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Anticoagulant rodenticide excretion in rats following median lethal dose (LD_{50}) administration

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Executive Summary

Rodenticide contamination is an emerging issue that has the potential to threaten the profitability and integrity of the Australian pork industry. Despite widespread use, there is a lack of data and scientific research pertaining to the acquisition of anticoagulant rodenticides in livestock species such as pigs. In order to maintain consumer confidence in Australian pork and to protect market access to Singapore, China and other countries, it is vital that we understand the environmental fate of anticoagulant rodenticides deployed on farm and the risks associated with the deployment of specific compounds at specific concentrations.

Previous research, conducted by SARDI Food Sciences (APL 2016/2230), examined the risk of exposure of pigs to four rodenticides (first and second-generation). Rodenticides were repeatedly administered to rats at low, chronic doses. Rat movement and behaviour proved to be minimally affected and all rodenticide compounds tested were detectable in the faeces. The high concentration of anticoagulant compounds within rodenticide bait products means that on farm, rodents are likely to consume rodenticides at dose rates much higher than the LD_{10} dose (dose which is lethal to 10% of a test population) utilised in APL 2016/2230. Therefore this proposed feeding study examined the anticoagulant excretion and behavioural/mobility changes that occur when rodents consume anticoagulant rodenticides at a median lethal dose rate (LD_{50}), providing a more accurate model of field rodenticide excretion

Approval to use animals for research was obtained from the University of Adelaide Medical Science Animal Ethics Committee. As a condition of animal ethics approval the study was conducted in two rounds, the first serving as a pilot study with a limited number of rats (n=4) and the second with greater animal numbers (n=22) to identify key results.

The rodenticide compounds used in this study, brodifacoum and bromadiolone, are examples of second-generation anticoagulant rodenticides (SGARs) and were specifically selected due to widespread use in the pork industry. Compounds were purchased in raw powdered form and mixed into acetone and water based aliquots of a fixed concentration. Several of these aliquots were analytically tested by the National Measurement Institute (NMI) to ensure accurate dosing.

Rodenticide dosages were based on oral LD_{50} values for each of the compounds reported in scientific literature. As there are a range of LD_{50} values reported for both brodifacoum (e.g. 0.26-0.49 mg/kg) and bromadiolone (e.g. 0.65-1.125 mg/kg), the pilot study utilised doses at the lower end of the reported ranges – 0.26 mg/kg for brodifacoum and 0.65 mg/kg for bromadiolone. Strict animal monitoring protocols and clearly defined humane endpoints were instituted to maximise the welfare of experimental animals.

Four (4) female Sprague Dawley rats (mean bodyweight 209 g), housed in individual cages at the University of Adelaide G11 Animal Housing Facility, were orally administered either 0.26 mg/kg brodifacoum or 0.65 mg/kg bromadiolone via oral gavage. No signs of anticoagulant toxicoses or adverse animal health were observed in any of the rats for up to 12 days following oral administration of brodifacoum or bromadiolone at the dose rates stated above. As a result, for the larger scale trial, rodenticide dose rates were elevated to the higher range of the reported LD_{50} values – 0.49 mg/kg for brodifacoum and 1.125 mg/kg for bromadiolone.

Twenty-two (22) female Sprague Dawley rats (mean bodyweight 225 g) were randomly assigned to one of three anticoagulant rodenticide treatments; no-dose control (n=6), 0.49 mg/kg brodifacoum

(n=8) or 1.125 mg/kg bromadiolone (n=8). Following a week of acclimatisation, animals received their rodenticide treatments via oral gavage and were monitored for up to seven (7) days. Rats were placed in metabolic cages for 16-hour periods overnight, which allowed the collection of urine and faeces for anticoagulant analysis. Open Field (OF) testing was performed daily to quantify parameters of rat movement. At day 7, all remaining animals were humanely euthanised using isoflurane anaesthetic/carbon dioxide and livers were collected post-mortem for anticoagulant analysis.

Rodenticide dosed rats were found to retain normal activity until clinical signs of anticoagulant toxicoses emerged, which occurred between 3-5 days after oral administration. Brodifacoum and bromadiolone dosed rodents were not found to excrete detectable levels of rodenticides through urine. Analysis of rat faecal pellets for both rodenticide treatments revealed that they contained detectable residues of 0.72-2.1mg/kg for brodifacoum and 0.14-0.82mg/kg for bromadiolone. Detectable residues of brodifacoum (4.55-8.05mg/kg) and bromadiolone (1.95-4.0mg/kg) were also detected in the livers of dosed rats.

This research has highlighted some issues with the use of second generation anticoagulant rodenticides. Due to the high-persistence and long liver half-lives of these compounds in target and non-target animals, baited rats become reservoirs of large active concentrations of rodenticides. When the incapacitating symptoms associated with anticoagulant toxicoses appear, rat mobility is impacted significantly. Unfortunately, this makes baited rodents more susceptible to predation from pigs, increasing the risk of acquisition of rodenticide residues in livestock. Furthermore, baited rats excrete high levels of active rodenticide in their faeces, establishing another potential pathway for the acquisition of rodenticide residues in livestock.

In light of these research findings, it is recommended that producers using second-generation anticoagulant rodenticides place a greater emphasis on the detection of rodent activity and ensure that rodent carcases are routinely removed from production areas to minimise the risk of contamination of livestock.

It is important to remember that due to animal ethics conditions, this research was conducted with a relatively modest sample size (n=8) for each rodenticide treatment and laboratory Sprague Dawley rats were used in this study, an example of a brown/Norway rat species (*Rattus norvegicus*). As there are currently no current laboratory rat strains that belong to the species *Rattus rattus* (black rat), care must be taken in extrapolating these results to this related species.

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1. Background to Research

Rodenticide contamination poses a serious threat to the profitability and integrity of the Australian pork industry. It has the potential to impact market access (domestic and export) and reduce consumer confidence and demand for Australian pork products. Despite their widespread use in agricultural enterprises around the world, there is a lack of data and scientific research pertaining to the acquisition of rodenticides in livestock species such as pigs. Those within the Australian pork industry have raised concerns regarding the potential pathways of contamination and the lack of clear and detailed biosecurity guidelines for producers with regard to best practice use and implementation of rodenticides.

Previous industry investigations (2012-2013) found that some pigs in a small number of piggeries had measurable levels of first-generation anticoagulant rodenticides (warfarin and coumatetralyl) in their livers. In one case, this was associated with increased bleeding time. APL 2016/2230, undertaken by SARDI in 2017 investigated the risk of exposure of pigs to four rodenticides (first and second-generation). Rodenticides were repeatedly administered to rats at low, chronic doses. Rat movement and behaviour proved to be minimally affected and all rodenticide compounds tested were detectable in the faeces. This finding, when combined with the fact that rats tend to defecate on their feed source, confirms that rat faeces are a potential pathway for anticoagulant rodenticides to enter the pig food value chain.

Whilst rodenticide residue levels are relatively low and therefore unlikely to affect the safety of pork, there is a consumer perception of food safety risk. In order to maintain consumer confidence in Australian pork and to protect market access to Singapore, China and other countries, it is vital that we understand the environmental fate of anticoagulant rodenticides deployed on farm and the risks associated with the deployment of specific compounds at specific concentrations.

The high concentration of anticoagulant compounds within rodenticide bait products means that on farm, rodents are likely to consume rodenticides at dose rates much higher than the LD_{10} dose (dose which is lethal to 10% of a test population) utilised in a previous study APL 2016/2230. For example, a typical brodifacoum wax block rodenticide contains 0.05 mg of active brodifacoum per gram of total bait, equating to a concentration of 50 mg/kg. The LD_{50} of brodifacoum for a typical adult brown rat weighing 320 grams is between 0.0832-0.1568 mg. Rats of this size consume 20-30 g of dry food daily, and would only need to consume between 1.7-3.1 g of brodifacoum wax bait to accumulate an LD_{50} dose.

Therefore this feeding study examined the anticoagulant excretion and behavioural/mobility changes that occur when rodents consume anticoagulant rodenticides at a median lethal dose rate (LD50), providing a more accurate model of field rodenticide excretion. This information is critical for managing the risks associated with rodenticide use and developing best practice guidelines for the use and placement of rodenticide products.

