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PRIORITY CONTRIBUTION

Evidence of pesticide resistance in medium-sized mammalian pests: a case study with 1080 poison and Australian rabbits

LAURIE E. TWIGG, GARY R. MARTIN and TIM J. LOWE
Vertebrate Pest Research Section, Department of Agriculture Western Australia, 100 Bougainvillea Avenue, Forrestfield, WA 6058, Australia

Summary

1. Toxicant-resistance is a potential, or very real, problem with many pest-control programmes world-wide. However, apart from rodents, pesticide-resistance has not been well documented in vertebrates. We assessed the potential impact of developing resistance to 1080 in rabbit populations with differing levels of historical exposure to 1080-baiting programmes in south-western Australia.

2. The sensitivity to 1080 of three out of the four populations of rabbits *Oryctolagus cuniculus* examined had decreased significantly since Australian rabbits were last tested over 25 years ago. The lethal dose$_{50}$ (LD_{50}) values for these populations, as determined from formal toxicity trials, ranged from 0·744 to 1·019 mg pure 1080 kg^{-1}, and were significantly greater ($P < 0·05$) than the previously reported values for Australian rabbits (LD_{50} range 0·34–0·46 mg pure 1080 kg^{-1}). The LD_{50} value for the fourth population (0·584 mg pure 1080 kg^{-1}), which has had the least exposure to 1080, did not differ from that reported previously ($P > 0·05$).

3. The lethal dose$_{99}$ (LD_{99}) values for the four rabbit populations tested ranged from 1·181 to 1·666 mg pure 1080 kg^{-1}, and suggested that, theoretically, all rabbits should be killed during routine baiting campaigns provided that there is no loss of active ingredient from the bait. In reality, the efficacy of 1080 poison bait laid in trails for controlling free-ranging rabbits was reduced in those populations where rabbits had decreased sensitivity to 1080. Mean reductions in rabbit numbers 7–9 days after trail baiting of resistant and sensitive populations ranged from 51·2% to 65·2%, and from 76·4% to 76·5%, respectively.

4. These findings suggest that genetic resistance to 1080 is developing in at least some populations of Australian rabbits. This has world-wide implications for agricultural protection and wildlife conservation programmes that rely on a 1080-baiting strategy for reducing the impact of vertebrate pests.

Key-words: baiting, efficacy, fluoroacetate, *Oryctolagus*, pest management

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Introduction

The development of resistance to rodenticides, particularly first and second generation anticoagulants, by commensal rodents and the associated impact of this on pest-control programmes world-wide is well documented (Redfern & Gill 1980; Misenheimer *et al*. 1994; Cowan *et al*. 1995). However, although genetic resistance against a variety of diseases and parasites is well recognized (e.g. rabbits and myxomatosis; Fenner & Ratcliffe 1965; Anderson & May 1982; Parer *et al*. 1994) little is known about the extent to which such resistance has developed against vertebrate pesticides in vertebrate groups other than rodents. The development of resistance to vertebrate pesticides by such animals would have serious implications for those control programmes that rely on these toxicants as part of their overall management strategy. For example, both Australia and New Zealand rely heavily on baiting programmes with Compound 1080 (sodium

Correspondence: Dr Laurie Twigg, Vertebrate Pest Research Section, Department of Agriculture Western Australia, 100 Bougainvillea Avenue, Forrestfield, WA 6058, Australia (fax +61 08 93662342; e-mail ltwigg@agric.wa.gov.au).

monofluoroacetate; NaFAc; hereafter referred to as 1080) for reducing the impact of introduced species on natural ecosystems and agricultural production (Seawright & Eason 1994; Saunders *et al*. 1995; Williams *et al*. 1995). Any loss of efficacy with these baiting programmes would have serious consequences for wildlife conservation and agricultural production in both countries. Consequently, as a case study we investigated whether resistance to 1080 could be developing in one of these introduced species, the European rabbit *Oryctolagus cuniculus* (L.).

The European rabbit is a major economic pest of agricultural industries in Australia and New Zealand, and this species can also have a significant impact on biodiversity, particularly the regeneration of native plants (Williams *et al*. 1995). In Australia, 1080 baits have been used to control *O. cuniculus* since the 1950s (Gooding & Harrison 1964; Wheeler & Hart 1979; McIlroy 1981; Oliver, Wheeler & Gooding 1982; Williams *et al*. 1995). In the intervening 50-plus years since the initial development of the 1080-based baiting strategies, the sensitivity of Australian populations of *O. cuniculus* to 1080 has only been assessed on two occasions, and both of these studies were conducted more than 25 years ago (Wheeler & Hart 1979; McIlroy 1981, 1982). At that time, the sensitivity of Australian rabbits to 1080 had not changed appreciably since the 1960s. However, when the efficacy of these baiting programmes was reassessed in Western Australia (WA) in the 1970s, there was a suggestion that the effectiveness of some 1080-bait products had decreased since the late 1950s/early 1960s, but the possible mechanisms for this were not well understood (Oliver, Wheeler & Gooding 1982).

