toxicology laboratory report No. T2648



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Toxicology Laboratory Analysis Report

Report No: T2648

CLIENT: Penny Fisher, Landcare Research, Lincoln, , ,
CLIENT REFERENCE No.:
Telephone No: 325 6700
SAMPLES: 18 egg and ovary samples
REQUIREMENT: Examine for brodifacoum
RECEIVED: 10 May 2005 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 18

LabNo.	Description	Brodifacoum, µg/g
8425	Ovaries, Chicken #229, Day 1	<MDL
8426	Ovaries, Chicken #247, Day 1	0.39
8427	Ovaries, Chicken #868, Day 1	0.053, 0.025
8428	Ovaries, Chicken #883, Day 1	0.025
8429	Ovaries, Chicken #886, Day 1	0.13
8430	Egg, Chicken #566, Day 1	0.032
8431	Egg, Chicken #883, Day 1	0.002
8482	Ovaries, Chicken #852, Day 4	0.051
8483	Ovaries, Chicken #881, Day 4	0.061
8484	Ovaries, Chicken #890, Day 4	0.001, <MDL
8485	Ovaries, Chicken #893, Day 4	0.061
8486	Ovaries, Chicken #895, Day 4	0.039
8487	Egg, Chicken #866, Day 4	0.010
8488	Egg, Chicken #581, Day 4	0.017
8489	Egg, Chicken #893, Day 4	0.002
8490	Egg, Chicken #895, Day 4	0.028

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM070, the determination of brodifacoum in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.001µg/g and the uncertainty (95% c.l.) is ~8%

TESTED BY: lhb **WORKBOOK REF:** 6/9
TEST PERIOD: 25-30/5/05

AUTHORISED BY:
L.H.Booth, L.E. Brown, G.R.G. Wright

Date: 31/05/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Brodifacoum concentrations detected in faeces samples, MDL 0.005 µg/g
Landcare Research toxicology laboratory report No. T2787

Toxicology Laboratory Analysis Report

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Report No: T2787

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: **Telephone No:** 325 6700
SAMPLES: 19 faeces samples
REQUIREMENT: Examine for brodifacoum
RECEIVED: 10 May 2005 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 19

LabNo.	Description	Brodifacoum, µg/g
8405	Faeces, Chicken #229. Day 1.	<MDL
8406	Faeces, Chicken #247. Day 1.	0.22
8407	Faeces, Chicken #699. Day 1.	0.21
8408	Faeces, Chicken #688. Day 1.	0.11, 0.10
8409	Faeces, Chicken #686. Day 1.	0.15
8462	Faeces, Chicken #662. Day 4. 13/5/05.	0.033
8463	Faeces, Chicken #681. Day 4. 13/5/05.	0.028
8464	Faeces, Chicken #690. Day 4. 13/5/05.	0.010
8465	Faeces, Chicken #693. Day 4. 13/5/05.	<MDL
8466	Faeces, Chicken #695. Day 4. 13/5/05.	<MDL
8501	Faeces, Chicken #238. Day 7. 16/5/05.	<MDL
8502	Faeces, Chicken #668. Day 7. 16/5/05.	<MDL
8503	Faeces, Chicken #689. Day 7. 16/5/05.	<MDL
8504	Faeces, Chicken #677. Day 7. 16/5/05.	<MDL
8505	Faeces, Chicken #685. Day 7. 16/5/05.	<MDL
8567	Faeces, Chicken #205. Day 14. 23/5/05.	<MDL
8568	Faeces, Chicken #215. Day 14. 23/5/05.	<MDL
8569	Faeces, Chicken #653. Day 14. 23/5/05.	<MDL
8570	Faeces, Chicken #700. Day 14. 23/5/05.	<MDL

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM073, the determination of coumarin rodenticides in animal tissue by HPLC. The method limit of detection is 0.1µg/g for warfarin, 0.01 µg/g for coumatetralyl and 0.005µg/g for the rest.

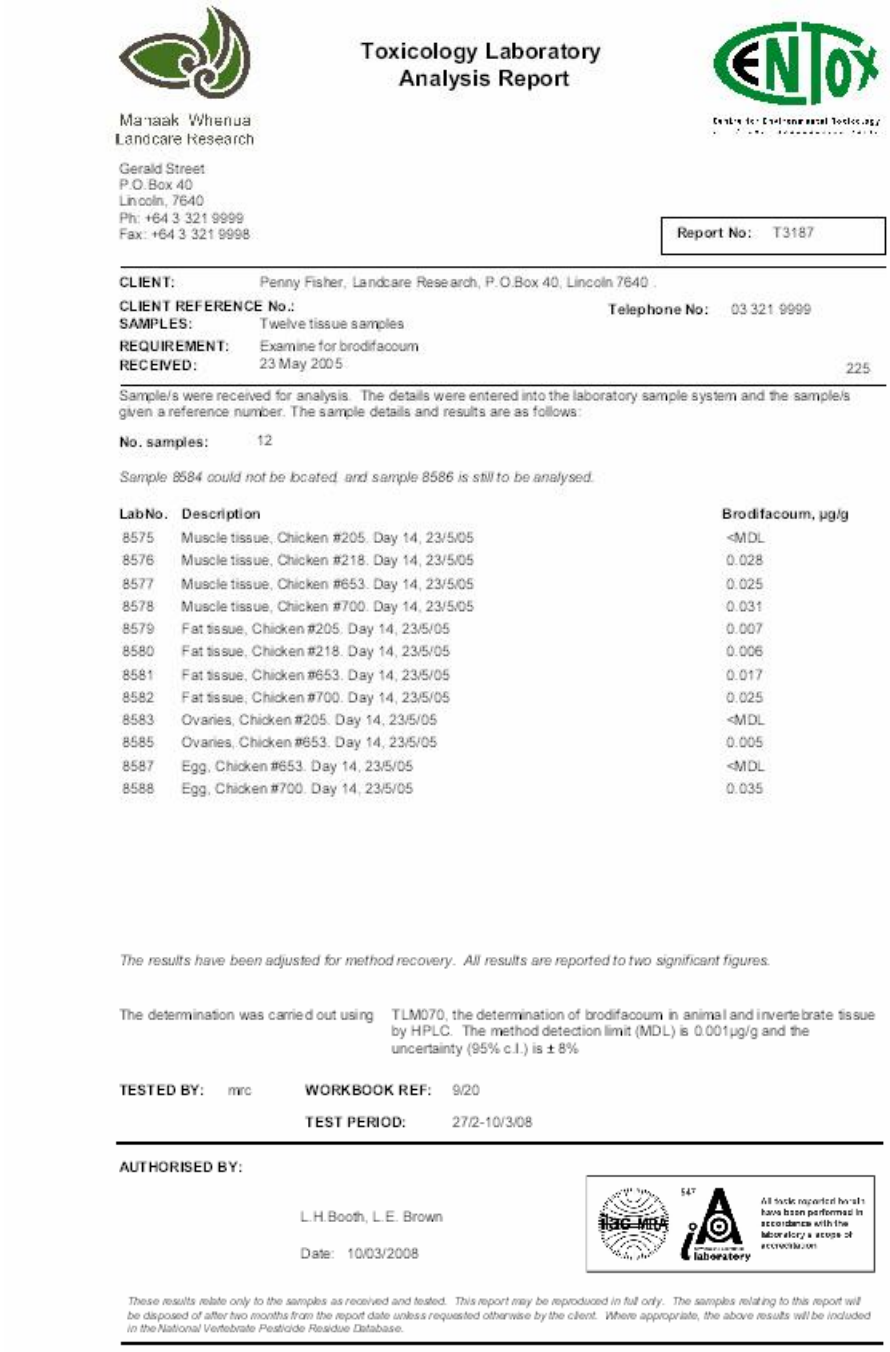
TESTED BY: leb **WORKBOOK REF:** 45/2
TEST PERIOD: 27/10-6-12/06

AUTHORISED BY: L.H.Booth, L.E. Brown

Date: 6/12/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

Brodifacoum concentrations detected in muscle, fat, ovary and egg samples MDL 0.001 µg/g. Landcare Research toxicology laboratory report No. T3187



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Toxicology Laboratory Analysis Report

Report No: T3187

CLIENT: Penny Fisher, Landcare Research, P.O.Box 40, Lincoln 7640 .
CLIENT REFERENCE No.: **Telephone No:** 03 321 9999
SAMPLES: Twelve tissue samples
REQUIREMENT: Examine for brodifacoum
RECEIVED: 23 May 2005 225

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 12

Sample 8584 could not be located, and sample 8586 is still to be analysed.