2. Objectives of the Research Project

1.) To compare the effect that rodenticide dose frequency (single LD_{50} dose vs three doses totalling LD_{50}) has on rodenticide excretion and behavioural changes.
2.) To measure the feeding and behavioural changes of rodents, following oral administration of second generation anticoagulant rodenticides (SGARs) at LD_{50}.
3.) To quantify the amount of anticoagulant rodenticides that are excreted in the faeces and urine of rodents, following oral administration of anticoagulant rodenticides at LD_{50}.

3. Introductory Technical Information

Anticoagulant rodenticides are a class of chronic pesticides that are used to target and kill rodents. There are several different anticoagulant rodenticides, but all share the same mode of action (Silverman, 1980). When a rodent consumes the bait, the active anticoagulant blocks the epoxide reductase enzyme and stops the recycling of activated vitamin K. This severely reduces the production of blood clotting factors and eventually when the existing supply of clotting factors are degraded, the clotting mechanism fails and haemorrhaging begins.

With all anticoagulant rodenticides, there is a considerable delay between ingestion of a lethal dose and the onset of symptoms. Rodents, particularly rats (*Rattus* sp.), are intelligent enough to associate the new food source with the onset of distressing physical symptoms. This is known as bait avoidance or bait shyness (Buckle et al., 1987). Therefore, the delay between consumption of a lethal dose of anticoagulant bait and the onset of symptoms is an advantageous feature of these compounds that has contributed to a long history of successful use (Hadler & Buckle, 1992). Anticoagulant toxicoses develops progressively, causing anorexia, haemorrhage, shock, loss of consciousness and eventually death (Petterino & Paolo, 2001).

Early commercial examples of anticoagulant rodenticides were referred to as first-generation compounds. These include warfarin and coumatetralyl, which dominated the practice of rodent control in the 1950s and 1960s. The emergence of resistance within a decade of first-generation rodenticide use (Rowe and Redfern, 1965; Greaves and Ayres, 1969; Hadler and Shadbolt, 1975) stimulated the development of a new class of compounds known as second-generation of anticoagulant rodenticides (SGARs). Commonly used SGARs include brodifacoum, bromadiolone and flocoumafen. These compounds have a far greater potency (meaning less is required for a lethal dose) and have been shown to be effective at controlling previously resistant strains whilst maintaining the delayed onset of symptoms required to prevent bait avoidance (Hadler & Buckle, 1992; Buckle et al., 2012; Meerberg et al., 2014). However, the greater potency and persistence of these compounds in the tissues of baited rodents mean that they carry a greater level of secondary poisoning risk.

This project investigated the excretion of selected SGARs, namely brodifacoum and bromadiolone, under conditions that model field rodenticide exposure. An Open Field (OF) test was used regularly to assess the impact of rodenticide ingestion on animal mobility compared to control animals.

4. Research Methodology

4.1 Animal ethics and husbandry

All experiments were conducted in accordance with the Australian Code for the care and use of animals for scientific purposes (8th Edition 2013). Approval to use animals for research was obtained from the University of Adelaide Medical Science Animal Ethics Committee (M-2018-104).

Animal Ethics approval was granted with the requirement that a pilot study with a limited number of animals (n=4) be undertaken to determine an appropriate experimental timeline. The Animal Ethics Committee (AEC) were concerned with the animal welfare implications of orally administering known lethal doses of rodenticides to experimental animals. Strict animal monitoring protocols and clearly defined humane endpoints were instituted to maximise the welfare of experimental animals. Outcomes of this pilot study were required to be reported to the AEC before approval of a full scale trial (n=22).

Young adult (approximately 8 weeks old / > 200 g bodyweight) female Sprague Dawley rats (*Rattus norvegicus*) were housed in group cages of up to 6 at the University of Adelaide G11 Animal Housing Facility. The facility was temperature (22°C ± 2°C) and light controlled (12hr light/dark). Rats had free access to both water and a pelleted cereal feed. Rats were acclimatised (including handling) for at least 7 days before the start of the trial. During the trial, rats were monitored and weighed twice daily from day 0-4 and four times daily from day 5 onwards or earlier for rats showing clinical signs of anticoagulant toxicoses.

4.2 Preparation of rodenticide dosages

The second-generation anticoagulant rodenticides (SGARs) selected for use in this study were brodifacoum and bromadiolone. Selection was based on their common use in the pig industry. Some examples of commercial rodenticides with these active compounds include:

- Brodifacoum – RATSAK®, TALON®, TOMCAT II®, THE BIG CHEESE®
- Bromadiolone – BROMAKIL®, CONTRAC®, MOUSEOFF®, TOMCAT®

Compounds were purchased in raw analytical standard form (supplier – Sigma-Aldrich) and dissolved into aliquots of a fixed concentration (0.05 mg/mL) with 1% acetone (propan-2-one) and 99% reverse osmosis (RO) water. Aliquots were stored at -20°C in the SARDI Food Sciences laboratories for up to 72 hours. Single aliquots for each rodenticide compound were sent to the National Measurement Institute (NMI) for verification of rodenticide concentrations via liquid chromatography with tandem mass spectrometry (LC-MS-MS) and results were received within 48 hours.

4.3 Pilot study

Four (4) female Sprague Dawley rats (mean bodyweight 209 g) were randomly assigned to one of four SGAR treatments; brodifacoum (0.26 mg/kg in a single dose), brodifacoum (0.26 mg/kg total across three doses), bromadiolone (0.65 mg/kg single dose) or bromadiolone (0.65 mg/kg total across three doses). Rodenticide dosages were based on oral LD_{50} values for each of the compounds reported in scientific literature and commercial rodenticide product documentation. There are a range of LD_{50} values reported for both brodifacoum (e.g. 0.26-0.49 mg/kg) and bromadiolone (e.g. 0.65-1.125 mg/kg) (Table 1). In the interests of animal welfare, for this pilot study, doses at the lower end of the reported ranges were used – 0.26 mg/kg for brodifacoum and 0.65 mg/kg for bromadiolone.

Table 1: Summary of acute toxicity of two second-generation anticoagulant compounds – brodifacoum and bromadiolone.

Anticoagulant compound	Reported rat oral LD_{50} range	Active concentration in rodenticide bait	Amount of bait required for an LD_{50}	References for oral LD_{50} values
Brodifacoum	0.26-0.49 mg/kg	50 mg/kg	1.7-3.1 g*	Redfern et al., 1976 & FAO, 2015
Bromadiolone	0.65-1.125 mg/kg	50 mg/kg	4.2-7.2 g*	Grand, 1976 & Meehan, 1978

*Calculated using the bodyweight of a typical adult brown rat (320 grams).

Following a week of acclimatisation, rodenticide solutions were administered by oral gavage with a soft plastic feeding tube at day 0 (for single dose treatments) and at days 0, 2 and 4 (for multiple dose treatments). Rats were monitored for up to twelve days. On day -2, 0, 2, 4, 6 and 8, rats were placed in metabolic cages for 24-hour periods. The design of these cages (Image 1 & Image 2, Appendix 12.1) allows for the separation and collection of faeces and urine for rodenticide analysis. At day 12, all remaining rats (n=3) were humanely euthanised using isoflurane anaesthetic/carbon dioxide and livers were collected post-mortem for anticoagulant analysis. Blood was collected from each animal at death via cardiac puncture for the purpose of Prothrombin Time (PT) and Partial Thromboplastin Time (PTT) testing at Idexx Laboratories, Adelaide.

4.4 Full scale study

Twenty-two (22) female Sprague Dawley rats (with mean ± SD bodyweight of 225 g ± 9 g) were randomly assigned to one of three anticoagulant rodenticide treatments; no-dose control (n=6), 0.49 mg/kg brodifacoum (n=8) or 1.125mg/kg bromadiolone (n=8). Rodenticide dosages were elevated to the higher range of the reported LD_{50} values for brodifacoum (0.49 mg/kg) and bromadiolone (1.125 mg/kg) based on the lack of clinical anticoagulant toxicoses observed in the pilot study. Analysis of rodenticide aliquots via LC-MS-MS was performed by NMI prior to the study to ensure accurate dosing.

