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# Usefully Persistent? Anticoagulants as Quantitative Markers of Bait Uptake in Brushtail Possums

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**ABSTRACT:** Prolonged persistence of second-generation anticoagulant rodenticides (SGARs) in animal tissues facilitates trophic transfer of residues, with exposure of predatory and scavenging non-target wildlife now widely reported. In many instances, anticoagulant residue levels measured in wildlife are apparently sublethal, although longer-term effects of such exposures are currently not well understood. Conversely, prolonged metabolic persistence is of practical utility when compounds are used as biological markers to determine food uptake by animals. We required two effective and distinct marker compounds to progress field-based research on optimal baiting strategies to manage introduced brushtail possums in New Zealand. Two SGARs (flocoumafen and bromadiolone) were evaluated, as neither are currently registered for application as rodenticides in areas typically subject to possum management. All captive possums ingesting small, sublethal (2-6 g) quantities of food containing 0.0005% (by weight) of either SGAR were reliably marked by the presence of residual concentrations in liver as measured by HPLC analysis. This marking persisted for at least six weeks, and liver concentrations in marked possums did not decline between three and six weeks after the marker was ingested. Marking was also quantitative for both SGARs with a correlation between liver residue concentration and the amount of marker ingested. Using such marker baits would provide at least a six-week period in which to recover possums in field research. By recording bodyweight and testing liver samples from such possums for both markers, regression equations generated from the work reported here will enable back-estimation, with confidence intervals, of the amounts of marker bait eaten by each possum.

**KEY WORDS:** anticoagulant, bait marker, bromadiolone, brushtail possum, flocoumafen, *Trichosurus vulpecula*

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## INTRODUCTION

In New Zealand (NZ), introduced brushtail possums (*Trichosurus vulpecula*) are vectors of bovine tuberculosis and also threaten endemic biodiversity (Montague 2000). Possum populations are currently managed over large areas by aerial application of bait containing the vertebrate toxic agent sodium fluoroacetate (Compound 1080) following an earlier application of non-toxic pre-feed bait (Fisher et al. 2011). In refining practice to ensure consistently high efficacy of aerial 1080 control, key questions include why some possums survive such operations - they may not encounter toxic bait or eat a lethal dose of toxic bait if they encounter it. Addressing these questions in field studies would be facilitated by the use of two distinct bait markers (one for prefeed and one for toxic bait) that persist for a period that allows an adequate sample of animals to be captured from the population to determine whether they are marked. For possums in New Zealand, this period was defined as at least six weeks after bait ingestion. Quantitative bait markers, where the "extent" of marking allows estimation of the amount of bait eaten, would provide useful additional data. The dye Rhodamine B has previously been used as a systemic marker of possum hair (Fisher and Tremblay 2005), but it is unlikely to provide reliable persistence over six weeks and cannot indicate the amount of bait eaten. The serum marker iophenoxic acid has extended persistence in eutherian mammals and can act as a quantitative marker (Ballesteros et al. 2012). However it appears to be metabolised and excreted relatively quickly by marsupials (Fisher and Marks 1997), which greatly reduces its utility as a bait marker for possums. Other

potential markers with suitable persistence include radioactive or stable isotopes and the calciphilic antibiotic tetracycline (Fry and Dunbar 2007). However, using these compounds in field studies involve operational and environmental hazards; require considerable sampling effort and analytical resourcing to detect marking; and they have not been tested for their reliability and utility as markers in possums.

In considering other potential markers of bait uptake in possums we noted the persistence of the second-generation anticoagulant rodenticide (SGAR) brodifacoum of >252 days (Eason et al. 1996) and a strong correlation in rats (*Rattus norvegicus*) between the amount of brodifacoum ingested in bait and resulting liver concentrations (Fisher 2009). This led us to consider SGARs as potential bait markers in possums, because other SGAR compounds have chemical structures and toxicity/persistence profiles similar to brodifacoum (Watt et al. 2005). Two of these, bromadiolone (BR) and flocoumafen (FL), are used for commensal rodent control in NZ but not for field applications in NZ, and were therefore unlikely to occur as "background" residual burdens in possum populations. However, there are no published estimates of the hepatic half-life of BR or FL in possums. Hepatic half-life estimates available for Norway rats, 170 days for BR (Parmar et al. 1987) and 220 days for FL (Huckle et al. 1989), suggested both SGARs may also have a relatively prolonged persistence in possum liver.