Our current study therefore had three main aims: (i) to assess the sensitivity to 1080 of European rabbit populations from south-western Australia with known long-term exposure to rabbit control programmes that employ 1080; (ii) to determine whether any decrease in the sensitivity to 1080 displayed by these rabbits has actually impacted on the efficacy of 1080-baiting programmes against free-ranging rabbit populations; and (iii), on the basis of the above findings, to postulate whether any loss in the effectiveness of 1080-baiting programmes was due to either genetic resistance or learned behaviour, or both. The implications of our findings for vertebrate pest-control programmes, and the possible ecological basis for the development of resistance to vertebrate pesticides, are then discussed.

Methods

AVAILABLE BAITING METHODS

Two different baiting strategies are available for controlling rabbits with 1080 bait in Australia; 'conventional' oat baiting and the one-shot oat baiting technique (Gooding & Harrison 1964; Williams *et al*. 1995). The conventional 1080 oat bait technique was developed in the early 1950s. This baiting technique requires landholders to provide three pre-feeds of unpoisoned oats to rabbits over an 8–12-day period prior to the introduction of the poisoned oat bait. With this method all oats in the poison bait contain 1080 (0·02–0·04 mg 1080 oat^{-1}) and bait is laid at around 5–7 kg km^{-1}, depending upon rabbit density. The 1080 one-shot oat baiting technique was first used in WA in 1960. Prior to this, the conventional 1080 oat baiting technique was used. With the 1080 one-shot, both the poisoned and unpoisoned oats are laid simultaneously in the same trail. Poisoned oats nominally contain 4·5 mg 1080 oat^{-1} (theoretically enough to kill two to three adult rabbits) and these are diluted with filler (unpoisoned) oats to obtain a 0·5% or 1·0% bait mix (i.e. the ratio of poisoned to unpoisoned oats). The 1·0% mix is mainly used in the higher rainfall areas in the south-west of WA. Depending upon rabbit density, the rate of lay is around 6 kg km^{-1} trail^{-1}, usually with two to three parallel trails laid. Although baiting with one-shot oats is an effective control technique, overall kill rates with this technique can be slightly less than those achieved with conventional oat bait.

THE RABBIT POPULATIONS

Four separate populations of rabbits from WA were investigated: Dalyup (Esperance; 33°52′ S; 121°54′ E), Chapman Valley (28°20′ S; 115°00′ E), Boxwood Hill (34°17′ S; 118°46′ E) and Mt Barker (34°38′ S; 117°40′ E; the same population as studied by Wheeler & Hart (1979)). Three of these populations were deliberately chosen from areas with a history of regular use of 1080 to control rabbits. Based upon historical records, the use of 1080 oats for rabbit control was: Dalyup (Esperance) ≥ Chapman Valley > Mt Barker > Boxwood Hill (C. Parry & R. Gwynne, Department of Agriculture, WA, personal communication).

The four rabbit populations had passed through at least one to three breeding seasons (*c.* 10–36 months) since their last known exposure to 1080-baiting programmes prior to their inclusion in the toxicity and/or efficacy trials. At present, there are no reliable techniques to age rabbits once they obtain adult weight (*c.* 1200 g), although free-ranging rabbits in south-western Australia can live up to 5+ years (Wheeler & King 1980; Twigg *et al*. 1998, 2000). However, only adult rabbits were used in the toxicity trials, and due to the timing (i.e. trials conducted well after the breeding season was completed) the majority, if not all, rabbits, including the new recruits, on site during the efficacy trials were also adult.

TOXICITY TRIALS

To enable the determination of the sensitivity of the four populations to 1080, rabbits were collected from each population by live-trapping with cage traps between March and June 2000. There was evidence

(e.g. fresh fur growth around the eyes) that all four populations had been exposed to myxomatosis prior to the live-capture being undertaken. All rabbits were acclimatized to captivity for at least 2 weeks prior to their inclusion in the trials. Rabbits were held in individual cages in an animal house maintained at 23 ± 2 °C with a 12 : 12 h photoperiod. Rabbits were fed with commercial 'rabbit and guinea-pig' pellets (Thompson & Redwood Produce Supplies, WA) and, occasionally, carrots and a rabbit lucerne mix. Food and water were provided *ad libitum*. Except for rabbits from Mt Barker, which were tested outside the known period of activity for rabbit haemorrhagic disease (RHD), all other rabbits were inoculated with Cylap vaccine (Websters) as a preventative measure against RHD.

The toxicity trials were undertaken between April and July 2000. Dose groups included only adult (> 1199 g) individuals of both sexes, and excluded any known pregnant females (determined by palpation). 1080 was administered in aqueous solution by intraperitoneal injection (IP). Dosing solutions were corrected for the 97% purity of the parent Tenate Brand of 1080 powder used (as determined by HPLC) and were therefore equivalent to 100% pure NaFAc. The same glass syringe was used for all dosing, and the same two dosing solutions were used for the four populations tested: 2·0 mg NaFAc ml^{-1} for the lower doses and 5·0 mg NaFAc ml^{-1} for the higher dose levels. In addition, other rabbits from each population were dosed with deionized water only. Dose volumes ranged from 0·2 to 0·6 ml. All dosing was undertaken between 09:00 and 12:00 hours. The dosing solutions were stored at 23 °C. These solutions are known to be stable under such conditions for at least 6, and probably 12, months (Mead 1980; L.E. Twigg, unpublished data). Frequent observations were undertaken for the first 5–7 h after dosing, and all rabbits were inspected at least daily thereafter for a total of 10 days. Any mortality and signs of poisoning during this period were considered to have been caused by 1080. Most mortality in rabbits dosed with 1080 occurs within 24–48 h (McIlroy 1981; this study). Approximately 4 months were required to complete all four toxicity trials.