Following a week of acclimatisation, rodenticide solutions were administered by oral gavage with a soft plastic feeding tube at day 0. To minimise handling stress at the higher dose rates, it was decided to dose all rats via a single gavage. Rats were monitored for up to seven days. Rats were placed in individual metabolic cages (with dimensions of 34 x 31 x 31 cm) for 16-hour periods overnight, allowing for the separation and collection of urine and faeces for anticoagulant analysis. Each morning, metabolic cage samples were collected, pooled for each treatment (to ensure sufficient material for analysis) and frozen in preparation for anticoagulant analysis.

Open Field (OF) testing was performed daily to quantify parameters of rat movement. Rats were individually placed in a circular OF test arena one-metre in diameter (Image 3, Appendix 12.2), and their activity was recorded using a webcam (Logitech C510 HD) for a minimum of five minutes. Video footage was processed using an open source image processing program called ImageJ and open field analysis software called MouseMove (program links and step-wise instructions available in Samson *et al.,* 2015). These software programs were used to measure and compare the mobility and exploratory behaviours of the rats. The test was enriched using food treats (fruit loops) to alleviate the acclimatisation/boredom effect that had been previously observed in APL Project 2016/2230.

At day 7, all remaining animals (n=10) were humanely euthanised using isoflurane anaesthetic/carbon dioxide and livers were collected post-mortem for anticoagulant analysis.

5. Results

5.1 Pilot study

Analytical testing, performed by NMI, validated the concentration of rodenticides in solution. Results, received at day 2, indicated that rats had received between 65-70% of the intended dose. On day 5, all rats were given a top up dose to ensure that a full LD_{50} had been administered. During this top up dosing, one of the rats chewed through the plastic feeding tube which then became lodged in its oesophagus. As a result, this rat was not able to receive a complete LD_{50} dose. Attempts were made to recover the feeding tube and clear the blockage but were unsuccessful. After discussion with the AEC Animal Welfare Officer, the animal was euthanised. No data were subsequently available from this rat. No clinical signs of anticoagulant toxicity were observed in any of the remaining rats up to day 12. At day 12, the study was terminated and all rats were humanely euthanised.

Blood was collected from the heart of each rat at death and sent to Idexx Laboratories, Adelaide for Prothrombin Time (PT) and Partial Thromboplastin Time (PTT) testing. Prothrombin Time for all animals was within, or slightly lower than, the reference range for a healthy rat while only 1/3 rats (bromadiolone (0.65mg/kg across three doses)) recorded a slightly prolonged Partial Thromboplastin Time (Table 2). These blood coagulation results indicate that none of the rats were at immediate risk of haemorrhage despite having consumed anticoagulant rodenticides at a median lethal dose.

Table 2: Pilot study rat blood coagulation and liver rodenticide concentration results, rats were orally administered either 0.26mg/kg brodifacoum (n=1) or 0.65mg/kg bromadiolone (n=2); reference range values for a normal rat are also included to provide a comparison.

Treatment	**Dose frequency**	**Prothrombin Time (PT)**	**Partial Thromboplastin Time (PTT)**	**Liver rodenticide concentration**
Brodifacoum 0.26 mg/kg	Single dose	*no data	*no data	*no data
Brodifacoum 0.26 mg/kg	Multiple dose (3x)	8.7 secs	12 secs	1.30 mg/kg
Bromadiolone 0.65 mg/kg	Single dose	10.1 secs	27 secs	2.05 mg/kg
Bromadiolone 0.65 mg/kg	Multiple dose (3x)	12.3 secs	45 secs	1.80 mg/kg
Reference range values		**12.6-14.4 secs**	**9-28 secs**	**0 mg/kg**

*no data available as this rat was euthanised due to an animal welfare issue.

As the pilot study only involved two rats for each compound, there was insufficient material (faeces and urine) collected from the metabolic cages at each collection interval for rodenticide analysis. However, the weight of the samples indicated that a full-scale trial with five rats per treatment would produce sufficient material for analysis of pooled samples.

5.2 Full scale study

5.2.1 Changes to experimental protocol

Due to the lack of evidence of clinical anticoagulant toxicoses in the pilot study, rodenticide dosages were elevated to the higher range of the reported LD_{50} values for brodifacoum (0.49 mg/kg) and bromadiolone (1.125 mg/kg) for the full scale study. The experimental protocol was also amended so that analysis of rodenticide aliquots was performed by NMI immediately prior to the commencement of the study to ensure accurate dosing. The comparison of dose frequency (single vs multiple doses) was removed from the experimental protocol after discussions with the AEC regarding the ethics of orally gavaging rats that may be showing clinical signs of anticoagulant toxicity.

5.2.2 Clinical observations, rodenticide excretion and metabolism

Analytical testing performed by NMI, validated the concentration of rodenticides in solution and ensured that rats were dosed accurately. Rat faecal pellets and urine samples were pooled for the rats in each treatment group to ensure sufficient material for sample analysis. No-dose control liver samples were also pooled. Observations for rats in each treatment were as follows:

Brodifacoum 0.49 mg/kg

- One-hundred percent of brodifacoum dosed rats (8/8) were euthanised due to the emergence of clinical signs of anticoagulant toxicoses between 3-5 days following oral consumption of 0.49 mg/kg of brodifacoum.
- Brodifacoum was not present at levels above the limit of detection (> 0.005 mg/kg) in rat urine.
- Brodifacoum was present at detectable levels in the faeces of dosed rats within one day (24 hours) of consumption.
- Faecal brodifacoum concentration peaked at 2.1 mg/kg, 8-24 hours (Day 1) after consumption and steadily declined up to Day 5, where the last of the brodifacoum dosed rats were euthanised due to animal welfare concerns (Table 3).

Bromadiolone 1.125mg/kg

- Fifty percent of bromadiolone dosed rats (4/8) were euthanised due to the emergence of anticoagulant related toxicoses between 4-5 days following oral consumption of 1.125 mg/kg bromadiolone. The remaining four bromadiolone dosed rats showed no clinical signs of anticoagulant toxicoses up to seven days post consumption.
- Bromadiolone was not present at levels above the limit of detection (> 0.005 mg/kg) in rat urine.
- Bromadiolone was present at detectable levels in the faeces of dosed rats within one day (24 hours) of consumption.
- Bromadiolone concentration in rodent faeces peaked at 2.6 mg/kg during Day 2, 32-48 hours after consumption before steadily declining up to the conclusion of the trial at Day 7 (Table 3).

No-dose control

- No adverse clinical signs
- Brodifacoum detected in pooled liver (0.25 mg/kg), bromadiolone not detected
- Neither rodenticide found at levels above the limit of detection (> 0.005 mg/kg) in rat faeces or urine throughout the trial.

Table 3: Full scale trial summary of mean daily rat faecal rodenticide concentration (mg/kg) and mean ± standard error (SE) liver rodenticide concentration (mg/kg), rats were orally administered either 0.49 mg/kg brodifacoum (n=8) or 1.125 mg/kg bromadiolone (n=8).

Treatment	Mean daily faecal rodenticide concentration (mg/kg)							Mean ± SE liver rodenticide concentration (mg/kg)
	D1	D2	D3	D4	D5	D6	D7	
Brodifacoum	2.10	0.98	0.80	0.72	0.75	-	-	6.64 ± 0.45
Bromadiolone	0.80	2.60	0.82	0.43	0.28	0.16	0.14	2.59 ± 0.24

Anticoagulant related clinical signs developed progressively and included stress tears, lack of interest in food, reduction in bodyweight, visibly pale eyes, ears and limbs, hunched posture, ruffled coat, dried blood around the nose and eyes, impaired coordination and a reluctance/inability to move. Whilst no external haemorrhages were observed, post-mortem examinations revealed minor internal haemorrhaging in some rats. Animals were required to be humanely euthanised if they showed four or more clinical signs, appeared visually ill or lost greater than 15% of their starting bodyweight during the course of the trial.