Practical use of BR or FL as bait markers requires sublethal oral exposures, to ensure later recovery of live animals for euthanasia and testing of liver for residual concentrations. The minimum lethal exposure for BR in

possums has been estimated as 0.38 mg/kg/day but most deaths occurred from consuming 1 mg/kg/day; for FL the minimum lethal exposure was 0.27 mg/kg/day and most deaths were from 0.4 mg/kg/day (Bell et al. 1988). On this basis, we estimated concentrations of up to 0.001% (by weight) of BR or FL in bait would present a low mortality risk to possums eating <20 g of bait over consecutive days. To identify suitable bait markers for field research, we conducted trials with captive possums to confirm whether ingestion of small quantities (up to 6 g) of bait containing low concentrations of BR or FL (0.001% and 0.0005% respectively) would produce detectable residual concentrations in their liver (as a marker of bait ingestion) three and six weeks after bait ingestion.

## METHODS

Wild-caught possums were housed in the Landcare Research Animal Facility, Lincoln, NZ. They were acclimatised for at least three weeks to housing in individual cages (1.0 × 0.4 × 0.55 m) with a nest box, with constant room temperature (18-22°C) and a natural lighting cycle. They were fed a maintenance diet of possum pellets (CRT Reliance Feeds, Rolleston, NZ), vegetables and fruit, and had access to water at all times.

### Trial 1: Bromadiolone or Flocoumafen (0.001%) Presented in Peanut Butter

Individually-housed possums were pre-fed with 5 g of peanut butter daily for two days to familiarise them with a palatable food. Possums were randomly allocated to one of seven treatment groups (each  $n = 6$ ) and weighed before treatment food was offered. Treatments were peanut butter containing either FL or BR at a nominal concentration of 0.001% (w/w) and offered in 2-, 4-, or 6-g amounts. A control group was offered 4 g plain peanut butter.

Normal food was removed while treatment food was available overnight and consumption of treatment (by weight) was measured the following morning. Possums that did not eat the treatment food overnight were excluded from the trial. If the treatment food was partially eaten the remainder was weighed to estimate the amount ingested. Table 1 shows the number of possums that ate some treatment food in each group.

Possums were returned to normal diet and housing after ingesting treatment food. Three weeks post-treatment, half (or nearest to half) the possums in each group were anaesthetised with isoflurane, then euthanased by cardiac injection of sodium pentobarbitone. Whole livers were dissected out and stored in labelled plastic ziplock bags at -20°C until analysis for BR or FL concentration. Because we did not know whether BR or FL residues would persist in possums, three-week liver samples were tested within two days of collection. No detectable residual concentration of either anticoagulant in these three-week samples, would indicate they were not suitably persistent as marker of bait uptake. However, liver residues were present at three weeks post-ingestion (see Results) so remaining possums in each treatment group were euthanased at six weeks and their livers tested for BR or FL concentrations.

### Trial 2: Bromadiolone or Flocoumafen (0.0005%) Presented in Cereal Pellet Bait

Guided by the results of Trial 1 (BR or FL at 0.001% in peanut butter), we sought to determine (i) whether presentation of BR or FL in a field-type cereal pellet bait also produced quantitative marking in possum liver and (ii) whether lower concentrations of BR or FL (0.0005%, half of those in Trial 1) in food could produce reliable quantitative marking. This trial aimed to minimise the potential residue burden in natural environments if either compound was eventually used in field applications as markers of bait uptake.

Forty wild-caught possums were housed and acclimatised as described for Trial 1, and fed for three days with 10 g of cereal pellet baits of a proprietary 'RS5' formulation (Animal Control Products, Whanganui, NZ) as a standard bait type used for possum management. They were randomly allocated to five treatment groups (each  $n = 8$ ) and offered 2 g or 6 g cereal pellet bait containing 0.0005% of either FL or BR, or control treatment of 2 g plain cereal pellet overnight (Table 2). Three and six weeks after ingestion of treatments, three possums from each treatment (20 possums at each interval) were euthanased and their livers sampled as described for Trial 1.