Working within the recommendations stipulated by the Animal Ethics and Experimentation Committee (AEEC) regarding animal welfare concerns, the dosing protocol used was a modification of the formal lethal $dose_{50}$ (LD_{50}) procedure (the amount of toxin that will theoretically kill 50% of test subjects). On day 1, six rabbits (three males and three females) were dosed at the published formal LD_{50} value of 0·456 mg NaFAc kg^{-1} for WA rabbits (Wheeler & Hart 1979). The mortality that occurred within 24 h of administering this dose was then used as the basis for determining the next dose levels. Observation and dosing continued until the level at which no deaths occurred, and the level which resulted in 90–100% mortality, were determined. Once these patterns were established, all remaining rabbits were dosed at the appropriate level until six to 10 rabbits were tested per dose group, with approximately equal sexes per group. Thus, rather than dosing all rabbits on the same day as per standard LD_{50} procedure, because of the restriction of needing to observe prior mortalities, our procedure meant that about 2–3 weeks were required to complete the toxicity trial for each population. A 1·375 dose progression was used and, based upon their sex, all rabbits were allocated to a dose group at random. Care was also taken to ensure that the location of individuals within the animal room was stratified. Virtually all rabbits were used only once. However, because we were restricted in the number of rabbits we could collect, and because of the unexpectedly high tolerance of some populations, five rabbits from Dalyup (Esperance) and four rabbits from Mt Barker, which survived an earlier low dose of NaFAc, were redosed at a higher level after allowing at least 3 weeks to ensure their full recovery (Twigg & King 1991). In living animals, fluoroacetate (1080) is mainly detoxified in the liver via an enzymatic process involving a unique glutathione transferase and glutathione (Mead, Moulden & Twigg 1985; Twigg, Mead & King 1986; Twigg & King 1991). Most fluoroacetate is excreted within 2–3 days, and the half-life in mammals is 2–3 h (Twigg & King 1991; Gooneratne *et al*. 1994).

LD_{50} values and their 95% confidence limits (CL) were calculated using two procedures: (i) moving averages and interpolation (Thomson 1947), which is the same procedure as used by Wheeler & Hart (1979), and (ii) probit analysis with a binomial distribution (Finney 1964). The response variate was the number of deaths within a population. This enabled examination of any differences between the analytical techniques commonly used to determine lethal dose estimates. The lethal $dose_{99}$ (LD_{99}; the amount of toxin that will theoretically kill 99% of test subjects), possible difference between the sexes, and the interaction of dose and population were also determined using the probit procedure and deviance ratios. Deviance ratios approximate *F*-tests (Zar 1984).

There are two main procedures currently recognized for determining the sensitivity of animals to 1080. First, the more formal toxicity trials (Wheeler & Hart 1979; McIlroy 1981, 1982), including the approximate lethal dose (ALD) procedure (Calver *et al*. 1989; Martin & Twigg 2002). These procedures provide estimates for ALD, LD_{50} and LD_{99}, etc. Secondly, the plasma citrate technique can be used to compare the relative sensitivity to 1080 of animals with differing degrees of exposure to fluoroacetate-bearing vegetation (Twigg & King 1991). The latter relies on a dose–response increase in the concentration of citrate in the plasma of animals administered known amounts of 1080. At the same dose level, the more sensitive animals accumulate significantly greater amounts of citrate in their plasma in response to the administration of 1080. Thus, to demonstrate further that dosed rabbits were in fact affected by 1080, and to establish if this technique was suitable for detecting relatively small differences in

sensitivity between populations, changes in plasma citrate were also determined for some rabbits from each population after the formal toxicity trials were complete.

Increases in plasma citrate concentration were determined after rabbits from each population were administered known amounts of 1080, mostly via the IP route. Dosed rabbits were killed by cervical fracture approximately 3 h after dosing, or once they displayed obvious signs of poisoning. Blood samples (*c*. 0·5 ml) were immediately collected via cardiac puncture using heparinized syringes. After centrifugation (2500 ***g*** for 10 min), plasma was transferred into labelled vials and stored at –7 °C until analysis. Citrate analyses were undertaken using the Boehringer Mannheim citrate assay kit (catalogue no. 139 076; R-Biofarm GMBH, Darmstadt, Germany) and a Roche, Cobas Mira-S autoanalyser (Roche Australia, Castle Hill, New South Wales, Australia). The samples (25 µl) were deproteinized with two equivalent volumes of 1 M perchloric acid, and then neutralized with 0·3 M potassium carbonate before analysis. Plasma citrate concentrations are expressed as µg ml^{-1}, and changes in these levels in response to dosing are presented as increases above the base level (time 0) concentration for each individual (Twigg & King 1991).