Anticoagulant analysis of rat livers revealed detectable levels of brodifacoum and bromadiolone in all rodenticide dosed rats. Brodifacoum dosed rats had mean ± SE liver concentrations of 6.64 mg/kg ± 0.45 mg/kg, up to 2.5 times higher than bromadiolone dosed rats (mean ± SE liver concentration of 2.59 mg/kg ± 0.24 mg/kg) (Table 3 & Figure 1). The weight of individual rat livers was used to convert rodenticide concentration (mg of rodenticide per kg of liver tissue) into liver rodenticide content (mg) (Figure 2). The weight of liver samples were similar for each rat, therefore liver rodenticide content (mg) was reflective of concentration (mg/kg).

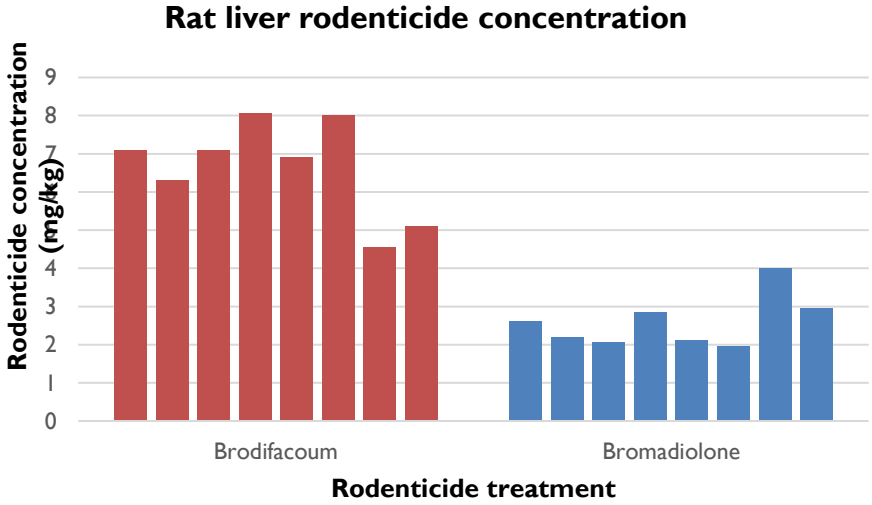

Figure 1: Post-mortem brodifacoum/bromadiolone concentration (mg/kg) in the livers of individual rats following oral consumption of either 0.49 mg/kg brodifacoum (n=8) or 1.125 mg/kg bromadiolone (n=8).

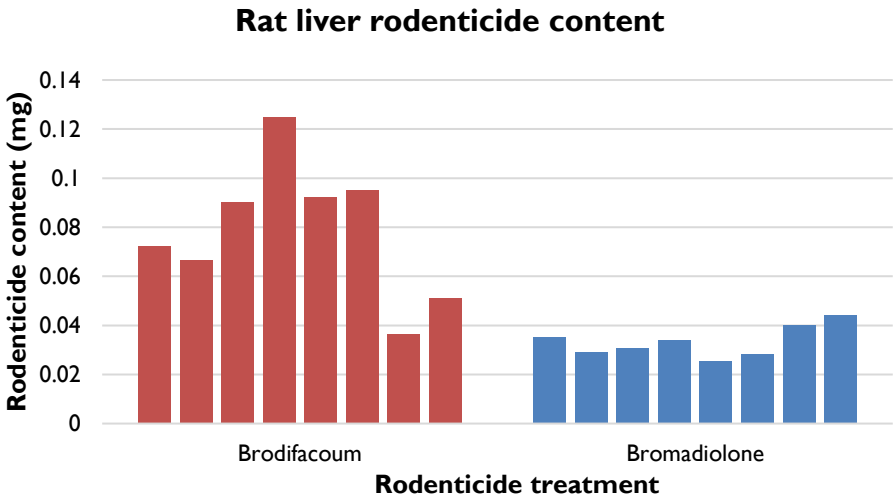

Figure 2: Post-mortem brodifacoum/bromadiolone content (mg) in the livers of individual rats following oral consumption of either 0.49 mg/kg brodifacoum (n=8) or 1.125 mg/kg bromadiolone (n=8).

Analysis of rat faecal material revealed detectable levels of brodifacoum and bromadiolone at all collection intervals (Table 3, Figure 3). As with the rat liver results, average weight of faecal material produced by each rat during the metabolic cage collection intervals was used to convert rodenticide concentration (mg/kg) into faecal rodenticide excretion (mg) (Figure 4). Cumulative rodenticide excretion was also calculated and is shown in Figure 5. Bromadiolone dosed rats excreted a greater cumulative amount of rodenticide through faeces over the course of the study, although no data was available for brodifacoum dosed rats after Day 5 as by this point, all rats had been euthanised.

As faecal samples were pooled for each treatment group, it was not possible to determine the level of total faecal rodenticide excretion for individual rats. However, analysis of pooled samples did allow for an estimation of total faecal rodenticide excretion per rat (Table 4). For each brodifacoum dosed rat, it was estimated that 23.1% of the amount of rodenticide consumed at Day 0 was excreted through faeces over a five-day window (Day 1-5). For bromadiolone dosed rats, 12.4% of rodenticide consumed at Day 0 was excreted over a seven-day period (Day 1-7).

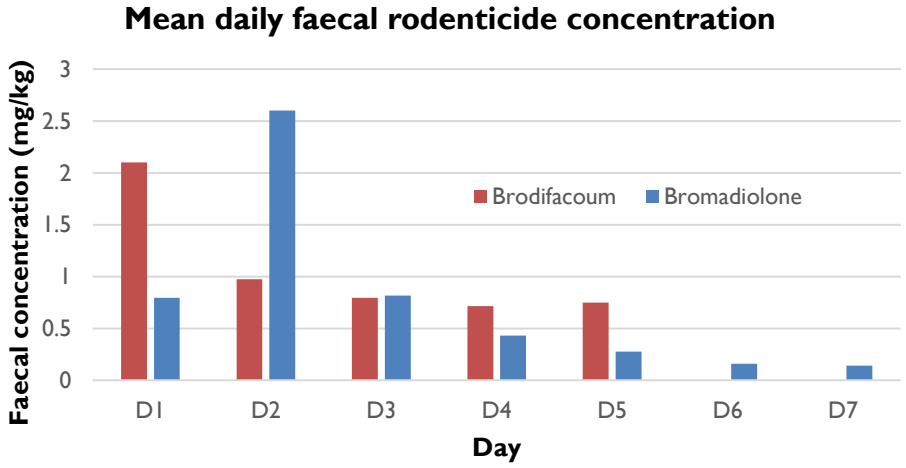

Figure 3: Mean daily faecal brodifacoum/bromadiolone concentration in rats following oral consumption of either 0.49 mg/kg brodifacoum (n=8) or 1.125 mg/kg bromadiolone (n=8).

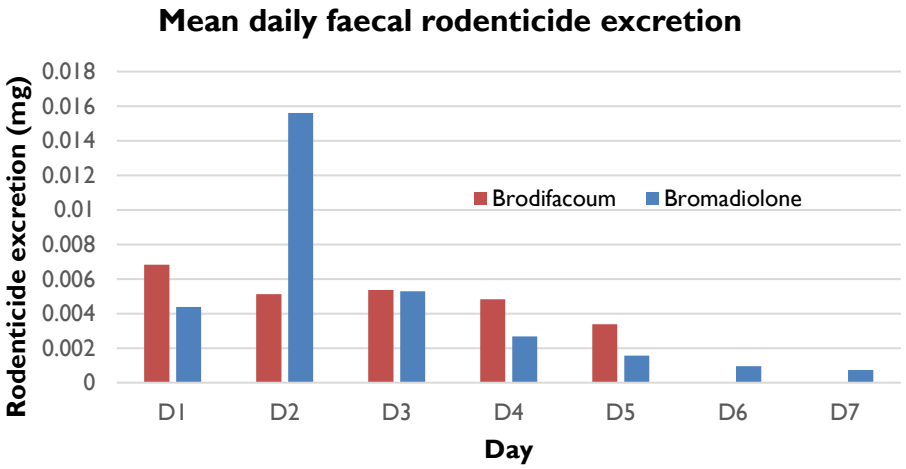

Figure 4: Daily faecal brodifacoum/bromadiolone excretion (mg) in rats following oral consumption of either 0.49 mg/kg brodifacoum (n=8) or 1.125 mg/kg bromadiolone (n=8).