**Table 1. Number of possums that ate the bait offered in each of seven peanut butter treatments in Trial 1.**

Treatment	Amount of Bait Offered		
	2 g	4 g	6 g
0.001% Bromadiolone in peanut butter	4/6	5/6	5/6
0.001% Flocoumafen in peanut butter	4/6	4/6	5/6
Plain peanut butter (control)		6/6	

**Table 2. Number of possums that ate the bait offered in each of cereal pellet treatments in Trial 2.**

Treatment	Amount of Bait Offered	
	2 g	6 g
0.0005% Bromadiolone in cereal pellet	8/8	7/8*
0.0005% Flocoumafen in cereal pellet	8/8	7/8*
Plain cereal pellet (control)	8/8	

\* one possum in the group ate approximately half of the treatment bait

### Treatment Preparation and Chemical Analyses

All preparation of treatment foods containing BR and FL, and analyses of liver tissue for these compounds, were undertaken by the Landcare Research toxicology laboratory (Lincoln, New Zealand). Treatment food and liver tissue were analysed for concentrations of BR and FL using an HPLC-fluorescence detection method and post-column pH switching technique developed by Jones (1996). In each batch of liver samples tested, 1-g blank and 1-g spiked sample were prepared using difenacoum as an internal standard. The method limit of detection (MDL) for both compounds in liver was 0.005 µg/g and

method uncertainty (95% confidence interval) was  $\pm 4\%$  for BR and  $\pm 7\%$  for FL. For analysis of bait the method uncertainty was  $\pm 4\%$ .

### Statistical Analyses

Data were analysed using the lm procedure in the R statistical computing environment (R Development Core Team 2012). Linear regressions forced through the origin were fitted to the data to generate slope relationship equations, with data from the three-week and six-week sample points compared for flocoumafen and bromadiolone. Also using R, the Shapiro-Wilks test was applied to testing of normality for residuals around bromadiolone and flocoumafen regressions.

## RESULTS

### Trial 1: Bromadiolone or Flocoumafen (0.001%) Presented in Peanut Butter

Not all possums ate all the treatment food offered (Table 1). In the six weeks following ingestion of the treatments, no signs of illness or behavioural change in the possums were observed. In control possums sampled at three ( $n = 3$ ) and six weeks ( $n = 3$ ), liver concentrations were below the MDL for both BR and FL.

Concentrations of BR and FL in the peanut butter preparations were analysed as 0.001% and 0.0009% respectively, and these concentrations were used in calculating amounts ingested by possums. Amounts of FL or BR ingested were expressed relative to each

possum's body weight as 'dose' (mg anticoagulant per kg possum weight). All possums that ingested some BR or FL had detectable liver concentrations of the corresponding anticoagulant and there was a correlation for both anticoagulants between the amount ingested and the resultant concentration in possum liver at both three and six weeks (Figures 1 and 2).

In possums that ingested BR (Figure 1), the difference between the slopes of the curves calculated for the three- and six-week sampled groups was not significantly different from zero (Difference = -1.4075,  $SE_{diff} = 1.169$ ,  $t_7 = -0.93$ ,  $P = 0.37$ ). Residuals from the BR group regression were normally distributed ( $W = 0.9135$ ,  $p\text{-value} = 0.09929$ ). In possums that ingested FL (Figure 2), the difference between the slopes of the curves calculated for the three-week and six-week sampled groups was marginally significantly different from zero (Difference = -2.5247,  $SE_{diff} = 1.1641$ ,  $t_{13} = 2.17$ ,  $P = 0.05$ ). Residuals from flocoumafen regression were normally distributed ( $W = 0.9027$ ,  $p\text{-value} = 0.07546$ ). As a highly palatable food, peanut butter was first used to present BR and FL to determine whether they had potential as markers. While the results of the peanut butter trial were not intended to be used in estimates of cereal pellet bait uptake, the 95% confidence and predictive intervals for the BR and FL in peanut butter regression data are shown in Figures 1 and 2, respectively.