LabNo.	Description	Brodifacoum, µg/g
8575	Muscle tissue, Chicken #205. Day 14, 23/5/05	<MDL
8576	Muscle tissue, Chicken #218. Day 14, 23/5/05	0.028
8577	Muscle tissue, Chicken #653. Day 14, 23/5/05	0.025
8578	Muscle tissue, Chicken #700. Day 14, 23/5/05	0.031
8579	Fat tissue, Chicken #205. Day 14, 23/5/05	0.007
8580	Fat tissue, Chicken #218. Day 14, 23/5/05	0.006
8581	Fat tissue, Chicken #653. Day 14, 23/5/05	0.017
8582	Fat tissue, Chicken #700. Day 14, 23/5/05	0.025
8583	Ovaries, Chicken #205. Day 14, 23/5/05	<MDL
8585	Ovaries, Chicken #653. Day 14, 23/5/05	0.005
8587	Egg, Chicken #653. Day 14, 23/5/05	<MDL
8588	Egg, Chicken #700. Day 14, 23/5/05	0.035

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM070, the determination of brodifacoum in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.001µg/g and the uncertainty (95% c.l.) is ± 8%

TESTED BY: mrc **WORKBOOK REF:** 9/20
TEST PERIOD: 27/2-10/3/08

AUTHORISED BY:

L.H.Booth, L.E. Brown

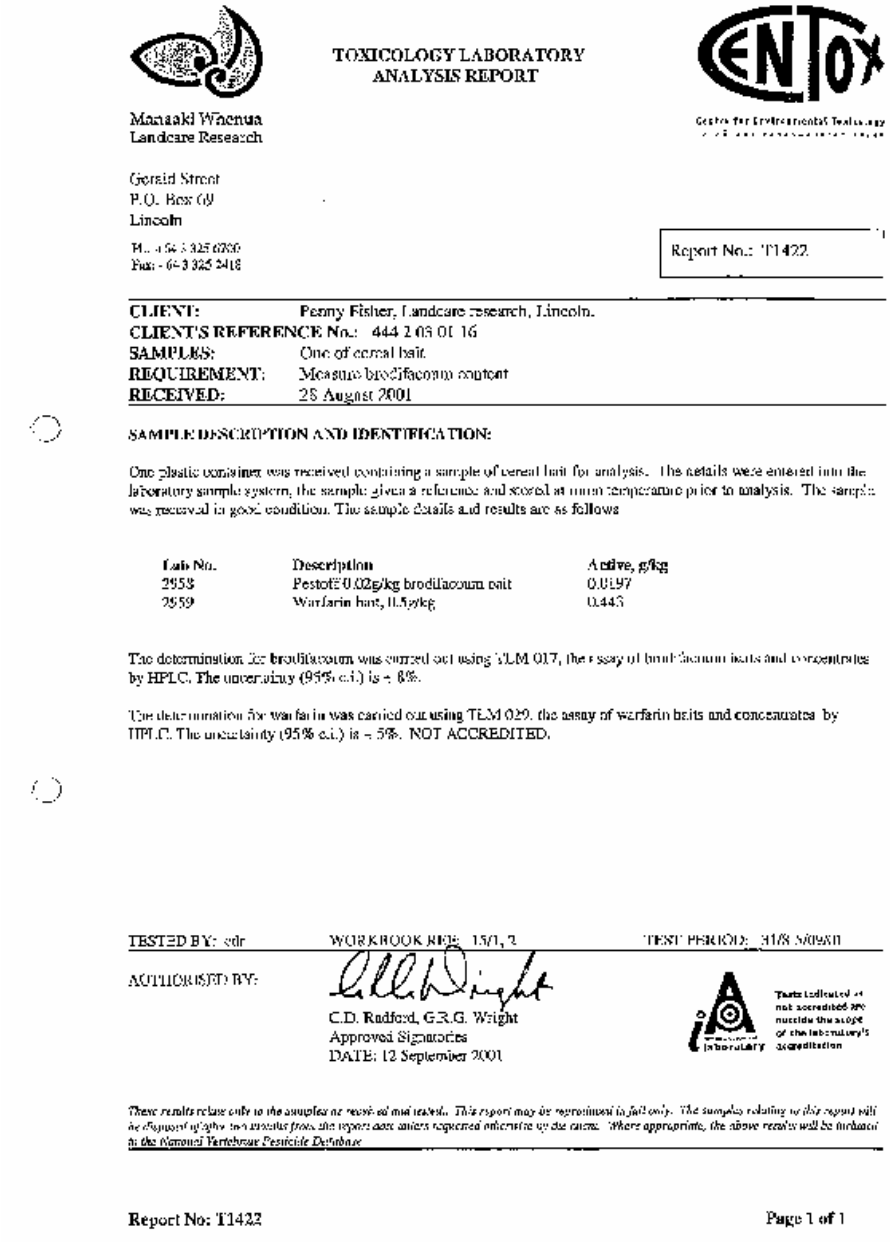
Date: 10/03/2008

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Appendix 3. Laboratory reports for rat brodifacoum liver, muscle and faeces

Concentration of brodifacoum in Pestoff® 20R rodent bait used in Trial 1, Landcare Research toxicology laboratory report T1422



TOXICOLOGY LABORATORY
ANALYSIS REPORT

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Report No.: T1422

CLIENT:	Penny Fisher, Landcare research, Lincoln.
CLIENT'S REFERENCE No.:	444 2 08 01 16
SAMPLES:	One of cereal bait.
REQUIREMENT:	Measure brodifacoum content
RECEIVED:	28 August 2001

SAMPLE DESCRIPTION AND IDENTIFICATION:

One plastic container was received containing a sample of cereal bait for analysis. The details were entered into the laboratory sample system, the sample given a reference and stored at room temperature prior to analysis. The sample was received in good condition. The sample details and results are as follows

Lab No.	Description	Active, g/kg
2958	Pestoff 0.02g/kg brodifacoum bait	0.0197
2959	Warfarin bait, 0.5g/kg	0.443

The determination for brodifacoum was carried out using TLM 017, the assay of brodifacoum baits and concentrates by HPLC. The uncertainty (95% c.i.) is ± 8%.

The determination for warfarin was carried out using TLM 029, the assay of warfarin baits and concentrates by HPLC. The uncertainty (95% c.i.) is ± 5%. NOT ACCREDITED.

TESTED BY: sdr WORKBOOK REF: 15/1, 3 TEST PERIOD: 31/8 5/09/01

AUTHORISED BY:

C.D. Radford, G.R.G. Wright
Approved Signatories
DATE: 12 September 2001

Tests indicated as not accredited are outside the scope of the laboratory's accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Database

Report No: T1422 Page 1 of 1

Concentration of brodifacoum in rat liver, Trial 1. Landcare Research toxicology laboratory report T1475

TOXICOLOGY LABORATORY ANALYSIS REPORT

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Report No.: T1475

CLIENT: Penny Fisher, Landcare Research, Lincoln.
CLIENT'S REFERENCE No.: 4 44 2 09 01 16
SAMPLES: Rat liver
REQUIREMENT: Measure brodifacoum content
RECEIVED: 4 October 2001

SAMPLE DESCRIPTION AND IDENTIFICATION:

12 plastic bags were received with samples of rat liver for analysis. The details were entered into the laboratory sample system, the samples given a reference and stored at -18 °C prior to analysis. All of the samples were received in good condition. The sample details and results are as follows:

Lab No.	Description	Brodifacoum, µg/g
3170	Rat liver #154, DOC Step 1, 20/9/01	1.7
3171	Rat liver # 155, DOC Step 1, 17/9/01	1.9
3172	Rat liver # 156, DOC Step 1, 17/9/01	1.5
3173	Rat liver # 157, DOC Step 1, 18/9/01	1.7
3174	Rat liver # 158, DOC Step 1, 17/9/01	1.9
3175	Rat liver # 159, DOC Step 1, 16/9/01	2.1
3176	Rat liver # 160, DOC Step 1, 17/9/01	1.9
3177	Rat liver # 161, DOC Step 1, 20/9/01	1.8
3178	Rat liver # 162, DOC Step 1,	2.1, 3.1
3179	Rat liver #163, DOC Step 1, 15/9/01	2.2
3180	Rat liver #164, DOC Step 1, 19/9/01	1.5
3181	Rat liver # 63, DOC Step 1, 16/9/0.	2.0

The results have been checked for the recovery of the method by simultaneously running a known reference material.