FIELD EFFICACY TRIALS

The efficacy trials were undertaken during the summer–autumn on farming properties at Boxwood Hill/Wellstead and Esperance in the southern agricultural region (during 2000–01) and at Chapman Valley (Yuna) in the northern agricultural region (2001–02) of WA. Most broadacre control of rabbits in WA is undertaken during this summer drought period (Oliver, Wheeler & Gooding 1982; Williams *et al*. 1995; Twigg *et al*. 1998). The major agricultural enterprises in these regions are merino wool, cereal grain, canola and beef cattle production. Land systems in these areas mainly comprise aeolian sands over laterite cap-rock, and are often interspersed with remnants of native vegetation (Twigg *et al*. 1998). Climate is typically mediterranean, with an annual rainfall of about 450 mm (Boxwood Hill/Wellstead and Chapman Valley) to 621 mm (Esperance). However, rainfall in these regions can be highly unpredictable, and some summer rainfall occurred prior to the Esperance trials that resulted in a small amount of 'green-pick' (feed) at these sites. Very little, if any, rainfall occurred during the trials in the other two regions. Six properties, of at least 1000 ha each, were used at each location in the southern agricultural region, and one property (Yuna; same population as used in the toxicity trials) was used in the northern agricultural region. Myxomatosis and RHD were not active during any of the trials.

A 1·0% 1080 one-shot oat bait mix was used for all poison bait trails. All trails were laid at approximately 6 kg km^{-1} per trail using a disk-style bait layer. Depending upon rabbit density, two to three parallel trails approximately 20 m apart were used when laying the one-shot bait. These trails were placed in the rabbit-feeding areas approximately 20 m, 40 m and 60 m out from the rabbit refuge. All paddocks were destocked during the 1080 one-shot trail-baiting treatments. All trails were checked over the first 7 days to ensure that they were not eaten out, which would necessitate their relaying. 'Null' trails, containing only unpoisoned (filler) oats, were laid on each property in a similar way, except for the exclusion of the poisoned oats and the use of only one to two trails that were laid approximately 20–40 m from the rabbit refuge.

Rabbit numbers on all sites (poisoned or unpoisoned oats) were monitored by before and after treatment spotlight counts along permanently marked transects (700–3800 m long). Counts were undertaken for three consecutive nights before, and 7 days after (i.e. over nights 7, 8 and 9 post-baiting), treatment began. These counts commenced 1 h after dusk, and the same observer/counter and 4-wheel drive vehicle (travelling at 15 km h^{-1}) were used. The maximum number of rabbits over the counts for the three consecutive nights, standardized to rabbits km^{-1}, was used as the index of abundance for that census for each site (Twigg *et al*. 1998). Efficacy was then determined as the percentage reduction in rabbit numbers between the before and after treatment surveys. The reductions on the poisoned sites were adjusted relative (proportionally) to those changes that occurred on the unpoisoned sites (Twigg *et al*. 2001).

Results

TOXICITY TRIALS AND LD VALUES

The probit analysis undertaken fitted probits for all four rabbit populations simultaneously. The effect of dose (d.f. 1, 11, deviance ratio = 61·9, $P < 0·001$) and population (d.f. 3, 11, deviance ratio = 7·3, $P < 0·001$) were significant factors affecting the survival of rabbits. However, the interaction of dose and population was not significant (d.f. 3, 11, deviance ratio = 1·1, $P = 0·261$), indicating that the effect of dose rate was the same in all populations but death rates were higher in some populations than in others. Therefore, as there were no significant differences in the overall response of rabbits to 1080, the effect of sex (d.f. 1, 8, deviance ratio = 0·9, $P = 0·343$) and the interaction of dose and sex (d.f. 1, 8, deviance ratio = 0·2, $P = 0·667$) on the response of rabbits were also examined. Neither factor was significant, indicating that the response of male and female rabbits was similar. Consequently, the LD values for each population were determined separately using the pooled results for male and female rabbits.

The mortality of rabbits resulting from the administration of 1080, and the associated LD_{50} and LD_{99}, are given in Tables 1 and 2. All rabbits that died during these trials did so within 24 h. The rabbit population from Boxwood Hill has had the least amount of past

Table 1. Mortality data for populations of European rabbits from various locations in Western Australia. Dose rates are mg pure 1080 kg^{-1} (NaFAc; i.e. corrected for purity of powder). 1080 was administered via IP injection and the resulting mortality was recorded over 10 days. F/M: ratio female to male