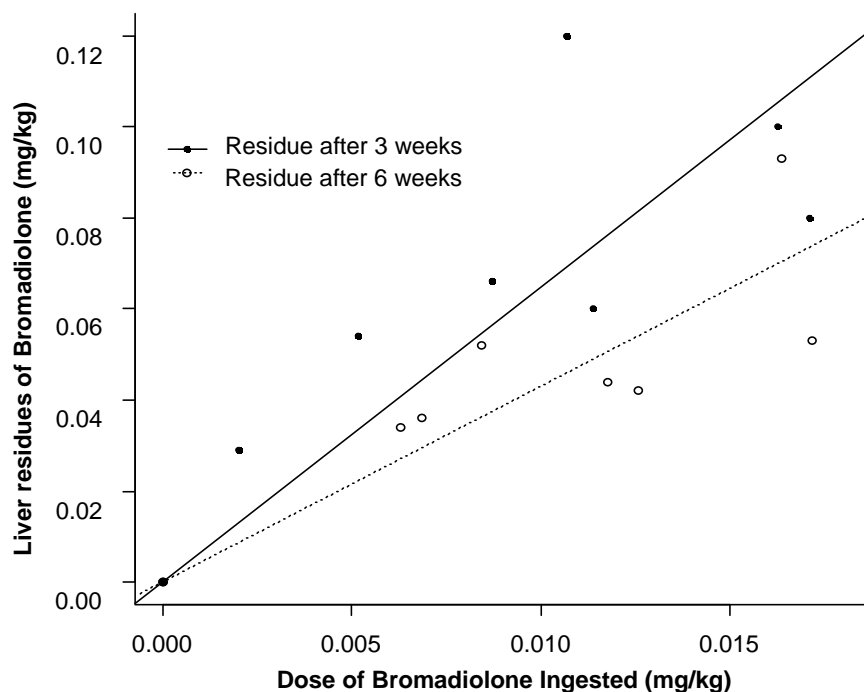


Figure 1. Regressions of residual liver concentrations of bromadiolone in possums at three weeks ( $R^2 = 0.472$ ,  $y = 0.034 + 3.795 * \text{dose}$ ) and six weeks ( $R^2 = 0.463$ ,  $y = 0.015 + 3.115 * \text{dose}$ ) after the ingestion of 2-6 g of peanut butter containing 0.001% bromadiolone.