The determination was carried out using TLM 009, the determination of brodifacoum in liver tissue by HPLC. The least detectable level (LDL) is 0.02µg/g.

TESTED BY: rdr WORKBOOK REF: 16/1 TEST PERIOD: 15/10-24/10/01

AUTHORISED BY:

C.D. Radford, G.R.G. Wright
Approved Signatories
DATE: 7 November 2001

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after five months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Database.

Concentration of brodifacoum in rat muscle, Trial 1
Landcare Research toxicology laboratory report T1491

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TOXICOLOGY LABORATORY ANALYSIS REPORT

Centre for Environmental Toxicology

Report No.: T1491

CLIENT:	Penny Fisher, Landcare Research, Lincoln.
CLIENT'S REFERENCE No.:	4 44 2 03 01 16
SAMPLES:	Rat muscle
REQUIREMENT:	Measure brodifacoum content
RECEIVED:	4 October 2001

SAMPLE DESCRIPTION AND IDENTIFICATION:

12 plastic bags were received with samples of rat muscle for analysis. The details were entered into the laboratory sample system, the samples given a reference and stored at -18°C prior to analysis. All of the samples were received in good condition. The sample details and results are as follows:

Lab No.	Description	Brodifacoum, µg/g
3182	Rat muscle #154, DOC Step 1, 20/9/01	0.11
3183	Rat muscle #155, DOC Step 1, 17/9/01	0.14
3184	Rat muscle #156, DOC Step 1, 17/9/01	0.12
3185	Rat muscle #157, DOC Step 1, 19/9/01	0.14, 0.16
3186	Rat muscle #158, DOC Step 1, 17/9/01	0.20
3187	Rat muscle #159, DOC Step 1, 16/9/01	0.21
3188	Rat muscle #160, DOC Step 1, 17/9/01	0.24
3189	Rat muscle #161, DOC Step 1, 20/9/01	0.16
3190	Rat muscle #162, DOC Step 1,	0.13
3191	Rat muscle #163, DOC Step 1, 15/9/01	0.14
3192	Rat muscle #164, DOC Step 1, 19/9/01	0.17
3193	Rat muscle #165, DOC Step 1, 16/9/01	0.13

The results have been checked for the recovery of the method by simultaneously running a known reference material.

The determination was carried out using TLM 009, the determination of brodifacoum in muscle tissue by HPLC. The limit of detection level (LDL) is 0.02µg/g.

TESTED BY: cd; WORKBOOK REF: 16/19 TEST PERIOD: 15/29/11/01

AUTHORISED BY:

C.D. Radford, C.R.G. Wright
Approved Signatories
DATE: 09 November 2001

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Database.

Concentration of brodifacoum in Pestoff® 20R rodent bait used in Trials 2 & 3
Landcare Research toxicology laboratory report T1588

TOXICOLOGY LABORATORY
ANALYSIS REPORT

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Centre for Environmental Toxicology

Report No.: T1588

CLIENT:	Penny Fisher, Landcare Research, Lincoln.
CLIENT'S REFERENCE No.:	4 44 2 03 01 16
SAMPLES:	One of cereal bait
REQUIREMENT:	Measure brodifacoum content
RECEIVED:	14 March 2002

SAMPLE DESCRIPTION AND IDENTIFICATION:

One plastic container was received with a sample of cereal bait for analysis. The details were entered into the laboratory sample system, the sample given a reference and stored at room temperature prior to analysis. The sample was received in good condition. The sample details and results are as follows:

Lab No.	Description	Brodifacoum, mg/kg
3901	Pestoff Batch 01.08.17, 0.02g/kg	18.3

The results have been adjusted for the recovery of the method by simultaneously running a known reference material.

The determination was carried out using TFM 017, the assay of brodifacoum baits and concentrates by HPLC. The uncertainty (95% c.i.) is ± 8%.

TESTED BY: edr WORKBOOK REF: 18/15 TEST PERIOD: 29/4-2/05/02

AUTHORISED BY:

C.D. Radford, G.R.G. Wright
Approved Signatories
DATE: 2 May 2002

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Pesticide Residue Database.

Report No: T1588 Page 1 of 1

Concentration of brodifacoum in rat liver, Trial 2
Landcare Research toxicology laboratory report T1705

TOXICOLOGY LABORATORY ANALYSIS REPORT

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Report No.: T1705

CLIENT: Penny Fisher, Landcare Research, Lincoln.
CLIENT'S REFERENCE No.: 444209 0107
SAMPLES: 15 rat liver samples
REQUIREMENT: Examine for brodifacoum
RECEIVED: 18 April 2002

SAMPLE DESCRIPTION AND IDENTIFICATION:

15 plastic bags were received with samples of rat liver for analysis. The details were entered into the laboratory sample system, the samples given a reference and stored at -18°C prior to analysis. All of the samples were received in good condition. The sample details and results are as follows:

Lab No.	Description	Brodifacoum, µg/g
4336	Rat liver, DoC Step 2, 18/4/02, rat No.310, control	<MDL
4337	Rat liver, DoC Step 2, 18/4/02, rat No.311, control	<MDL
4338	Rat liver, DoC Step 2, 18/4/02, rat No.312, control	<MDL
4339	Rat liver, DoC Step 2, 18/4/02, rat No.313	2.9
4340	Rat liver, DoC Step 2, 18/4/02, rat No.314	5.7
4341	Rat liver, DoC Step 2, 18/4/02, rat No.315	4.5
4342	Rat liver, DoC Step 2, 18/4/02, rat No.316	6.7
4343	Rat liver, DoC Step 2, 18/4/02, rat No.317	3.2
4344	Rat liver, DoC Step 2, 18/4/02, rat No.318	5.9, 5.9
4345	Rat liver, DoC Step 2, 18/4/02, rat No.319	11
4346	Rat liver, DoC Step 2, 18/4/02, rat No.320	6.0
4347	Rat liver, DoC Step 2, 18/4/02, rat No.321	1.6
4348	Rat liver, DoC Step 2, 18/4/02, rat No.322	4.8
4349	Rat liver, DoC Step 2, 18/4/02, rat No.323	7.7
4350	Rat liver, DoC Step 2, 18/4/02, rat No.324	0.07

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM 009, the determination of brocifacoum in liver tissue by HPLC. The method detection limit (MDL) is 0.01 µg/g%) and the uncertainty (95% c.l.) is ± 30%.

TESTED BY: cd: WORKBOOK REF: 20/10, 11 TEST PERIOD: 17/7-23/08/02

AUTHORISED BY:

C.D. Radford, G.R.G. Wright
Approved Signatories
DATE: 26 August 2002

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after one month from the report date, unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Chemicals Database.

Report No: T1705 **Page 1 of 1**

Concentration of brodifacoum in rat liver, Trial 3
Landcare Research toxicology laboratory report T1751

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TOXICOLOGY LABORATORY
ANALYSIS REPORT

Report No.: T1751

CLIENT: Penny Fisher, Landcare Research.
CLIENT'S REFERENCE No.: 444 209 0107
SAMPLES: Rat liver
REQUIREMENT: Examine for brodifacoum
RECEIVED: 4 June 2002

SAMPLE DESCRIPTION AND IDENTIFICATION:

14 plastic containers were received with samples of liver for analysis. The details were entered into the laboratory sample system, the samples given a reference and stored at −18°C prior to analysis. All of the samples were received in good condition. The sample details and results are as follows:

Lab No.	Description	Brodifacoum, µg/g
4640	Rat liver, DoC step 3, 4/6/02, rat No. 385 control	0.05
4641	Rat liver, DoC step 3, 4/6/02, rat No. 386 control	0.05
4642	Rat liver, DoC step 3, 4/6/02, rat No. 387 control	0.05
4643	Rat liver, DoC step 3, 4/6/02, rat No. 388	17
4644	Rat liver, DoC step 3, 4/6/02, rat No. 389	10
4645	Rat liver, DoC step 3, 4/6/02, rat No. 390	7.7
4646	Rat liver, DoC step 3, 4/6/02, rat No. 391	6.7
4647	Rat liver, DoC step 3, 4/6/02, rat No. 392	8.7
4648	Rat liver, DoC step 3, 4/6/02, rat No. 393	24, 10
4649	Rat liver, DoC step 3, 4/6/02, rat No. 394	11
4650	Rat liver, DoC step 3, 4/6/02, rat No. 395	6.7
4651	Rat liver, DoC step 3, 4/6/02, rat No. 396	14
4652	Rat liver, DoC step 3, 4/6/02, rat No. 397	7.6
4653	Rat liver, DoC step 3, 4/6/02, rat No. 399	16

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM 009, the determination of brodifacoum in liver tissue by HPLC. The method detection limit (MDL) is 0.01 µg/g.