		Dose (mg NaFAc kg^{-1})						
Location/parameter	F/M	0	0·332	0·456	0·628	0·864	1·188	1·634
Dalyup (Esperance)	16/15							
No. dosed		3	0	7	7	7	8	7
No. deaths		0	0	0	0	3	5	6
Chapman Valley	14/21							
No. dosed		3	0	7	9	11	7	
No. deaths		0	0	0	1	6	7	
Boxwood Hill	20/17							
No. dosed		2	8	8	11	8	5	
No. deaths		0	0	2	8	6	5	
Mt Barker	17/24							
No. dosed		3	4	10	12	11	7	
No. deaths		0	0	0	5	6	7	
Mt Barker*	31 males	0	0·261	0·326	0·408	0·510	0·637	
No. dosed		6	5	5	5	5	5	
No. deaths		0	1	0	3	3	4	

*Rabbits only used once, same population/area as our study; from Wheeler & Hart (1979).

exposure to 1080-baiting campaigns and this population was also the most sensitive to 1080. While it is acknowledged that our dose group sizes could not be identical for all populations examined, the 95% CLs did not overlap for many of the tests undertaken. This indicates that the LD_{50} values for populations from Mt Barker, Chapman Valley and Dalyup (Esperance) were all significantly greater ($P < 0·05$) than that recorded for the Mt Barker population in the late 1970s. However, the LD_{50} value for the Boxwood Hill rabbits was similar to that recorded for the Mt Barker rabbits irrespective of when the sensitivity of the latter population was determined. The LD_{50} values for the Dalyup (Esperance) and Chapman Valley rabbit populations were also significantly greater ($P < 0·05$) than those of the Boxwood Hill population (Table 2). The LD_{99} values ranged from 1·181 to 1·666 mg 1080 kg^{-1} for the four populations. The greatest upper CL was for the rabbits from Dalyup (1·89 mg kg^{-1}; Table 2), and this population has had considerable past exposure to 1080-baiting programmes. The single Dalyup rabbit surviving the 1·634 mg kg^{-1} dose was female. Although not significant ($P > 0·05$), the LD values for female rabbits were higher than those of males. The LD_{50} values estimated with probit procedure were also marginally higher than the corresponding values estimated using the moving average technique (Table 2).

PLASMA CITRATE

Mean pre-dosing levels of citrate in the plasma of rabbits ranged from 57 µg ml to 94 µg ml (Table 3) and were similar to those of other mammals (Twigg & King 1991). Post-dosing blood samples were collected either when animals displayed obvious signs of 1080 poisoning or approximately 3 h after the administration of 1080. All rabbits that succumbed during the plasma citrate trials displayed obvious signs of 1080 poisoning.

The mean changes in plasma citrate concentration 3 h after rabbits were given deionized water only was $-1·3 \pm 7·6$ µg ml^{-1} (SEM; $n = 3$ for Chapman Valley, and 3 Boxwood Hill rabbits). Although the Dalyup rabbits had the smallest increase, the elevation in plasma citrate 3 h after the administration of 1080 was similar between the rabbits from the three populations that were administered 0·59 mg 1080 kg^{-1} (Table 3; ANOVA, d.f. 2, 33, $F = 0·30$, $P = 0·74$). This suggested that the plasma citrate technique was not suitable for determining small changes in the sensitivity to 1080 between different rabbit populations. However, the route of administration did influence the level of citrate recorded in the plasma 3 h after dosing. The levels recorded in orally dosed rabbits were significantly less than those observed when 1080 was administered via IP injection (Table 3, *t*-test, d.f. 9, $t = 3·11$, $P = 0·013$). This was probably related to an increase in the lag phase associated with fluoroacetate poisoning in animals that are orally dosed (Buffa, Guarriero-Bobyleva & Costa-Tiozzo 1973; Twigg & King 1991).

FIELD EFFICACY TRIALS

On the Dalyup site at Esperance, where rabbits were less sensitive to 1080 (Table 2), the mean reduction in rabbit numbers 8 days after baiting was $51·2 \pm 11·1\%$ (SD, $n = 2$; Table 4). In contrast, the reduction in rabbit numbers for Boxwood Hill, the area with the more sensitive rabbits, was $76·5\% \pm 11·1\%$ (SD, $n = 3$; Table 4). However, the decrease in sensitivity to 1080 and/or the impact of this resistance on rabbit control programmes appeared to vary within a region. The subsidiary groups of properties used in each region were selected

Table 2. The LD_{50} and LD_{99} values, with 95% CL, for populations of European rabbits from various locations in Western Australia. Values are mg pure 1080 kg^{-1} (NaFAc; i.e. corrected for purity of powder) and were calculated with either the moving average or the probit procedure (see the Methods)

	mg pure NaFAc kg^{-1}			
Population	LD_{50}	95% CL	LD_{99}	95% CL
Dalyup (Esperance)				
Moving average	1·019	0·851–1·221		
Probit	1·095	0·953–1·237	1·666	1·445–1·887
Chapman Valley				
Moving average	0·820	0·730–0·922		
Probit	0·857	0·720–0·993	1·427	1·192–1·662
Boxwood Hill				
Moving average	0·584	0·496–0·687		
Probit	0·610	0·493–0·728	1·181	0·971–1·391
Mt Barker				
Moving average	0·744	0·652–0·849		
Probit	0·780	0·662–0·898	1·351	1·128–1·574
All females				
Moving average	0·878	0·670–1·151		
Probit	0·873	0·753–0·993	1·659	1·371–1·947
All males				
Moving average	0·754	0·673–0·844		
Probit	0·792	0·684–0·901	1·579	1·314–1·844
All rabbits				
Moving average	0·791	0·706–0·886		
Probit	0·829	0·749–0·909	1·618	1·361–1·875
Mt Barker*				
Moving average	0·46	0·34–0·61		

*Same population/area as used in our study; taken from Wheeler & Hart (1979).