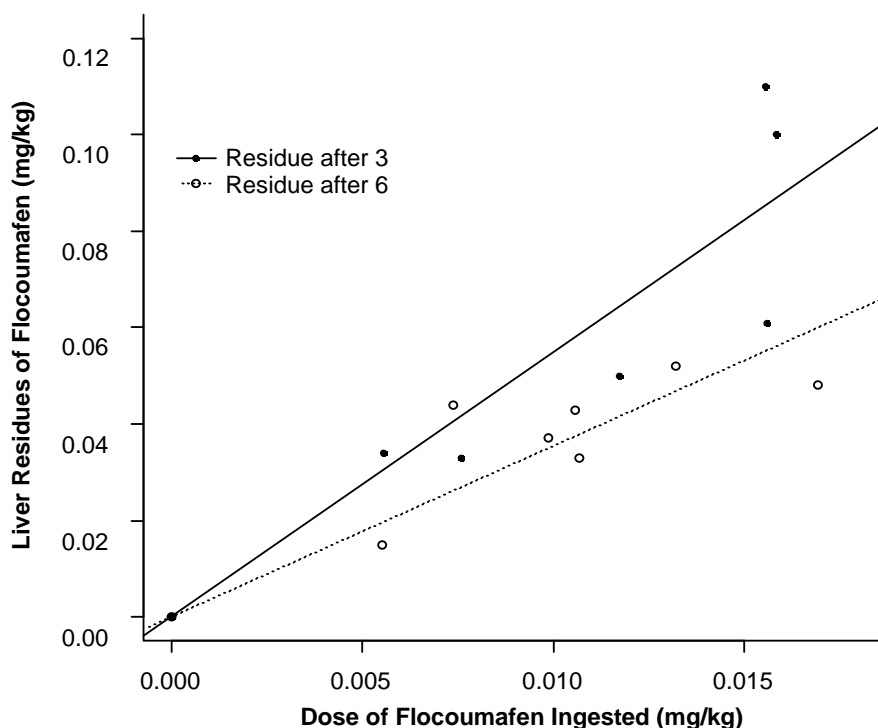


Figure 2. Regressions of residual liver concentrations of flocoumafen in possums at three weeks ( $R^2 = 0.704$ ,  $y = -0.001 + 6.152 * \text{dose}$ ) and six weeks ( $R^2 = 0.515$ ,  $y = 0.014 + 2.366 * \text{dose}$ ) after the ingestion of 2-6 g of peanut butter containing 0.001% flocoumafen.