TESTED BY: cdc WORKBOOK REF: 218 TEST PERIOD: 8/8-24/9/02

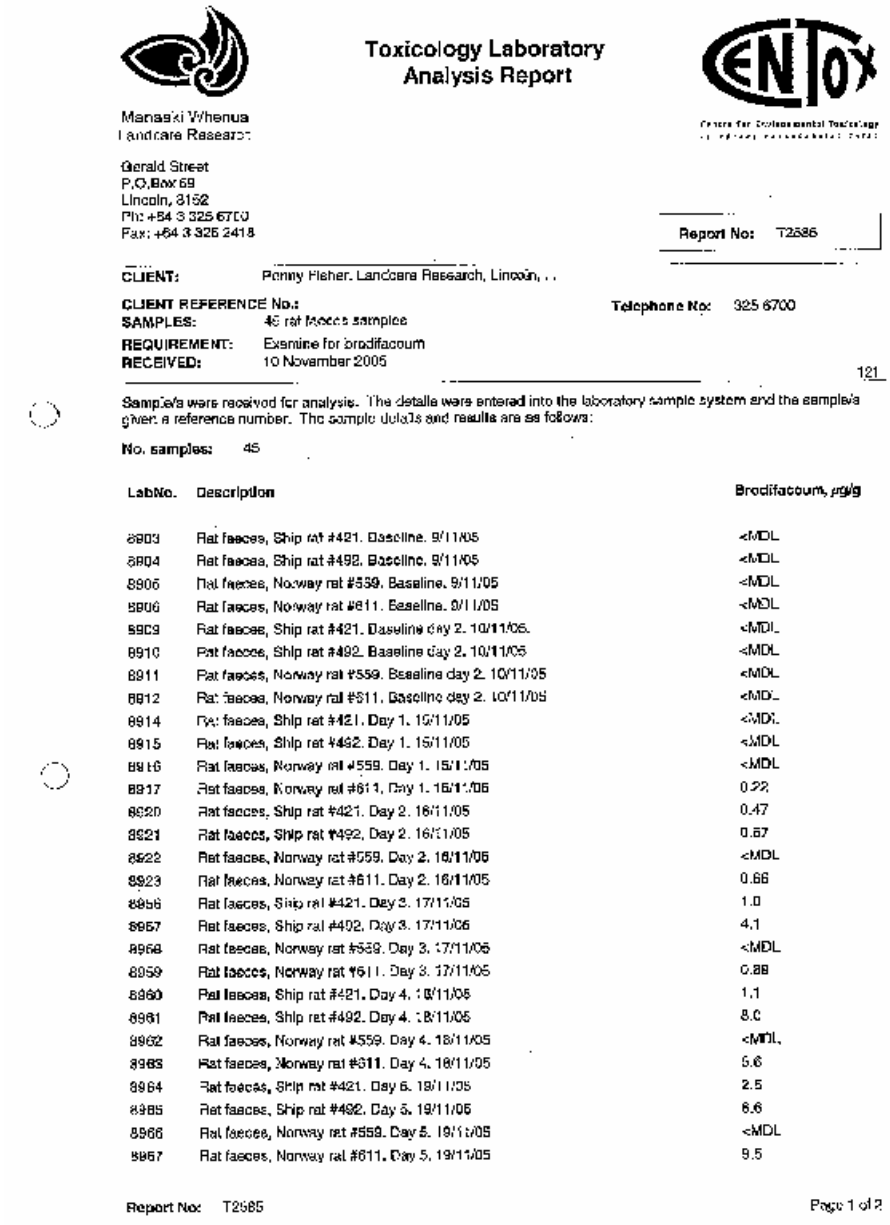
AUTHORISED BY:

C.D. Radford, G.R.G. Wright
Approved Signatories
DATE: 25 September 2002

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation.

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Database.

Concentration of brodifacoum in rat faeces. Landcare Research toxicology laboratory report T2585



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Toxicology Laboratory Analysis Report

Report No: T2585

CLIENT: Penny Fisher, Landcare Research, Lincoln, , ,

CLIENT REFERENCE No.: **Telephone No:** 325 6700

SAMPLES: 45 rat faeces samples

REQUIREMENT: Examine for brodifacoum

RECEIVED: 10 November 2005

121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 45

LabNo.	Description	Brodifacoum, µg/g
8903	Rat faeces, Ship rat #421. Baseline. 9/11/05	<MDL
8904	Rat faeces, Ship rat #492. Baseline. 9/11/05	<MDL
8905	Rat faeces, Norway rat #559. Baseline. 9/11/05	<MDL
8906	Rat faeces, Norway rat #611. Baseline. 9/11/05	<MDL
8909	Rat faeces, Ship rat #421. Baseline day 2. 10/11/05.	<MDL
8910	Rat faeces, Ship rat #492. Baseline day 2. 10/11/05	<MDL
8911	Rat faeces, Norway rat #559. Baseline day 2. 10/11/05	<MDL
8912	Rat faeces, Norway rat #611. Baseline day 2. 10/11/05	<MDL
8914	Rat faeces, Ship rat #421. Day 1. 15/11/05	<MDL
8915	Rat faeces, Ship rat #492. Day 1. 15/11/05	<MDL
8916	Rat faeces, Norway rat #559. Day 1. 15/11/05	<MDL
8917	Rat faeces, Norway rat #611. Day 1. 15/11/05	0.22
8920	Rat faeces, Ship rat #421. Day 2. 16/11/05	0.47
8921	Rat faeces, Ship rat #492. Day 2. 16/11/05	0.67
8922	Rat faeces, Norway rat #559. Day 2. 16/11/05	<MDL
8923	Rat faeces, Norway rat #611. Day 2. 16/11/05	0.66
8956	Rat faeces, Ship rat #421. Day 3. 17/11/05	1.0
8957	Rat faeces, Ship rat #492. Day 3. 17/11/05	4.1
8958	Rat faeces, Norway rat #559. Day 3. 17/11/05	<MDL
8959	Rat faeces, Norway rat #611. Day 3. 17/11/05	0.88
8960	Rat faeces, Ship rat #421. Day 4. 18/11/05	1.1
8961	Rat faeces, Ship rat #492. Day 4. 18/11/05	8.0
8962	Rat faeces, Norway rat #559. Day 4. 18/11/05	<MDL
8963	Rat faeces, Norway rat #611. Day 4. 18/11/05	5.6
8964	Rat faeces, Ship rat #421. Day 5. 19/11/05	2.5
8965	Rat faeces, Ship rat #492. Day 5. 19/11/05	6.6
8966	Rat faeces, Norway rat #559. Day 5. 19/11/05	<MDL
8967	Rat faeces, Norway rat #611. Day 5. 19/11/05	9.5

8958	Rat faeces, Ship rat #421. Day 6. 20/11/05	2.8
8959	Rat faeces, Ship rat #492. Day 6. 20/11/05	5.1
8970	Rat faeces, Norway rat #559. Day 6. 20/11/05	<MDL
8971	Rat faeces, Ship rat #421. Day 7. 21/11/05	3.0
8972	Rat faeces, Ship rat #492. Day 7. 21/11/05	3.8
8973	Rat faeces, Norway rat #559. Day 7. 21/11/05	<MDL
8976	Rat faeces, Ship rat #421. Day 8. 22/11/05	3.2
8977	Rat faeces, Ship rat #492. Day 8. 22/11/05	12.6
8978	Rat faeces, Norway rat #559. Day 8. 22/11/05	<MDL
8980	Rat faeces, Ship rat #421. Day 9. 23/11/05	2.2
8981	Rat faeces, Norway rat #559. Day 9. 23/11/05	<MDL
8982	Rat faeces, Ship rat #421. Day 10. 24/11/05	2.1
8983	Rat faeces, Norway rat #559. Day 10. 24/11/05	<MDL
8984	Rat faeces, Ship rat #421. Day 11. 25/11/05	2.7
8985	Rat faeces, Norway rat #559. Day 11. 26/11/05	<MDL
8986	Rat faeces, Ship rat #421. Day 12-13. 26-27/11/05	3.4
8987	Rat faeces, Norway rat #559. Day 12-13. 26-27/11/05	<MDL

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM017, the assay of brodifacoum baits and concentrates by HPLC. The method uncertainty (95% c.l.) is ± 7%.