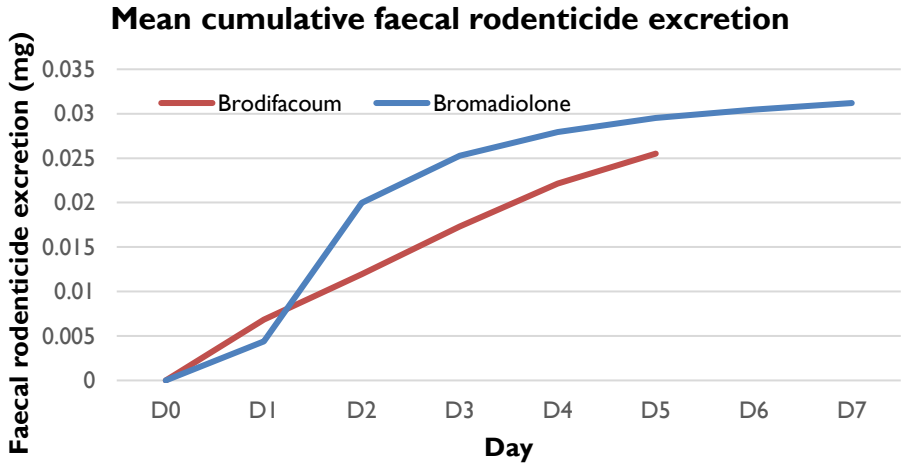

Figure 5: Mean cumulative faecal brodifacoum/bromadiolone excretion (mg) in rats following oral consumption of either 0.49 mg/kg brodifacoum (n=8) or 1.125 mg/kg bromadiolone (n=8).

Table 4: Summary of mean total liver rodenticide content and faecal rodenticide excretion relative to rodenticide consumption in rats following oral consumption of either 0.49 mg/kg brodifacoum (n=8) or 1.125 mg/kg bromadiolone (n=8).

Treatment	Mean dose administered	Mean total liver rodenticide content	Mean total faecal rodenticide excretion	% Faecal rodenticide excretion
Brodifacoum	0.11 mg	0.079 mg	0.026 mg	23.1%
Bromadiolone	0.25 mg	0.034 mg	0.031 mg	12.4%

5.2.3 Rat exploratory behaviour and mobility

Open Field (OF) testing was performed daily on six (6) rats from each rodenticide treatment to quantify parameters of rat exploration and mobility. Compared to the controls, brodifacoum dosed rats retained normal exploratory activity for at least 48 hours following consumption of a 0.49 mg/kg dose. The total travel distance (metres) during OF testing declined with the emergence of clinical signs of anticoagulant toxicoses (Figure 4). This was generally observed in the 24-48-hour window before euthanasia of the rat. Similarly, bromadiolone rats retained normal exploratory activity until the emergence of anticoagulant toxicoses (Day 3-5/ the final 48-hour window before euthanasia). Four out of the six (4/6) rats that received an oral dose of 1.125 mg/kg bromadiolone and were subjected to OF testing were found to retain exploratory activity at a level comparative to no-dose control rats for up to 7 days (Figure 5).

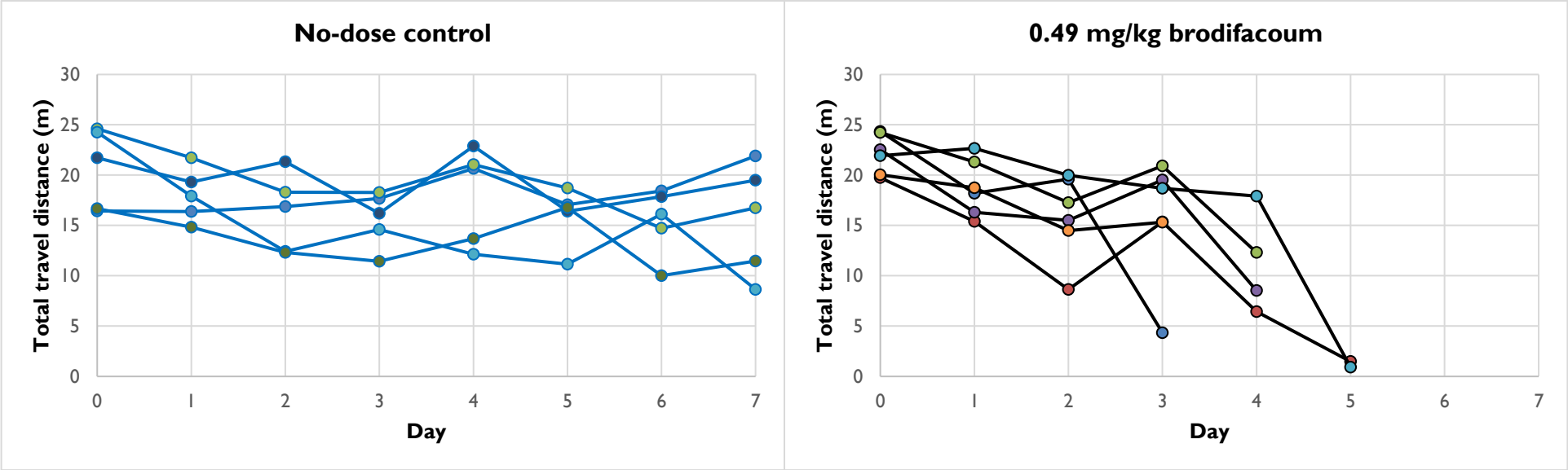

Figure 6: Total travel distance (metres) of no-dose control (left) and 0.49 mg/kg brodifacoum dosed (right) rats during daily Open Field (OF) testing. Each line represents the OF test results of an individual rat over the course of the 7-day trial.

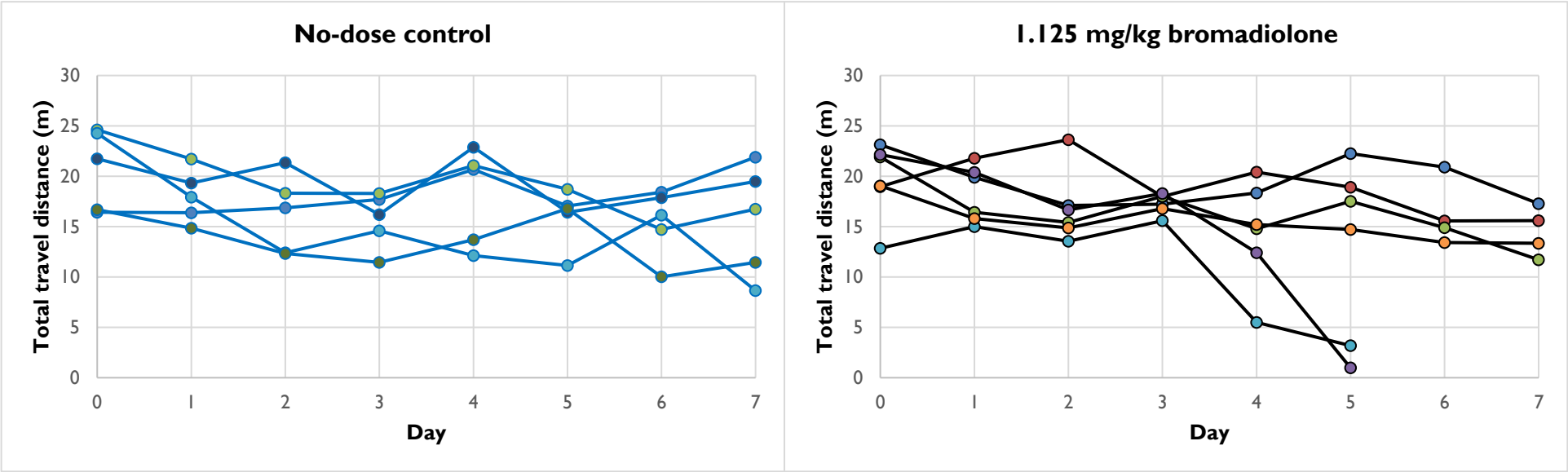

Figure 7: Total travel distance (metres) of no-dose control (left) and 1.125 mg/kg bromadiolone dosed (right) rats during daily Open Field (OF) testing. Each line represents the OF test results of an individual rat over the course of the 7-day trial.