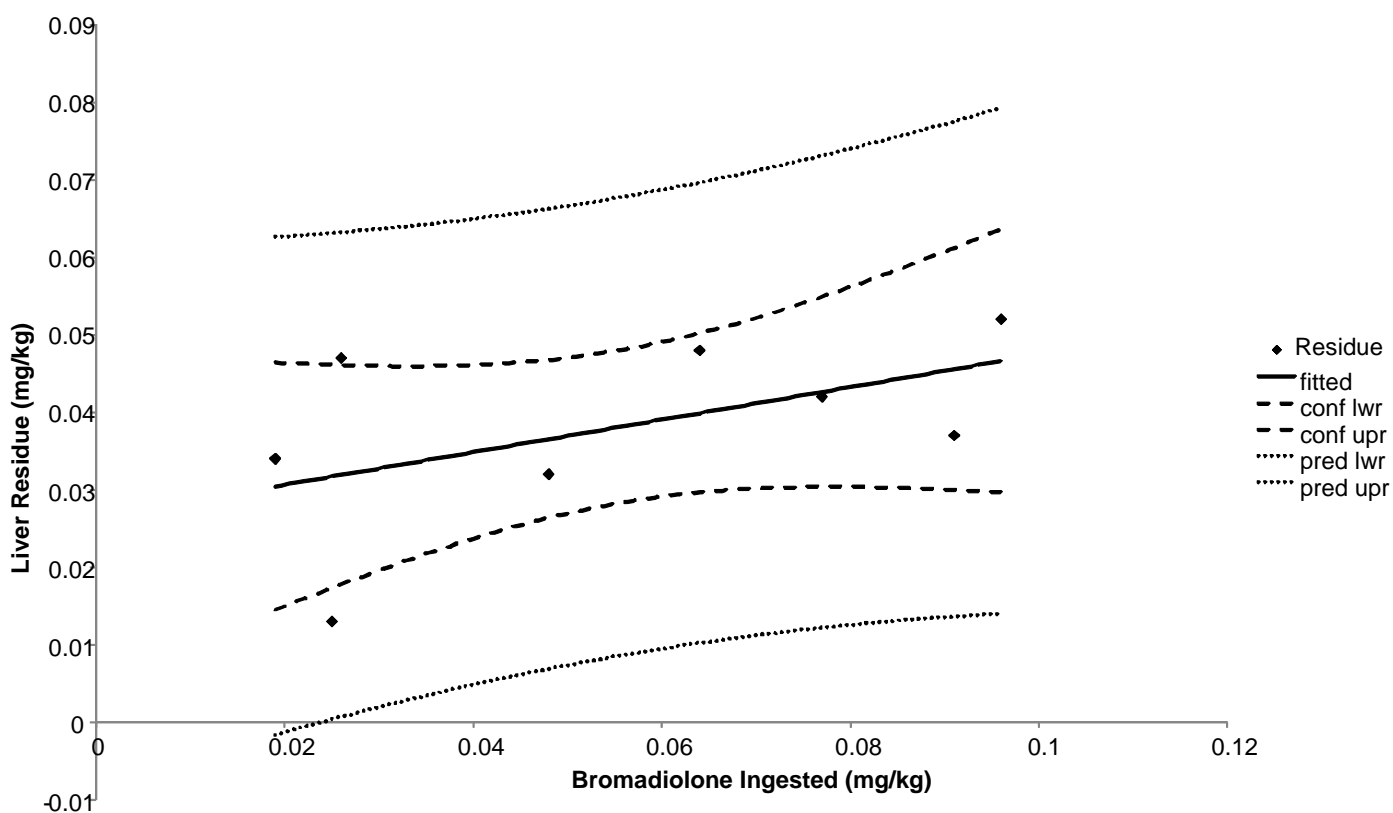


Figure 3. Fitted regression curve with 95% confidence intervals and 95% prediction intervals from data for possum livers sampled at three weeks ( $R^2 = 0.273$ ,  $y = 0.0264 + 0.210 * \text{dose}$ ) after ingesting bromadiolone in cereal pellet bait. Data for samples taken at six weeks ( $R^2 = 0.474$ ,  $y = 0.006 + 0.585 * \text{dose}$ ) not shown.