TESTED BY: lnb **WORKBOOK REF** 2/18, 3/15-17, 3/18-20

TEST PERIOD: 13/12/05-1/3/06

AUTHORISED BY:

L.H. Booth, L.E. Brown, G.R.G. Wright

Date: 2/03/2006

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Appendix 4. Raw data and laboratory reports for diphacinone in pig tissues

Raw coagulation time data from pig plasma samples in Trial 1 (100% / undiluted plasma): 2.5 mg/kg x 3 days treatment group

Dose group (mg/kg)	Pig ID	Sex	Sample day post dosing	Date sampled	Date tested	Replicate	PT (s)	APTT (s)
2.5 mg/kg/d * 3	27	F	0 (baseline)	20/08/2004	20/08/2004	1	13.7	33.4
2.5 mg/kg/d * 3	27	F	0 (baseline)	20/08/2004	20/08/2004	2	13.7	33.2
2.5 mg/kg/d * 3	27	F	2	1/09/2004	2/09/2004	1	47	97.9
2.5 mg/kg/d * 3	27	F	2	1/09/2004	2/09/2004	2	58	91.6
2.5 mg/kg/d * 3	27	F	7	6/09/2004		1	15.8	32.1
2.5 mg/kg/d * 3	27	F	7	6/09/2004		2	16.8	43.5
2.5 mg/kg/d * 3	27	F	14	13/09/2004		1	15.5	31.7
2.5 mg/kg/d * 3	27	F	14	13/09/2004		2	15.3	41.8
2.5 mg/kg/d * 3	26	F	0 (baseline)	20/08/2004	20/08/2004	1	14.4	31.2
2.5 mg/kg/d * 3	26	F	0 (baseline)	20/08/2004	20/08/2004	2	14.8	insufficient sample
2.5 mg/kg/d * 3	26	F	2	1/09/2004	2/09/2004	1	63	103.3
2.5 mg/kg/d * 3	26	F	2	1/09/2004	2/09/2004	2	69.4	105.4
2.5 mg/kg/d * 3	26	F	7	6/09/2004		1	25.3	41.2
2.5 mg/kg/d * 3	26	F	7	6/09/2004		2	25.1	45.3
2.5 mg/kg/d * 3	26	F	14	13/09/2004		1	15.1	34.7
2.5 mg/kg/d * 3	26	F	14	13/09/2004		2	15.2	51.6
2.5 mg/kg/d * 3	44	M	0 (baseline)	20/08/2004	20/08/2004	1	15.7	34.5
2.5 mg/kg/d * 3	44	M	0 (baseline)	20/08/2004	20/08/2004	2	15.6	insufficient sample
2.5 mg/kg/d * 3	44	M	2	1/09/2004	2/09/2004	1	61.2	124.9
2.5 mg/kg/d * 3	44	M	2	1/09/2004	2/09/2004	2	64	88.1
2.5 mg/kg/d * 3	44	M	7	6/09/2004		1	15.8	33.5
2.5 mg/kg/d * 3	44	M	7	6/09/2004		2	16.4	39

2.5 mg/kg/d * 3	44	M	14	13/09/2004		1	16.4	64.1
2.5 mg/kg/d * 3	44	M	14	13/09/2004		2	16.6	53.1

b) Raw coagulation time data from pig plasma samples in Trial 1 (100% / undiluted plasma): 0.5 mg/kg x 5 days treatment group

Dose group (mg/kg)	Pig ID	Sex	Sample day post dosing	Date sampled	Date tested	Replicate	PT (s)	APTT (s)
05 mg/kg/d x5	45	M	0 (baseline)	20/08/2004	20/08/2004	1	14.3	28.4
05 mg/kg/d x5	45	M	0 (baseline)	20/08/2004	20/08/2004	2		
05 mg/kg/d x5	45	M	2	1/09/2004	2/09/2004	1	74.4	114.2
05 mg/kg/d x5	45	M	2	1/09/2004	2/09/2004	2	77.1	120.5
05 mg/kg/d x5	45	M	6	5/09/2004	6/09/2004	1	322.7	176.6
05 mg/kg/d x5	45	M	6	5/09/2004	6/09/2004	2	299.8	123.1
05 mg/kg/d x5	42	M	0 (baseline)	20/08/2004	20/08/2004	1	13.9	na
05 mg/kg/d x5	42	M	0 (baseline)	20/08/2004	20/08/2004	2	14.3	
05 mg/kg/d x5	42	M	2	1/09/2004	2/09/2004	1	95.9	57.7
05 mg/kg/d x5	42	M	2	1/09/2004	2/09/2004	2		170.6
05 mg/kg/d x5	42	M	7	6/09/2004		1	16.8	40.8
05 mg/kg/d x5	42	M	7	6/09/2004		2	16.8	40.4
05 mg/kg/d x5	42	M	14	13/09/2004		1	15.9	45.3
05 mg/kg/d x5	42	M	14	13/09/2004		2	15.9	32.4
05 mg/kg/d x5	24	F	0 (baseline)	20/08/2004	20/08/2004	1	15.6	35
05 mg/kg/d x5	24	F	0 (baseline)	20/08/2004	20/08/2004	2	16.4	
05 mg/kg/d x5	24	F	2	1/09/2004	2/09/2004	1	67.5	91
05 mg/kg/d x5	24	F	2	1/09/2004	2/09/2004	2	61.6	85.6

c) Raw coagulation time data from pig plasma samples in Trial 1 (100% / undiluted plasma): 12.5 mg/kg treatment group

Dose group (mg/kg)	Pig ID	Sex	Sample day post dosing	Date sampled	Date tested	Replicate	PT (s)	APTT (s)
12.5	25	F	0 (baseline)	20/08/2004	20/08/2004	1	13.4	37.6
12.5	25	F	0 (baseline)	20/08/2004	20/08/2004	2	15.2	34.4

12.5	25	F	2	1/09/2004	2/09/2004	1	56.7	92.7
12.5	25	F	2	1/09/2004	2/09/2004	2	59.6	94.6
12.5	25	F	7	6/09/2004		1	16.2	48
12.5	25	F	7	6/09/2004		2	16.4	47.4
12.5	25	F	14	13/09/2004		1	15	40.7
12.5	25	F	14	13/09/2004		2	14.8	42
12.5	28	F	0 (baseline)	20/08/2004	20/08/2004	1	na	na
12.5	28	F	0 (baseline)	20/08/2004	20/08/2004	2	na	na
12.5	28	F	2	1/09/2004	2/09/2004	1	42.3	91.8
12.5	28	F	2	1/09/2004	2/09/2004	2	45.4	105.9
12.5	28	F	7	6/09/2004		1	15.7	47.7
12.5	28	F	7	6/09/2004		2	16.2	48.1
12.5	28	F	14	13/09/2004		1	-	35.2
12.5	28	F	14	13/09/2004		2	15.3	37.4
12.5	43	M	0 (baseline)	20/08/2004	20/08/2004	1	16.6	31.8
12.5	43	M	0 (baseline)	20/08/2004	20/08/2004	2	na	32.3
12.5	43	M	2	1/09/2004	2/09/2004	1	82	101.6
12.5	43	M	2	1/09/2004	2/09/2004	2	77.8	118.1
12.5	43	M	7	6/09/2004		1	17.2	45.5
12.5	43	M	7	6/09/2004		2	18	45.2
12.5	43	M	14	13/09/2004		1	16.4	31.9
12.5	43	M	14	13/09/2004		2	16.8	30

Raw coagulation time data from pig plasma samples in Trial 1 (100% / undiluted plasma): 2.5 mg/kg treatment group

Dose group (mg/kg)	**Pig ID**	**Sex**	**Sample day post dosing**	**Date sampled**	**Date tested**	**Replicate**	**PT (s)**	**APTT (s)**
2.5 mg/kg single	41	M	0 (baseline)	20/08/2004	20/08/2004	1	16.6	35.8
2.5 mg/kg single	41	M	0 (baseline)	20/08/2004	20/08/2004	2		
2.5 mg/kg single	41	M	2	1/09/2004	2/09/2004	1	39.4	89.9