6. Discussion

6.1 Reported LD_{50} values

In toxicology, the median lethal dose (or LD_{50}) is defined as the dose or concentration of a compound (in this case specifically a poison) that is required to kill half the members of a test population; a lower LD_{50} is indicative of increased toxicity (Duffus, 2009). Median lethal dose values reported in the literature are typically estimated from laboratory feeding studies conducted over 50 years ago, and with conflicting results and therefore had to be considered as general indicators of a substance's acute toxicity rather than an absolutely accurate dose.

Many of the anticoagulant rodenticides commonly used today (including brodifacoum and bromadiolone) were developed in the 1970's, with laboratory feeding studies of these compounds conducted around the same time (Grand, 1976; Redfern et al., 1976; Dubock, 1978). Modern scientific research appropriately carries an increased consideration for animal ethics and the care and use of animals for scientific purposes. Therefore, feeding trials of known poisons (including rodenticides) are more difficult to gain approval for and to conduct. In recent years, there has been very little published scientific research to verify these original findings. This is potentially problematic, as present-day users of rodenticides are forced to rely on 50-year-old research findings. The limited replication of this research that has occurred over the years has frequently yielded conflicting results, and as such, many of the commercially used anticoagulant rodenticides have a range of LD_{50} values rather than a single figure.

This study encountered this issue, as there are a range of LD_{50} values reported for both brodifacoum (e.g. 0.26-0.49 mg/kg) (Redfern, 1976; FAO, 2015) and bromadiolone (e.g. 0.65-1.125 mg/kg) (Grand, 1976; Meehan 1978). In the interests of animal welfare and to satisfy AEC approval conditions, for the pilot study, doses at the lower end of the reported ranges were used. These low range LD_{50} doses were found to cause no clinical anticoagulant toxicoses in rats (n=3) up to twelve days post ingestion. As a result of this finding, the dose rates for brodifacoum and bromadiolone were elevated to 0.49 mg/kg and 1.125 mg/kg respectively for the full scale study (n=22). In response to this increased dose rate, 8/8 brodifacoum dosed rats displayed signs of anticoagulant toxicoses between 3-5 days after oral administration and were subsequently euthanised. Four out of eight (4/8) bromadiolone dosed rats also presented with clinical signs (between days 4-5) and were euthanised. The remaining four bromadiolone dosed rats and six no-dose control rats showed no clinical signs up to seven days after dosing.

Researchers acknowledge that this research was conducted with a relatively modest sample size (n=8) for each rodenticide treatment. However, the results support the use of reported high range LD_{50} values (0.49 mg/kg and 1.125 mg/kg respectively), rather than the low range LD_{50} values for brodifacoum (0.26 mg/kg) and bromadiolone (0.65 mg/kg). Using a standard active concentration of 50mg/kg, the increased effect demonstrated by the high end LD_{50} results of this research indicate the need to increase in the amount of bait consumed by rats in the field for effective control. This could be achieved by improving the palatability of commercial bait formulations and by reducing the availability of preferred natural food sources of rodents. For a typical adult brown rat (*Rattus norvegicus*) weighing 320 g, high range LD_{50} consumption equates to 3.1 g of brodifacoum bait and 7.2 g of bromadiolone bait. Adult rats of this size typically consume 20-30 g of dry food daily, therefore the bait intake required for an effective dose remains a modest proportion of daily food intake. Therefore, these findings do not question the efficacy of rodenticides containing these active compounds.

6.2 Rodenticide excretion and metabolism

The liver is the predominant tissue site of rodenticide persistence, as this is the site at which all anticoagulants act in blocking the production of blood clotting factors (Silverman, 1980; Hadler et al., 1992). In the pilot study, liver residues were found to be 1.3 mg/kg for brodifacoum and 1.8-2.1 mg/kg for bromadiolone dosed rats. In the full-scale study, brodifacoum was detected at a low concentration (0.25 mg/kg) in the pooled no-dose control liver samples. These animals were not orally gavaged and were housed in separate cages to the dosed rats at all times during the study. No active rodenticide was detected in the faeces of no-dose control rats, therefore the detected residue in liver could have occurred as a result of cross-contamination of samples during post-mortem removal of the liver, despite vigorous cleaning of the instruments between individual rat post mortems. Liver residues for rodenticide dosed rats were much higher; 4.55-8.05 mg/kg in brodifacoum and 1.95-4.0 mg/kg for bromadiolone dosed rats. The difference between the liver residues for brodifacoum and bromadiolone dosed rats given the fact that the bromadiolone dose administered (1.125 mg/kg) was greater than that of brodifacoum (0.49 mg/kg) could be explained by the higher affinity of brodifacoum for VKORC1, reduced limited metabolism by the CYP450 enzyme family and significant enterohepatic recirculation compared to bromadiolone (Rubinstein et al., 2019). The livers of rodenticide dosed rats weighed between 10-15.5 g. Therefore, baited rats are effectively mobile contamination vectors containing 0.013-0.125 mg of active rodenticide.

If a pig were to consume one of these baited rodents, it could ingest up to 0.125 mg of a second-generation anticoagulant rodenticide. Given that the limit of detection for these compounds in pork matrices is > 0.005 mg/kg, it is unlikely that this dose would result in the acquisition of detectable rodenticide residues in pork products. However, due to the long half-life and high persistence of second-generation anticoagulant rodenticides – particularly brodifacoum – (Fisher et al., 2003; Vandenbroucke et al., 2008), a pig that consumes several baited rodents over a period of weeks or months could feasibly accumulate detectable residues in pork offal.

Although rat urine remains a hygiene issue, it was not found to pose a rodenticide contamination risk, with neither brodifacoum nor bromadiolone detected in any of the pooled urine samples. Brodifacoum and bromadiolone were present at detectable levels in rat faeces within 24 hours of ingestion of the rodenticide. Faecal rodenticide concentration peaked in the first two days before declining over the remainder of the trial, this was in contrast to the progression of anticoagulant toxicoses which advanced over the length of the trial. Dosed rats from both treatments had a consistent level faecal output throughout the study, therefore the total level of faecal rodenticide excretion was reflective of the faecal concentration. This is a crucial finding as it shows that baited rodents continue to excrete active rodenticide through faeces despite the emergence of incapacitating symptoms.

Bromadiolone dosed rats excreted a greater total amount of rodenticide through faeces over the course of the study, although no data was available for brodifacoum dosed rats after Day 5. This was not surprising, as the bromadiolone dose administered (1.125 mg/kg) was greater than that of the more potent brodifacoum (0.49 mg/kg). However, when considering the ratio of consumed to excreted rodenticide, the reverse was true. For bromadiolone dosed rats, 12.4 % of rodenticide consumed at Day 0 was excreted over a seven-day period (Day 1-7) while for each brodifacoum dosed rat, it was estimated that 23.1% of the amount of rodenticide consumed at Day 0 was excreted through faeces over a five-day window (Day 1-5).

6.3 Rat exploratory behaviour and mobility

The OF test relies on the principle that healthy rodents will innately explore novel surroundings (Samson *et al.,* 2015). Laboratory Sprague Dawley rats were used in this study, this strain is an example of a brown/Norway rat species (*Rattus norvegicus*). The black rat or roof rat (*Rattus rattus*) is another rat species commonly found in piggeries. Black rats have a unique set of behavioural features; they are agile climbers that often harbour in roof voids and ceiling cavities as opposed to the underground burrows of brown/Norway rats. As there are currently no current laboratory rat strains that belong to the species *Rattus rattus,* it is difficult to interpret from the results of this research, the impact that LD_{50} exposure would have on the foraging, exploratory and climbing behaviour of black rats.