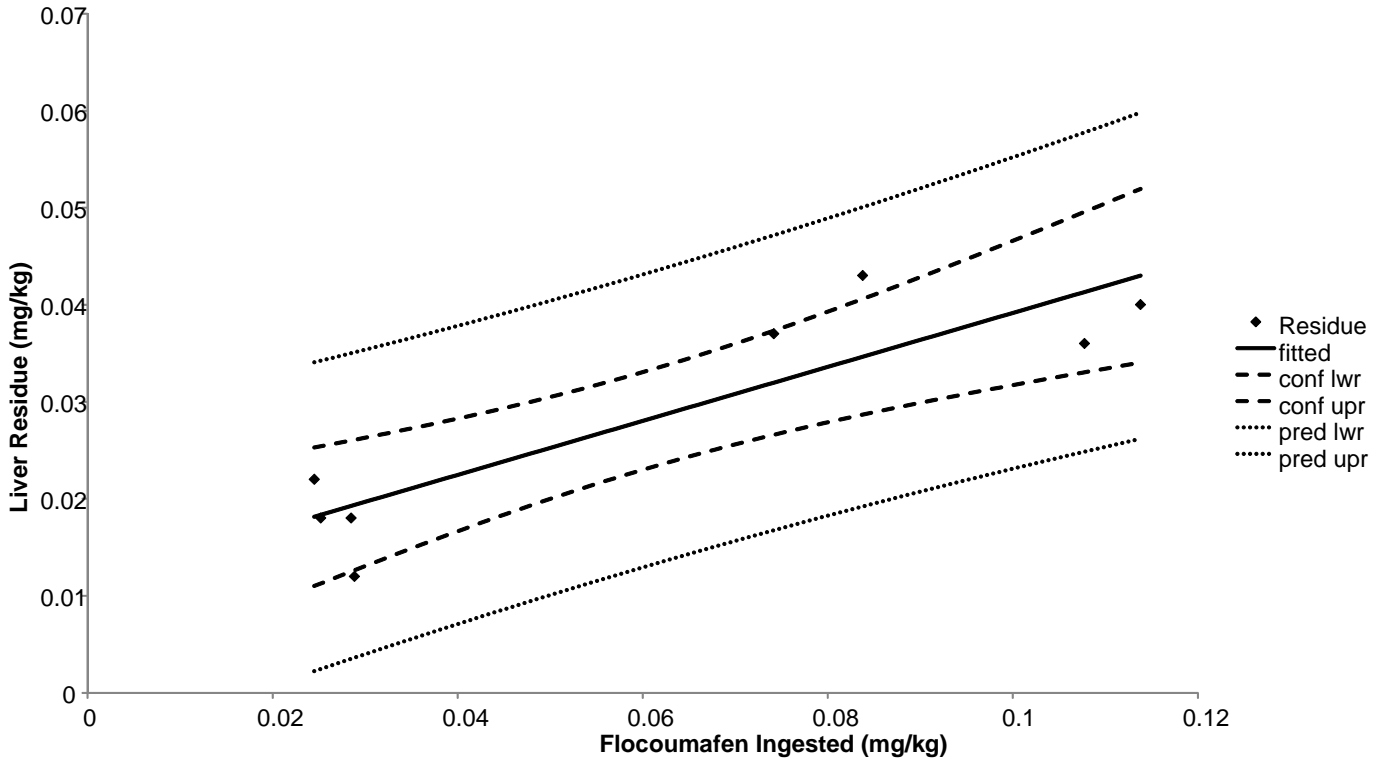


Figure 4. Fitted regression curve with 95% confidence intervals and 95% prediction intervals from data for possum livers sampled at three weeks ( $R^2 = 0.792$ ,  $y = 0.011 + 0.278 * \text{dose}$ ) after ingesting flocoumafen in cereal pellet bait. Data for samples taken at six weeks ( $R^2 = 0.709$ ,  $y = 0.003 + 0.276 * \text{dose}$ ) not shown.

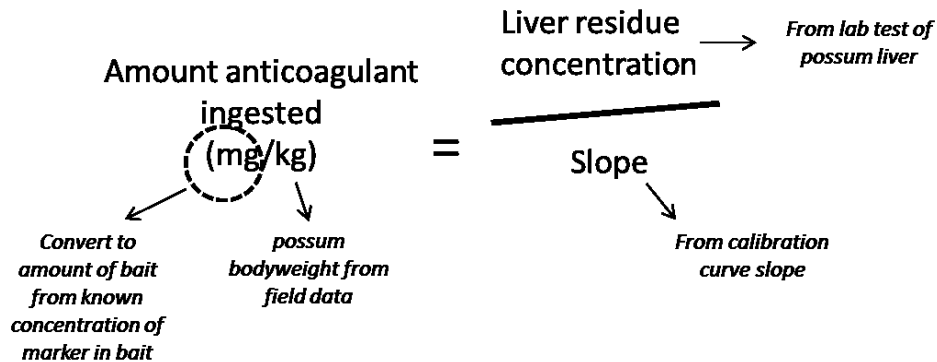


Figure 5. Equation for estimating bait intake by an individual possum.

Two female possums in BR treatment group euthanased at six weeks had pouch young approximately two weeks old. Analysis of the liver and internal organs from these pouch young found no detectable BR or FL, indicating there was no maternal transfer of anticoagulant residues to dependent young at the concentrations used.

### Trial 2: Bromadiolone or Flocoumafen (0.0005%) Presented in Cereal Pellet Bait

Not all possums ate all of the treatment food offered (Table 2). In the six weeks following ingestion of the treatments, no possums showed signs of illness or behav-

ioural change. In control possums sampled at three ( $n = 3$ ) and six weeks ( $n = 3$ ), liver concentrations were below the MDL for both BR and FL. Concentrations of BR and FL in the cereal pellet preparations were both measured as 0.0005% and these concentrations were used in calculating amounts ingested by possums.

All possums that ingested some BR or FL in cereal pellets had detectable liver concentrations of the corresponding anticoagulant and again there was a correlation for both anticoagulants between the amount ingested and the resultant concentration in possum liver at both three and six weeks. Fitted regression curves were used to es-

timate 95% confidence intervals around the data, and also the predictive confidence if these curves were to be used to back-estimate amounts of bait eaten, from liver residues in possums of known bodyweight. For BR at three weeks, the regression showed 0.2099 increase in residue for each unit increase in dose, and confidence intervals on the curve were 0.1400 ( $t_6 = 1.500$ ,  $p = 0.185$ ). If the curve was to be used to predict amounts of bait eaten, the confidence intervals on a single predicted value would be given by the outer intervals shown in Figure 3. For BR at six weeks (data not shown), the regression showed 0.5852 increase in residue for each unit increase in dose, and confidence intervals around the curve were 0.276 ( $t_6 = 2.122$ ,  $p = 0.087$ ).

For FL at three weeks, the regression showed 0.2782 increase in residue for each unit increase in dose, and confidence intervals around the curve were 0.0571 ( $t_6 = 4.87$ ,  $p = 0.003$ ). If the curve was to be used to predict amounts of bait eaten, the confidence intervals on a single predicted value would be given by the outer intervals shown in Figure 4. For FL at six weeks (data not shown), the regression showed 0.2759 increase in residue for each unit increase in dose, and confidence intervals around the curve were 0.0790 ( $t_6 = 3.49$ ,  $p = 0.017$ ).