2.5 mg/kg single	41	M	2	1/09/2004	2/09/2004	2	39.5	72.4
2.5 mg/kg single	41	M	7	6/09/2004		1	15.3	31.8
2.5 mg/kg single	41	M	7	6/09/2004		2	15.6	33.5
2.5 mg/kg single	41	M	14	13/09/2004		1	17.3	27.2
2.5 mg/kg single	41	M	14	13/09/2004		2	17.1	33.6
2.5 mg/kg single	23	F	0 (baseline)	20/08/2004	20/08/2004	1	13.3	32.3
2.5 mg/kg single	23	F	0 (baseline)	20/08/2004	20/08/2004	2	13.8	35.5
2.5 mg/kg single	23	F	2	1/09/2004	2/09/2004	1	66.7	121.8
2.5 mg/kg single	23	F	2	1/09/2004	2/09/2004	2	66.7	110.6
2.5 mg/kg single	23	F	7	6/09/2004		1	15.9	36
2.5 mg/kg single	23	F	7	6/09/2004		2	16.2	45.3
2.5 mg/kg single	23	F	14	13/09/2004		1	15.8	37.9
2.5 mg/kg single	23	F	14	13/09/2004		2	15.6	36.9
2.5 mg/kg single	40	M	0 (baseline)	20/08/2004	20/08/2004	1		
2.5 mg/kg single	40	M	0 (baseline)	20/08/2004	20/08/2004	2		
2.5 mg/kg single	40	M	2	1/09/2004	2/09/2004	1	34	88.8
2.5 mg/kg single	40	M	2	1/09/2004	2/09/2004	2	30.1	63.4
2.5 mg/kg single	40	M	7	6/09/2004		1	17.1	26.5
2.5 mg/kg single	40	M	7	6/09/2004		2	17.6	32.3
2.5 mg/kg single	40	M	14	13/09/2004		1	-	47.8
2.5 mg/kg single	40	M	14	13/09/2004		2	16.9	48.3

Concentration of diphacinone in pig liver, trial 1
Landcare Research toxicology laboratory report T2262

Toxicology Laboratory Analysis Report

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Report No: T2262

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: 444208 0136 **Telephone No:** 325 6700
SAMPLES: Twelve of pig liver
REQUIREMENT: Examine for diphacinone
RECEIVED: 7, 7, 15, 29/9/04 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 12

LabNo.	Description	Diphacinone, µg/g
7693	Liver tissue, pig #24, sample (1)	0.70
7698	Liver tissue, pig #45, sample (1)	0.12
7929	Liver tissue, pig #26, 15/9/04	0.45
7930	Liver tissue, pig #27, 15/9/04	0.30
7931	Liver tissue, pig #28, 15/9/04	0.18
7932	Liver tissue, pig #40, 15/9/04	0.58
7933	Liver tissue, pig #41, 15/9/04	0.59
7934	Liver tissue, pig #43, 15/9/04	0.41
8012	Liver tissue, pig #23, 15/9/04	0.40
8013	Liver tissue, pig #25, 15/9/04	0.40
8014	Liver tissue, pig #42, 15/9/04	0.71
8015	Liver tissue, pig #44, 15/9/04	0.30

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using **TLM007**, the determination of diphacinone in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.02µg/g for liver, and the uncertainty (95% c.l.) is ~30%.

TESTED BY: lab **WORKBOOK REF:** 39/7
TEST PERIOD: 24/11-3/12/04

AUTHORISED BY: L.E. Brown, G.R.G. Wright
Date: 8/12/2004

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Pesticide Residue Database.

Concentration of diphacinone in pig muscle, trial 1
Landcare Research toxicology laboratory report T2508

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Toxicology Laboratory Analysis Report

Report No: T2508

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: **Telephone No:** 325 6700
SAMPLES: Seven samples of pig muscle tissue
REQUIREMENT: Examine for diphacinone
RECEIVED: 15 September 2004 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 7

LabNo.	Description	Diphacinone, µg/g
7835	Muscle tissue, #27, 15/9/04	<MDL
7837	Muscle tissue, #40, 15/9/04	<MDL
7838	Muscle tissue, #41, 15/9/04	<MDL
8016	Muscle tissue, #23, 15/9/04	<MDL
8018	Muscle tissue, #26, 15/9/04	<MDL
8019	Muscle tissue, #42, 15/9/04	<MDL
8020	Muscle tissue, #44, 15/9/04	<MDL

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM067, the determination of diphacinone in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.02µg/g for liver, 0.2µg/g for inverts. The uncertainty (95% c.l.) is ± 20%.

TESTED BY: leh **WORKBOOK REF:** 42/17
TEST PERIOD: 28/10-3/11/05

AUTHORISED BY:
L.E. Brown, G.R.G. Wright
Date: 9/11/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Concentration of diphacinone in pig liver, trial 2
Landcare Research toxicology laboratory report T2299

Toxicology Laboratory Analysis Report

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Report No: T2299

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: 444209 0135 **Telephone No:** 325 6700
SAMPLES: Twelve samples of pig liver
REQUIREMENT: Examine for diphacinone
RECEIVED: 25, 28 January, 3 February 2005 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 12

Blanks and spikes were tested at the same time. The recovery for spikes was 102%.

LabNo.	Description	Diphacinone, µg/g
8116	Liver tissue, pig #7, day 1, 25/1/05	3.2
8120	Liver tissue, pig #12, day 1, 25/1/05	2.8
8124	Liver tissue, pig #2, day 1, 25/1/05	2.8
8128	Liver tissue, pig #4, day 1, 25/1/05	2.4
8139	Liver tissue, pig #11, day 4, 28/1/05	0.45
8143	Liver tissue, pig #6, day 4, 28/1/05	1.5
8147	Liver tissue, pig #3, day 4, 28/1/05	0.27
8151	Liver tissue, pig #8, day 4, 28/1/05	0.59
8164	Liver tissue, pig #1, day 10, 3/2/05	0.75
8169	Liver tissue, pig #5, day 10, 3/2/05	0.61
8174	Liver tissue, pig #10, day 10, 3/2/05	0.53
8179	Liver tissue, pig #9, day 10, 3/2/05	0.59

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM367, the determination of diphacinone in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.02µg/g for liver, and the uncertainty (95% c.l.) is ± 20%.

TESTED BY: leb **WORKBOOK REF:** 39/
TEST PERIOD: 16-21/2/05

AUTHORISED BY:
L.E. Brown, G.R.G. Wright
Date: 1/03/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after ten months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Concentration of diphacinone in pig plasma, trials 1 and 2
Landcare Research toxicology laboratory report T2509 & T2329

Toxicology Laboratory Analysis Report

Manaaki Whenua
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Report No: T2509

CLIENT: Penny Fisher, Landcare Research, Lincoln, --
CLIENT REFERENCE No.: - **Telephone No:** 325 6700
SAMPLES: Six plasma samples
REQUIREMENT: Examine for diphacinone
RECEIVED: 28 January, 3 February 2005 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 8

LabNo.	Description	Diphacinone, µg/mL
8157	Plasma, pig #8, Day 4, 21/1/05	0.038
8158	Plasma, pig #11, Day 4, 21/1/05	0.092
8168	Plasma, pig #1, Day 10, 3/2/05	<MDL
8173	Plasma, pig #5, Day 10, 3/2/05	0.027
8176	Plasma, pig #10, Day 10, 3/2/05	<MDL
8183	Plasma, pig #9, Day 10, 3/2/05	0.020

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM067, the determination of diphacinone in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.02µg/g for liver. 0.2µg/g for inverts. The uncertainty (95% c.l.) is ± 20%.

TESTED BY: lab **WORKBOOK REF:** 43/17
TEST PERIOD: 28/10-3/11/05

AUTHORISED BY: L.E. Brown, G.R.G. Wright

Date: 9/11/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation.