Table 3. Pre-dosing levels (base level), and increases above base-level, in plasma citrate concentration of European rabbits from various locations in Western Australia dosed with 1080 (mg pure 1080 kg^{-1}). 1080 was administered by IP injection except for the oral administration to some Boxwood Hill rabbits

			µg citrate ml^{-1} plasma				
Population	Dose rate (mg kg^{-1})	*n*	Maximum	Mean	SD	Mean time to sample (min)	'Natural' deaths
Dalyup (Esperance)	Base level	16	93·6	63·4	14·9	–	–
	0·59	8	82·5	59·2	18·0	194	1
	1·02	8	135·0	77·9	38·3	177	2
Chapman Valley	Base level	17	121·7	93·7	16·2	–	–
	0·81	13	127·0	53·7	37·8	205	1
Boxwood Hill	Base level	14	75·4	56·7	10·4	–	–
	IP – 0·59	5	124·4	69·1	35·0	186	2
	Oral – 0·59	6	49·6	20·5	15·0	211	0
Mt Barker	Base level	21	106·0	68·1	16·2	–	–
	0·59	21	162·0	73·5	51·5	155	3

to be the 'opposite' with respect to the known history of 1080 use for those rabbit populations used in the toxicity trials (i.e. the Dalyup and Boxwood Hill populations). Thus, the properties selected for the Gibson area (Esperance) would be expected to give higher kill rates than those achieved on the Dalyup property and, conversely, the reductions for the Wellstead property would be expected to be lower than those achieved at Boxwood Hill. This was, in fact, reflected in the percentage knockdowns achieved at these subsidiary sites. Mean reductions for the Gibson and Wellstead properties were 76·4% and 61·6%, respectively (Table 4). Due mainly to the activity of RHD in the preceding months, it was not possible to find any properties with sufficient rabbit densities for efficacy trials in the Chapman Valley region, except for the property at Yuna where the rabbits used in the toxicity trials had originated. On this property, rabbit numbers were only reduced by 65% within 7–9 days of baiting (Table 4). This provides further evidence of the impact of the

Table 4. The percentage reductions in rabbit numbers following the application of 1080 one-shot oats on properties in three regions in the agricultural region of Western Australia

Area	Mean rabbits per km		% Decrease relative to unpoisoned sites*				History of 1080 use
	Before	After	Mean	SD	Range	*n*	
Boxwood/Wellstead region							
Boxwood Hill†	116·8	14·9	76·5	16·2	58·5–89·9	3	Low/moderate
Wellstead	66·0	26·3	61·6	11·1	53·7–69·4	2	Moderate
Unpoisoned sites	56·0	47·9				5	
Esperance region							
Dalyup†	89·4	47·0	51·2	11·8	41·8–60·1	2	High
Gibson	82·5	27·9	76·4	21·4	61·3–91·5	2	Moderate/low
Unpoisoned sites	67·1	72·8				4	
Chapman Valley region							
Yuna†	15·4	7·0	65·2	2·0	63·8–66·6	2	High
Unpoisoned sites	15·0	19·7				2	

*The percentage decrease has been calculated relative to those changes that occurred on the unpoisoned experimental control sites that were situated on the same properties or adjacent properties (see the Methods). Rabbit numbers were resurveyed over days 7–9 after bait was laid.
†These populations are the same as the rabbits used in the LD_{50} trials.

development of 1080 resistance on the effectiveness of some rabbit control programmes in WA.

Discussion

FACTORS AFFECTING TOXICITY TRIALS WITH 1080

The conditions under which any toxicity trial is undertaken can influence its outcome, and unless the conditions are similar between test procedures it can be difficult to make meaningful comparisons between studies (Griffith 1964; McIlroy 1981; Oliver & King 1983; Martin & Twigg 2002). Factors that can affect the outcomes of toxicity trials involving 1080 are discussed fully by McIlroy (1981); however, ambient temperature probably has the greatest influence on trial outcomes (McIlroy 1981; Oliver & King 1983). The impact of temperature is mainly restricted to those temperatures outside the 'normal' thermal range of the species tested. Variables such as group size, dosing protocols and the origin of test subjects appear to have minimal effect on the results of these trials. Varying dose group sizes from three to five individuals per group has little effect on LD_{50} estimates, but the use of larger dose groups does result in narrower CLs (McIlroy 1981). The use of small dose groups also has little effect on the determination of the ALD of 1080 to a wide range of species (Calver *et al*. 1989).