## DISCUSSION

Trial 1 used a highly palatable food (peanut butter) and found that BR and FL both could act as reliable and persistent systemic markers of bait uptake in possums (i.e., all possums ingesting at least two grams of bait containing 0.001% of either anticoagulant had measurable concentrations of the corresponding anticoagulant in their livers at least six weeks afterwards). Further, Trial 1 results indicated that such marking was also quantitative, shown by correlations between the amount of BR or FL ingested and the resultant concentration in liver at three or six weeks afterwards.

We were confident that these exposures in possums were sub-lethal, the 0.001% concentration used is five times lower than active concentrations in currently-registered rodenticide bait formulations. The minimum lethal exposure for bromadiolone in possums has been estimated as 0.38 mg/kg/day but most deaths occurred from consuming 1 mg/kg/day; for flocoumafen, the minimum lethal exposure was 0.27 mg/kg/day and most deaths were from 0.4 mg/kg/day (Bell et al. 1988). In Trial 1, no visible signs were observed in possums over six weeks after they ingested single doses of 0.006-0.017 mg/kg FL or 0.002-0.017 mg/kg BR in peanut butter (i.e., marking could be reliably achieved by sublethal exposures).

Trial 2 confirmed that both BR and FL acted as reliable, persistent markers when delivered in cereal pellet bait typically used for operational possum control. So, theoretically these anticoagulants could be used in field research of bait uptake by possums, allowing back-estimation of amounts of bait eaten in animals trapped up to 6 weeks after marker bait application, provided the body weight and liver residue concentration of each possum was known (e.g., Figure 5).

Trial 2 also showed that the even lower concentration of 0.0005% of BR or FL in bait reliably produced mark-

ing, even with small sample sizes. The confidence interval P-values for the BR regressions (0.185 at three weeks and 0.087 at six weeks) indicate no significance, and thus a very low predictive power in using BR as a quantitative bait marker. It would be useful to determine whether this could be increased by using 0.005% BR in cereal pellet bait and increasing sample size in the regression curve data. For FL, the regression curves generated in Trial 2 indicated better confidence and predictive power than BR in use as a quantitative marker.

This was an encouraging result, which indicated that minimal quantities of these anticoagulants could be used in field applications as reliable and at least partly quantitative markers, while reducing the potential residue burden introduced into the environment. Mammalian and avian wildlife worldwide, particularly carnivorous and scavenging species, are known to carry sublethal liver burdens of residual concentrations of various anticoagulants (e.g., Elliott et al. 2014) because of the widespread use of anticoagulants and especially SGARs as rodenticides. This is also the case in NZ, where anticoagulant residue burdens are present in a range of wildlife (e.g., Booth et al. 2012). If BR or FL were to be used in field applications as bait markers, this would entail much lower concentrations than in rodenticide bait and present correspondingly lower residue risks to non-target wildlife.

Such research applications as bait markers would require permissions and approvals from regulatory agencies and relevant land managers which might limit their use. Because of their current applications as rodenticides in NZ, both bromadiolone and flocoumafen are classified as hazardous substances by the Environment Protection Agency and as vertebrate toxic agents by the Agricultural Compounds and Veterinary Medicines group of the Ministry for Primary Industries. Consequently, before these compounds can be used as bait markers, it will be necessary to establish the regulatory permissions are needed for manufacture of bait containing "marker" concentrations of BR and FL (0.001-0.0005%) and use in field research applications.

We consider it likely that BR and FL (and other second-generation anticoagulants) would also act as persistent quantitative markers in other mammal species. One obvious limitation is the requirement for lethal sampling to obtain liver tissue for residue testing, limiting use as markers to species of low conservation or pest status. The quantitative relationship between the amount of anticoagulant ingested and liver residue concentration (at least for FL) also raises interesting implications for ongoing monitoring of wildlife for residue burdens. Our trials simulated only single oral exposures to anticoagulants – quite different to what likely occurs in secondary exposure of predator and scavenger non-target wildlife. However it could be possible, with some known parameters around tissue elimination half-lives, to model potential exposure profiles (possible extent and timing since anticoagulant exposure) from liver residue burdens measured in wildlife.

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