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Toxicology Laboratory Analysis Report

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Report No: T2329

CLIENT: Penny Fisher, Landcare Research, Lincoln, --

CLIENT REFERENCE No.: 444209 0195 **Telephone No:** 325 6700

SAMPLES: 18 pig plasma samples

REQUIREMENT: Examine for diphacinone

RECEIVED: 25, 28/1, 30/3/05 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 18

LabNo.	Description	Diphacinone, µg/mL
8132	Plasma, pig #7, day 1, 25/1/05	2.4
8133	Plasma, pig #12, day 1, 25/1/05	2.6
8134	Plasma, pig #2, day 1, 25/1/05	2.1
8135	Plasma, pig #4, day 1, 25/1/05	2.1
8155	Plasma, pig #3, day 4, 28/1/05	0.26
8156	Plasma, pig #6, day 4, 28/1/05	<MDL
8339	Plasma, pig #3, day 0, 20/8/04	<MDL
8340	Plasma, pig #3, day 2, 1/9/04	<MDL
8341	Plasma, pig #3, day7, 6/9/04	<MDL
8342	Plasma, pig #3, day 14, 13/9/04	<MDL
8343	Plasma, pig #1, day 0, 20/8/04	<MDL
8344	Plasma, pig #1, day 2, 1/9/04	<MDL
8345	Plasma, pig #1, day 7, 6/9/04	<MDL
8346	Plasma, pig #1, day 14, 13/9/04	<MDL
8347	Plasma, pig #2, day 0, 20/8/04	<MDL
8348	Plasma, pig #2, day 2, 1/9/04	0.28
8349	Plasma, pig #2, day 7, 6/9/04	<MDL
8350	Plasma, pig #2, day 14, 13/9/04	<MDL

Concentration of diphacinone in pig kidney, trial 2
Landcare Research toxicology laboratory report T2335 & T2330

Toxicology Laboratory Analysis Report

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Report No: T2335

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: 444209 0135 **Telephone No:** 325 6700
SAMPLES: Six samples of tissue
REQUIREMENT: Examine for diphacinone
RECEIVED: 25/1, 3/2/05 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 6

LabNo.	Description	Diphacinone, µg/g
8148	Kidney tissue, pig #5, day4, 28/1/05	0.15
8152	Kidney tissue, pig #3, day4, 28/1/05	0.12
8166	Kidney tissue, pig #1, day10, 3/2/05	0.07
8171	Kidney tissue, pig #5, day10, 3/2/05	0.11
8176	Kidney tissue, pig #10, day10, 3/2/05	0.07
8181	Kidney tissue, pig #9, day10, 3/2/05	0.078

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM067, the determination of diphacinone in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.02µg/g for liver, 0.2µg/g for inverts. The uncertainty (95% c.l.) is ± 20%.

TESTED BY: leb **WORKBOOK REF:** 39/13
TEST PERIOD: 5-11/4/05

AUTHORISED BY: L.E. Brown, G.R.G. Wright
Date: 12/04/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Toxicology Laboratory Analysis Report

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Report No: T2330

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .

CLIENT REFERENCE No.: 444208 0135 **Telephone No:** 325 6700

SAMPLES: Six samples of pig kidney

REQUIREMENT: Examine for diphacinone

RECEIVED: 26, 29/1/05 121

Samples were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 6

LabNo.	Description	Diphacinone, µg/g
8118	Kidney tissue, pig #7, day 1, 25/1/05	1.8
8122	Kidney tissue, pig #12, day 1, 25/1/05	1.5
8126	Kidney tissue, pig #2, day 1, 25/1/05	1.4
8130	Kidney tissue, pig #4, day 1, 25/1/05	1.2
8140	Kidney tissue, pig #11, day 4, 28/1/05	0.26
8144	Kidney tissue, pig #8, day 4, 28/1/05	0.074

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TJM067, the determination of diphacinone in animal and invertebrate tissue by H.P.L.C. The method detection limit (MDL) is 0.02µg/g for liver, 0.2µg/g for invers. The uncertainty (95% c.i.) is ± 20%.

TESTED BY: leb **WORKBOOK REF:** 58/12

TEST PERIOD: 1-4/4/05

AUTHORISED BY: L.E. Brown, G.R.G. Wright

Date: 5/04/2005

A tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Concentration of diphacinone in pig muscle & fat, trial 2
Landcare Research toxicology laboratory report T2313

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Toxicology Laboratory Analysis Report

Centre for Environmental Toxicology

Report No: T2313

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: 444209 0135
Telephone No: 325 6700
SAMPLES: Pig muscle and fat
REQUIREMENT: Examine for diphacinone
RECEIVED: 25, 28 Jan, 3 Feb 2005

121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 27

Muscle recoveries averaged 87%, and fat recoveries averaged 103%. Fat blanks were <MDL.

LabNo.	Description	Diphacinone, µg/g
7836	Muscle tissue, Pig #28, 15/9/04	<MDL
7838	Muscle tissue, Pig #48, 15/9/04	<MDL
8007	Muscle tissue, Pig #26, 15/9/04	<MDL, <MDL
8117	Muscle tissue, Pig #7, Day 1, 25/1/05	0.36
8119	Fat, Pig #7, Day 1, 25/1/05	0.60
8121	Muscle tissue, Pig #12, Day 1, 25/1/05	0.29
8123	Fat, Pig #12, Day 1, 25/1/05	0.44
8125	Muscle tissue, Pig #2, Day 1, 25/1/05	0.22
8127	Fat, Pig #2, Day 1, 25/1/05	0.49
8129	Muscle tissue, Pig #4, Day 1, 25/1/05	0.37
8131	Fat, Pig #4, Day 1, 25/1/05	0.38
8141	Muscle tissue, Pig #11, Day 4, 28/1/05	0.10
8142	Fat, Pig #11, Day 4, 28/1/05	0.088
8145	Muscle tissue, Pig #8, Day 4, 28/1/05	0.049
8146	Fat, Pig #8, Day 4, 28/1/05	0.042
8149	Muscle tissue, Pig #3, Day 4, 28/1/05	0.049, 0.17
8150	Fat, Pig #3, Day 4, 28/1/05	0.046
8153	Muscle tissue, Pig #6, Day 4, 28/1/05	0.13
8154	Fat, Pig #6, Day 4, 28/1/05	0.037
8165	Muscle tissue, Pig #1, Day 10, 3/2/05	0.10
8167	Fat, Pig #1, Day 10, 3/2/05	0.025
8170	Muscle tissue, Pig #5, Day 10, 3/2/05	0.12
8172	Fat, Pig #5, Day 10, 3/2/05	0.028
8175	Muscle tissue, Pig #10, Day 10, 3/2/05	0.081
8177	Fat, Pig #10, Day 10, 3/2/05	<MDL
8180	Muscle tissue, Pig #9, Day 10, 3/2/05	0.10, 0.22
8182	Fat, Pig #9, Day 10, 3/2/05	<MDL

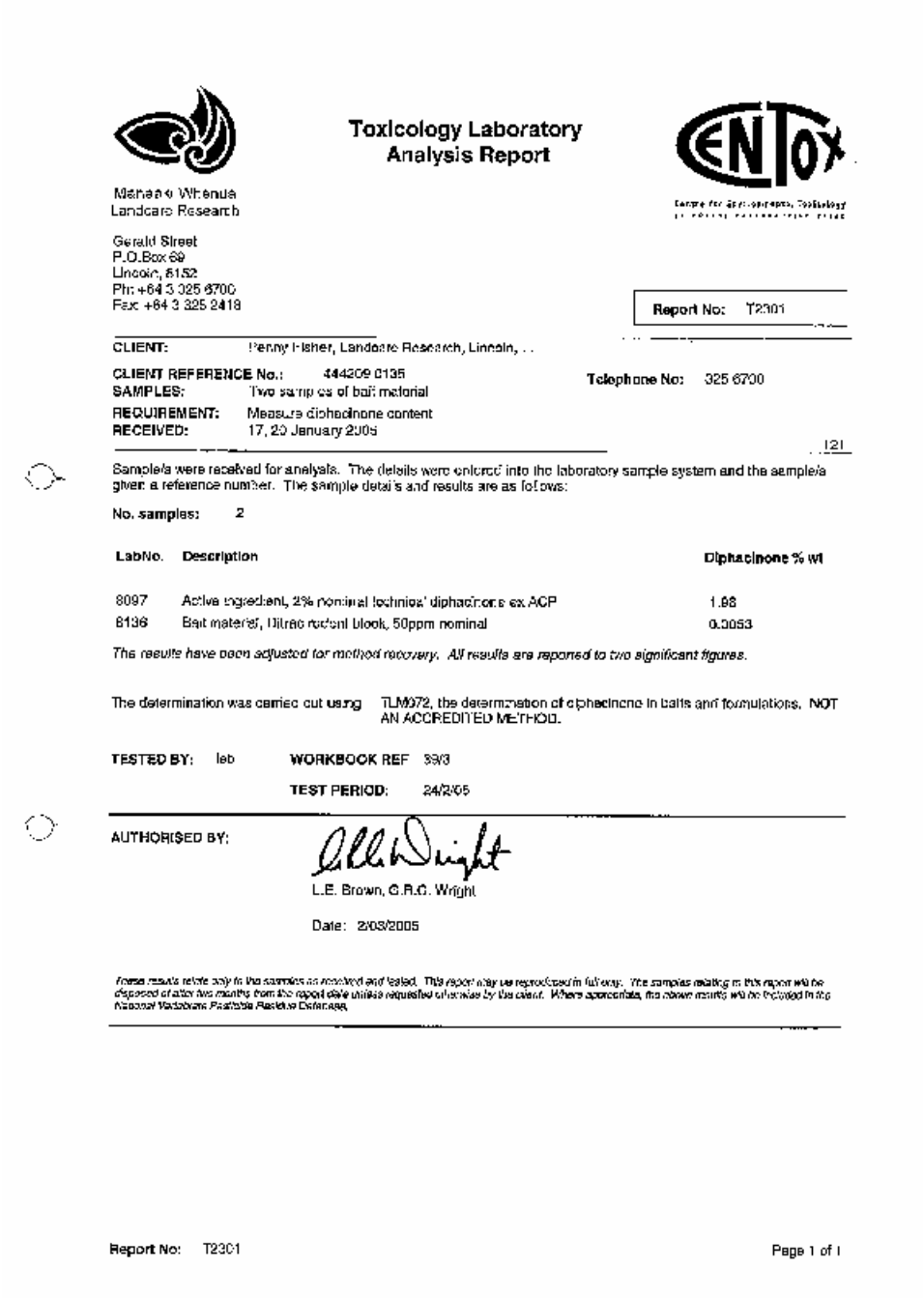
Table. Diphacinone residues detected over time in pig (*Sus scrofa*) liver, muscle, fat, kidney and plasma following a single oral exposure of 12.5 mg/kg. Samples taken at 'day 15' are from the three pigs dosed with 12.5 mg/kg diphacinone in Trial 1. F = female; M = male.