The conditions under which the sensitivity of rabbits to 1080 was determined during the laboratory-based toxicity trials in our study, and in those of Wheeler & Hart (1979) and McIlroy (1981), were very similar. The photocycle was 12 : 12 h, room temperature was approximately 22 °C, aqueous dosing solutions were used, dose volumes were similar, the observation periods were 7–10 days, only adult rabbits were used, and all LD_{50} estimates were calculated using the moving average/median dose procedure of Thomson (1947). One difference between the three studies was that we administered 1080 via IP injection but in the other two studies 1080 was given orally. However, the route of administration is not believed to unduly influence the ultimate sensitivity of animals to 1080 (Chenoweth & Gilman 1946; McIlroy 1981), rather, it can influence the lag-phase associated with 1080 poisoning, as orally administered 1080 appears to take longer to be absorbed (Buffa, Guarriero-Bobyleva & Costa-Tiozzo 1973). Furthermore, on those few occasions where small differences have been observed, IP injection gave slightly lower LD_{50} estimates than similar trials where individuals of the same species were dosed orally (McIlroy 1981).

Circadian rhythm can also influence the ability of some animals to metabolize toxins (Radzialowski & Bousquet 1967), so it is important that the administration of test drugs is undertaken at a similar time of day for each test animal (McIlroy 1981). Again, in the three studies investigating the sensitivity of Australian rabbits to 1080, rabbits were dosed at similar times each day. Furthermore, although there can be small differences in the LD_{50} estimates obtained with the moving average and the probit procedures, these differences are small and estimates from the two procedures are generally in close agreement (Table 2, this study; Bliss 1938). However, probit analysis has the added advantage that the response of separate populations can be compared statistically, and any interaction between the drug administered and the sex of test subjects can be examined using deviance ratios. Our probit analyses showed that none of these factors were adversely affecting the results of our toxicity trials. Thus, for the above reasons, we are confident that any differences observed in the sensitivity to 1080 of rabbits from various localities in Australia, or as indicated from earlier studies, are in fact due to real differences in the sensitivity of these populations to 1080.

SENSITIVITY OF RABBITS AND FIELD EFFICACY

The sensitivity to 1080 of three of the four populations we examined had decreased significantly compared with that reported previously for WA rabbits (Wheeler & Hart 1979). The degree to which this tolerance has developed was also correlated with the past use-patterns of 1080 in WA, with those populations having the greatest exposure to 1080 baiting also having the greatest tolerance. The Boxwood Hill population has had the least exposure to 1080-baiting campaigns, and the sensitivity of this population was not significantly different to that determined over 25 years ago for Australian rabbits (Wheeler & Hart 1979; McIlroy 1981). McIlroy (1981) determined the sensitivity to 1080 of several rabbit populations from eastern Australia. The LD_{50} and 95% CLs for these rabbits ranged from 0·34 to 0·40 and from 0·29 to 0·54 mg pure 1080 kg^{-1}, respectively. These values are not significantly different from those reported by Wheeler & Hart (1979) or from those found for the Boxwood Hill rabbits in our study. Wheeler & Hart (1979) also reported that the sensitivity of wild WA rabbits when tested in the field over a 48-h observation period, and with rabbits kept in cardboard boxes, was less than that which they obtained during their formal laboratory trials with wild rabbits from Mt Barker. However, they believed this difference to be related to differences in methodology, particularly the possible effect of uncontrolled environmental parameters on rabbits during their field-based toxicity trials.

Mean reductions in rabbit numbers obtained with 1080 one-shot bait trails over the summer–autumn drought period reported previously for the south-west agricultural region of WA range from 72% to 88% (unadjusted for untreated sites; Oliver, Wheeler & Gooding 1982). Similar unadjusted values for our rabbit populations ranged from 49% (Dalyup) to 84% (Boxwood Hill). This reinforces the notion that the reduced efficacy achieved at the Dalyup, Wellstead and Chapman Valley sites resulted from the development of genetic resistance to 1080 in these rabbit populations, as indicated by the outcomes of our toxicity trials. Maximum kills during 1080-baiting programmes are usually achieved within 8–12 days (Oliver, Wheeler & Gooding 1982; Williams *et al*. 1995), and this was also seen during a more detailed study over 7–21 days at the Boxwood Hill site (L.E. Twigg, unpublished data). However, mean rates of kill with 1080 one-shot oats can be as low as 50% during the wet winter months, and this has been attributed to the leaching of the water-soluble 1080 from the oat bait (Wheeler & Oliver 1978; Oliver, Wheeler & Gooding 1982).

The reduced sensitivity to 1080 of some rabbit populations from WA, and the relatively poor performance of 1080 trail baiting in corresponding areas, suggests that genetic resistance to 1080 has developed in some rabbit populations in Australia. It also suggests that this increased tolerance has developed over the 25-plus years since the sensitivity of WA rabbits was last determined. There are several possible causal mechanisms for the development of such a tolerance. Current baiting programmes that use 1080 in WA rely almost exclusively on the one-shot oat control technique. With this procedure not all oats contain the active ingredient, but the individual poisoned oats contain sufficient 1080 to kill, theoretically, about three 1500-g rabbits (assuming the LD_{50} for WA rabbits is still 0·46 mg 1080 kg^{-1}). However, the maximum upper CL for the LD_{99} in our study was 1·887 mg kg^{-1} for the Dalyup population, and 1·947 mg kg^{-1} with all females pooled (Table 2). This equates to 2·83 mg and 2·92 mg, and 3·77 mg and 3·89 mg of 1080 for a 1500-g and 2000-g rabbit, respectively. Adult rabbits in Australia range from 1200 to 2200 g (Williams *et al*. 1995; Twigg *et al*. 1998). Theoretically, there is enough 1080 on one poisoned oat to poison the largest rabbits (2200 g) but some rabbits could ingest sublethal amounts if the 1080 in the bait was reduced by some mechanism such as rainfall and/or physical abrasion.