In a previous rodenticide feeding study, APL project 2016/2230, replication of the OF test at day 0, 4, 7 and 13 resulted in experimental rats becoming acclimatised and bored with the relatively simple arena environment. This resulted in a decline in total travel distance during the OF test, even in control animals, over the duration of the study. For this study, the OF test arena was enriched with the placement of food treats at fixed positions. This change appeared to alleviate the acclimatised boredom effect, as no decline was observed in the travel distance for no-dose control rodents throughout the progression of the study.

For rodenticide dosed rats, exploratory and general locomotor activity of rats was only found to be impaired when clinical signs of anticoagulant toxicoses emerged. The delayed onset of incapacitating symptoms, that are associated with the mode of action of anticoagulant rodenticides, is widely credited as a key advantage over traditional poisons due to its role in preventing bait avoidance in target rodents. This finding is critical, as it demonstrates that there is a window of time before a lethal dose accumulates, in which baited rats are freely mobile and have the potential to contaminate feed stocks and the farm environment.

6.4 Conclusion

Take home messages:

- Rats consuming brodifacoum or bromadiolone at low range LD_{50} dose rates remained active and showed no clinical signs of anticoagulant toxicoses for up to 12 days – active brodifacoum and bromadiolone are excreted through faeces and are persistent in the liver tissues of rats throughout this time.
- Rats that consume brodifacoum or bromadiolone at high range LD_{50} dose rates retain normal exploratory behaviours until the clinical signs of anticoagulant toxicoses emerge, this can take 3-5 days after ingestion of the lethal dose - high concentrations of active brodifacoum and bromadiolone are excreted through faeces and are persistent in the liver tissues of rats at this time and therefore can directly contaminate animal feed and livestock.
- NB. Due to animal ethics conditions, this research was conducted with a relatively modest sample size (n=8) for each rodenticide treatment and laboratory Sprague Dawley rats were used in this study, an example of a brown/Norway rat species (*Rattus norvegicus*). As there are currently no laboratory rat strains that belong to the species *Rattus rattus* (black rat), care must be taken in extrapolating these results to this related species.

7. Implications & Recommendations

Issue

Rats exposed to field realistic doses of SGARs (LD_{50}) display no reduction in exploratory movement until the clinical signs of anticoagulant toxicoses emerge, this may take 3-4 days or longer following ingestion of a sufficiently lethal dose. Furthermore, during this active period, baited rats excrete high levels of active rodenticide through faeces. Due to the high-persistence and long liver half-lives of SGARs in animals, baited rats become reservoirs of active concentrations of rodenticides. When the incapacitating symptoms associated with anticoagulant toxicoses appear, rat mobility is impacted significantly. Unfortunately, this makes baited rodents more susceptible to predation from pigs, increasing the risk of acquisition of rodenticide residues in livestock.

Recommendation

- **Rat faecal pellets** should be considered a **high risk source** of rodenticide contamination.
- **Rat carcases** should be considered a **high risk source** of residue contamination and secondary poisoning.
- On farm, regular detection of rodent activity in production areas is vital and rodent carcases should be routinely removed.

Issue

There is scientific evidence linking intensive anticoagulant rodenticide (AR) use to the development of resistant rodent populations in central Europe and Asia (Takeda *et al* 2016) but to date, there is no data on the occurrence of AR resistance in Australia. The emergence of anticoagulant rodenticide resistance poses a serious threat to the profitability and integrity of the Australian pork industry. The continued use of ARs to combat potentially resistant rodent incursions unnecessarily exposes wildlife, domestic animals and livestock to the risk of non-target poisoning and the detection of rodenticide residues. The development of AR resistance in Australia would increase the burden of rodent infestations on farm, increase the risk of contamination of our food sources and threaten market access of Australian pork.

Recommendation

APL support an exploratory research project examining the genetic evidence for anticoagulant resistance in Australian rat populations.

Issue

This study has investigated the depuration of anticoagulant rodenticides in rats, but there is still lack of data and information on the contamination and depuration of anticoagulant rodenticides in pigs. With Pat Mitchell and APL's support, a project proposal was submitted by SARDI to a number of funding sources, to investigate the depuration of rodenticides in slaughter pigs and replacement gilts. Unfortunately, the project application was not successful, but the information gap still exists.

Recommendation

APL and SARDI to discuss the project proposal and how it fits within APL's priorities.

8. Intellectual Property

Not applicable.

9. Technical Summary

Title: Anticoagulant rodenticide excretion in rats following median lethal dose (LD_{50}) administration

The purpose of this research was to provide data relating to the excretion of rodenticides via rat faeces and urine and the mobility/foraging behaviours of rats following controlled exposure to common SGARs at dose rates reflective of farm exposure.

Take home messages:

- Rats that have consumed **sub-lethal doses** of SGARs will remain active for up to 12 days.
- Concentrations of active rodenticides are excreted through faeces and are persistent in the liver tissue of rats at this time.
- Rats that have consumed **lethal doses of SGARs** will retain normal activity until clinical signs of anticoagulant toxicoses emerge, this can take anywhere from 3-5 days after ingestion of the lethal dose.
- SGAR baited rodents do not excrete detectable levels of SGARs though urine. While not a high risk source of rodenticide contamination, rat urine is a hygiene issue.
- **Rat faecal pellets** should be considered a **high risk source** of rodenticide contamination.
- **Rat carcases** should be considered a **high risk source** of residue contamination and secondary poisoning.
- Ingestion of a sufficiently lethal dose of anticoagulant rodenticides (≥ high range LD_{50}) is required to cause death of baited rodents. However, this does not eliminate the risk posed by the faeces and liver tissue of baited rodents.
- It is important to remember that due to animal ethics conditions, this research was conducted with a relatively modest sample size (n=8) for each rodenticide treatment and laboratory Sprague Dawley rats were used in this study, an example of a brown/Norway rat species (*Rattus norvegicus*). As there are currently no current laboratory rat strains that belong to the species *Rattus rattus,* it is difficult to interpret from the results of this research, the impact that LD_{50} exposure would have on the foraging, exploratory and climbing behaviour of black rats.

10. Literature cited

Buckle, A. P., Klemann, N., & Prescott, C. V. (2012). Brodifacoum is effective against Norway rats (*Rattus norvegicus*) in a tyrosine139cysteine focus of anticoagulant resistance in Westphalia, Germany. *Pest management science*, 68(12), 1579-1585.

Buckle, A. P., Odam, E. M., & Richards, C. G. J. (1987). Chemical bait markers for the study of bait uptake by Norway rats. *Control of mammal pests/edited by CGJ Richards and TY Ku*.

Dubock, A. C., & Kaukeinen, D. E. (1978). Brodifacoum (Talon™ rodenticide), a novel concept. *Proceedings of the Eighth Vertebrate Pest Conference, Sacramento, California,* 127-137.

Duffus, J. (1993). Glossary for chemists of terms used in toxicology (IUPAC Recommendations 1993). *Pure and applied chemistry*, 65(9), 2003-2122.

Food And Agriculture Organisation of The United Nations. (2015). FAO Specifications and evaluations for agricultural pesticides – brodifacoum. http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/Specs/Brodifacoum_2015.pdf

Grand, M. (1976). [Experimental data on a new anticoagulant raticide: Bromadiolone].[French]. *Phytiatrie, Phytopharmacie*.

Greaves, J. H., & Ayres, P. (1969). Some rodenticidal properties of coumatetralyl. *Epidemiology & Infection*, *67*(2), 311-315.

Hadler, M. R., & Buckle, A. P. (1992). Forty-five years of anticoagulant rodenticides--past, present and future trends. *Proceedings of the Fifteenth Vertebrate Pest Conference*, 149-155.

Hadler, M. R., & Shadbolt, R. S. (1975). Novel 4-hydroxycoumarin anticoagulants active against resistant rats. *Nature*, *253*(5489), 275.

Meehan, A. P. (1978). Rodenticidal activity of Bromadiolone--a new anticoagulant. *Proceedings of the Eighth Vertebrate Pest Conference, Sacramento, California,* 122-126.