Pig ID /sex	Day post dosing	Diphacinone concentration in tissue (µg/g)				
		Liver	**Muscle**	**Fat**	**Kidney**	**Plasma**
400/F	1	2.45	0.37	0.38	1.2	2.1
395/M	1	2.81	0.22	0.43	1.4	2.1
398/F	1	2.82	0.29	0.44	1.5	2.6
393/M	1	3.22	0.36	0.6	1.8	2.4
392/M	4	0.45	0.1	0.088	0.26	0.092
397/F	4	1.46	0.043	0.042	0.07	0.02
396/M	4	0.27	0.17	0.046	0.15	0.26
351/F	4	0.59	0.13	0.037	0.12	0.04
352/F	10	0.59	0.2	< MDL[a]	0.08	0.02
394/M	10	0.53	0.081	< MDL[a]	0.07	0.02
339/F	10	0.61	0.12	0.028	0.11	0.03
391/M	10	0.75	0.1	0.025	0.07	0.02
28/F	15	0.18	< MDL[b]	-	-	-
43/M	15	0.41	< MDL[b]	-	-	-
25/F	15	0.4	< MDL[b]	-	-	-

[a] Method Detection Limit for diphacinone in fat = 0.02 µg/g.
[b] Method Detection Limit for diphacinone in muscle = 0.02 µg/g.

Appendix 5. Laboratory reports for weta/diphacinone trials

Concentration of diphacinone in Ditrac rodent block
Landcare Research toxicology laboratory report T2301



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Toxicology Laboratory Analysis Report

ENTOX

Report No: T2301

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .

CLIENT REFERENCE No.: 444209 0135
Telephone No: 325 6700
SAMPLES: Two samples of bait material
REQUIREMENT: Measure diphacinone content
RECEIVED: 17, 20 January 2005

121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 2

LabNo.	Description	Diphacinone % wt
8097	Active ingredient, 2% nominal technical diphacinone ex ACP	1.98
8136	Bait material, Ditrac rodent block, 50ppm nominal	0.0053

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM072, the determination of diphacinone in baits and formulations. NOT AN ACCREDITED METHOD.

TESTED BY: leb **WORKBOOK REF** 99/3
TEST PERIOD: 24/2/05

AUTHORISED BY:

L.E. Brown, G.R.C. Wright

Date: 2/03/2005

These results relate only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, this above month will be followed by the National Pesticides Available Residue Database.

Report No: T2301 Page 1 of 1

Concentration of diphacinone in whole bodies of weta, Days 1, 4, 8, 16 and controls
Landcare Research toxicology laboratory report T2318

Toxicology Laboratory Analysis Report

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Report No: T2318

CLIENT: Penny Fisher, Landcare Research, Lincoln, . .
CLIENT REFERENCE No.: 414008.0307
SAMPLES: 18 samples of weta
Telephone No: 325 6700
REQUIREMENT: Examine for diphacinone
RECEIVED: 22, 25/2, 1, 9, 14/3/05

121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 18

Spikes in 8283, 8289, 8285 gave recoveries of 86%, 82, and 76%. A positive in 8285, was not apparent when the sample was rerun.

LabNo.	Description	Diphacinone, µg/g
8200	Invertebrate, Weta #6, day 1, 22/2/05	3.8
8201	Invertebrate, Weta #11, day 1, 22/2/05	3.9
8202	Invertebrate, Weta #20, day 1, 22/2/05	1.8
8203	Invertebrate, Weta #29, day 1, 22/2/05	5.5
8206	Invertebrate, Weta #4, day 4, 25/2/05	5.9
8207	Invertebrate, Weta #10, day 4, 25/2/05	2.7
8208	Invertebrate, Weta #17, day 4, 25/2/05	5.4
8209	Invertebrate, Weta #27, day 4, 25/2/05	5.4
8230	Invertebrate, Weta #2, day 8, 1/3/05	3.3
8238	Invertebrate, Weta #22, day 8, 1/3/05	<MDL
8239	Invertebrate, Weta #32, day 8, 1/3/05	6.2
8279	Invertebrate, Weta #7, day 16, 9/3/05	1.9
8280	Invertebrate, Weta #15, day 16, 9/3/05	1.6
8281	Invertebrate, Weta #23, day 16, 9/3/05	1.3
8282	Invertebrate, Weta #26, day 16, 9/3/05	3.3
8283	Invertebrate, Weta #1, control, 15/12/04	<MDL, <MDL
8284	Invertebrate, Weta #9, control, 4/1/05	<MDL
8285	Invertebrate, Weta #24, control, 15/2/05	0.59, <MDL

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using **TLM067**, the determination of diphacinone in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.02µg/g for liver, 0.2µg/g for inverts. The uncertainty (95% c.i.) is ± 20%.

TESTED BY: rwh **WORKBOOK REF:** 1/2
TEST PERIOD: 15-29/3/05

AUTHORISED BY: L.E. Brown, G.R.G. Wright
Date: 29/03/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation

These results apply only to the samples as received and tested. This report may be reproduced in full only. The samples relating to this report will be disposed of after two months from the report date unless requested otherwise by the client. Where appropriate, the above results will be included in the National Vertebrate Pesticide Residue Database.

Concentration of diphacinone in whole bodies of weta, remaining samples
Landcare Research toxicology laboratory report T2351

Toxicology Laboratory Analysis Report

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Report No: T2351

CLIENT: Penny Fisher, Landcare Research, Lincoln, , ,
CLIENT REFERENCE No.: 444008 0307 **Telephone No:** 325 6700
SAMPLES: 9 weta samples
REQUIREMENT: Examine for diphacinone
RECEIVED: 23/9, 26/4/05 121

Sample/s were received for analysis. The details were entered into the laboratory sample system and the sample/s given a reference number. The sample details and results are as follows:

No. samples: 9

LabNo.	Description	Diphacinone, µg/g
8325	Invertebrate, weta, #10, day 31, 24/3/05	1.7, 1.9
8326	Invertebrate, weta, #14, day 31, 24/3/05	1.5
8327	Invertebrate, weta, #25, day 31, 24/3/05	1.2
8330	Invertebrate, weta, #31, day 31, 24/3/05	1.9
8364	Invertebrate, weta, #3, day 64, 26/4/05	0.20
8365	Invertebrate, weta, #8, day 64, 26/4/05	1.8
8366	Invertebrate, weta, #16, day 64, 26/4/05	0.85
8367	Invertebrate, weta, #23, day 64, 26/4/05	<MDL
8368	Invertebrate, weta, #30, day 64, 26/4/05	2.5

The results have been adjusted for method recovery. All results are reported to two significant figures.

The determination was carried out using TLM067, the determination of diphacinone in animal and invertebrate tissue by HPLC. The method detection limit (MDL) is 0.02µg/g for liver, 0.2µg/g for inverts. The uncertainty (95% c.l.) is ± 20%.

TESTED BY: leb **WORKBOOK REF:** 4015
TEST PERIOD: 26/4-3/5/05

AUTHORISED BY:

L.E. Brown, G.P.G. Wright

Date: 19/05/2005

All tests reported herein have been performed in accordance with the laboratory's scope of accreditation