Meerburg, B. G., van Gent-Pelzer, M. P., Schoelitsz, B., & van der Lee, T. A. (2014). Distribution of anticoagulant rodenticide resistance in Rattus norvegicus in the Netherlands according to Vkorc1 mutations. *Pest management science*, *70*(11), 1761-1766.

Petterino, C., & Paolo, B. (2001). Toxicology of various anticoagulant rodenticides in animals. *Veterinary and human toxicology*, *43*(6), 353-360.

Redfern, R., Gill, J. E., & Hadler, M. R. (1976). Laboratory evaluation of WBA 8119 as a rodenticide for use against warfarin-resistant and non-resistant rats and mice. *Epidemiology & Infection*, *77*(3), 419-426.

Rowe, F. P., & Redfern, R. (1965). Toxicity tests on suspected warfarin resistant house mice (Mus musculus). *Epidemiology & Infection*, *63*(3), 417-425.

Rubinstein, I., van Breemen, R., Nosal, D. G., Weinberg, G., Hershow, R. C., & Feinstein, D. L. (2019). Should Cytochrome P450 Inducers be Used to Accelerate Clearance of Brodifacoum from Poisoned Patients?. *Drugs in R&D*, *19*(1), 67-71.

Samson, A. L., Ju, L., Kim, H. A., Zhang, S. R., Lee, J. A., Sturgeon, S. A., Sobey, C. G., Jackson, S. P. & Schoenwaelder, S. M. (2015). MouseMove: an open source program for semi-automated analysis of movement and cognitive testing in rodents. *Scientific reports*, *5*, 16171.

Silverman, R. B. (1980). A model for a molecular mechanism of anticoagulant activity of 3-substituted 4-hydroxycoumarins. *Journal of the American Chemical Society*, 102(16), 5421-5423.

Takeda, K., Ikenaka, Y., Tanikawa, T., Tanaka, K. D., Nakayama, S. M., Mizukawa, H., & Ishizuka, M. (2016). Novel revelation of warfarin resistant mechanism in roof rats (Rattus rattus) using pharmacokinetic/pharmacodynamic analysis. *Pesticide biochemistry and physiology*, *134*, 1-7.

11. Publications Arising

No publications have arisen from this report at the time of completion.

12. Appendix

12.1 Rat metabolic cages

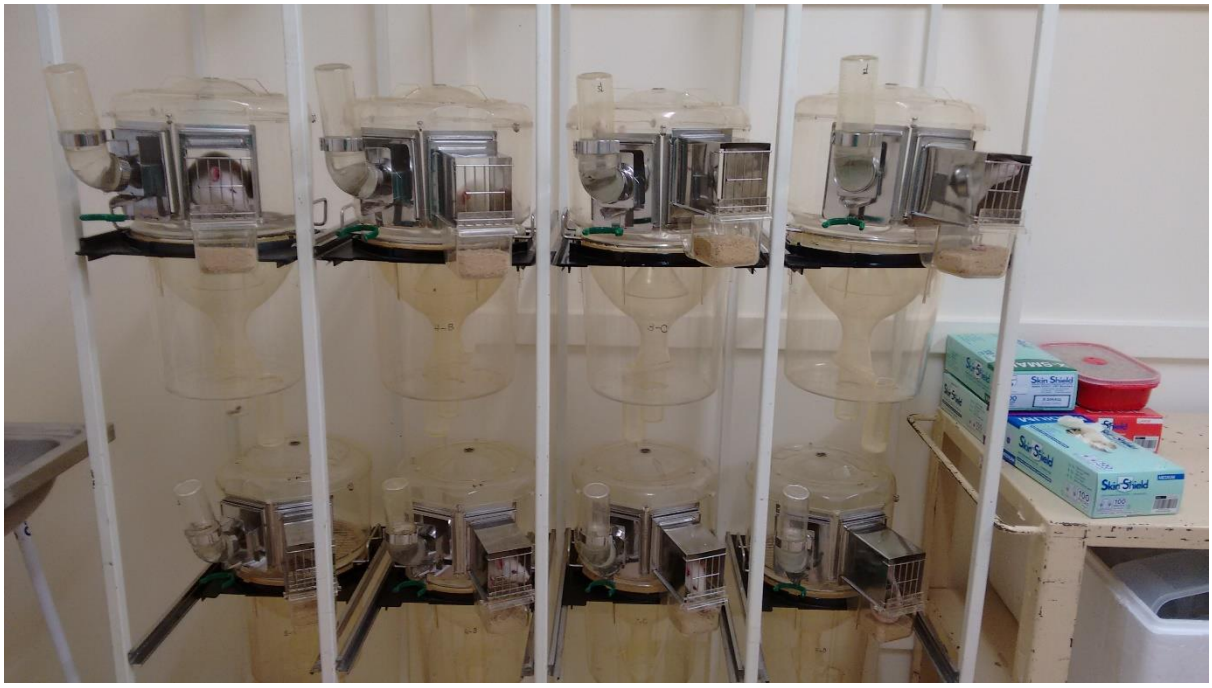

Image 1: Rat metabolic cages housed within a rack, rats within the cage have access to water and feed, two sample collection tubes are inserted from beneath the cage and the funnel cage design allows for the separation of faeces and urine.

Image 2: Faeces and urine is separated into two collection tubes.

12.2 Open Field (OF) test arena

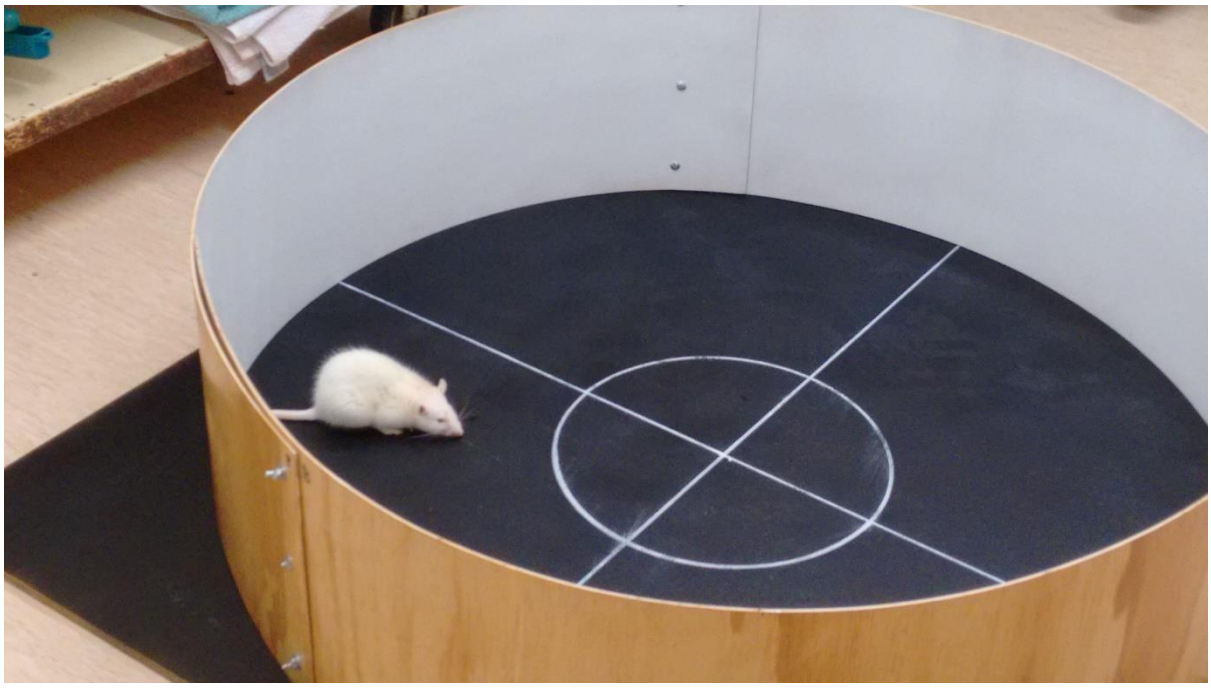

Image 3: Open Field (OF) test arena, the arena is circular with a one-metre diameter, the walls of the arena are 30cm high to prevent the rat from being distracted by objects or movement in the room.