There are three plausible mechanisms regarding how the ingestion of sublethal doses by rabbits could occur. (i) 1080 is highly water soluble and can readily leach from baits (Wheeler & Oliver 1978). (ii) Rabbits and some non-target species are known to readily dehusk oats, consuming only the kernel. Approximately 80% of the 1080 in one-shot oats is in or on the husk, with the remainder in the kernel (L.E. Twigg, unpublished data). (iii) Some rabbits may only consume a relatively small proportion of a poisoned grain and survive. All three mechanisms could cause a reduction in the amount of 1080 ingested by some rabbits, thereby resulting in the sublethal dosing of some individuals. This in turn could lead to increased selection for the development of tolerance to 1080 in these rabbit populations. That is, frequent baiting campaigns select for increased tolerance to 1080 under these conditions such that future breeding stocks may be limited locally to only those individuals that survive routine baiting programmes. In a manner similar to this, the development of tolerance to fluoroacetate (1080) has been demonstrated in laboratory rats *Rattus* sp. and house flies *Musca domestica* by dosing successive generations at LD_{75}–LD_{90} levels. The LD_{50} was increased from 2·0 to 3·5 mg 1080 kg^{-1} (1·8-fold) over five generations for the rats (Howard, Marsh & Palmateer 1973), and from 0·6 to 4·2 µg fly^{-1} (seven-fold) over 25 generations for the flies (Tahori 1963). The development of bait/poison shyness could provide an alternative hypothesis for explaining the relatively poor efficacy we observed at those sites with the more tolerant rabbits; however, such selection would not result in the development of genetic resistance to 1080, as seen in some of our rabbit populations.

It is possible that the green-pick (which could make some rabbits less inclined to take bait) may have influenced the poor result obtained at the Dalyup site at

Esperance. However, this is unlikely because the summer rainfall that occurred did so across most of this region and, although there were site differences, a small amount of green feed was present at all sites. Thus, any effects resulting from the presence of the green-pick should have occurred at some level at all sites. Further, the sites at Wellstead and Chapman Valley, with a known history of 1080 use, also displayed reduced efficacy compared with those sites with a more limited past-use of 1080. However, to understand just how widespread the phenomenon of 1080 resistance is, the efficacy of 1080 rabbit control programmes would need to be determined on a number of properties with differing historical patterns of 1080 use in a variety of regions across Australia. Nevertheless, if the detrimental impact of developing resistance to 1080 on rabbit control programmes is widespread, then this will impinge upon the effectiveness of such control programmes in at least some regions of Australia, particularly, for example, those areas with unpredictable 'out-of-season' summer rainfall.

We know of no studies other than those with rodents (Redfern & Gill 1980; Misenheimer *et al*. 1994; Cowan *et al*. 1995) that have demonstrated the development of genetic resistance to pesticides in free-ranging populations of medium-sized vertebrates. However, resistance to diseases and parasites, and the coevolution of these 'pathogens' and their hosts, are known to be related in part to the frequency of exposure of the host to a particular causative agent (Anderson & May 1982). The interactions of disease virulence and transmission are equally important factors in the evolutionary response of these pathogens and their hosts. It has been argued that such responses account for much of the polymorphism found in natural populations (Anderson & May 1982), and that resistance may ultimately occur with many biological control agents (Cooper 1999). With European rabbits, genetic resistance to the myxoma virus developed within 20 years of its introduction into rabbit populations in both Australia and England, where rabbit mortalities have been reduced from 99% to around 50–70%. Attenuated strains of the virus have also appeared within this period (Fenner & Ratcliffe 1965; Anderson & May 1982; Flowerdew, Trout & Ross 1992; Parer *et al*. 1994). This demonstrates that European rabbits are capable of rapidly developing the appropriate mechanisms for overcoming the effects of 'harmful' agents. Interestingly, both 1080 poison and the myxoma virus were introduced into rabbit control programmes in Australia in the early 1950s, so many rabbit populations have been exposed to both 'agents' for a similar period. Further, resistance to the anticoagulant rodenticides displayed by rodents first appeared within 10 years of the introduction of these pesticides. This resistance is known to be genetically inheritable, and the proportion of resistant individuals within geographically separated populations can range from 20% to more than 80% (Cowan *et al*. 1995; Quy *et al*. 1995). At least with the anticoagulant bromadiolone and house mice *Mus musculus*, this resistance involves the genetic selection for biochemical pathways that are less sensitive to bromadiolone (i.e. less sensitive hepatic vitamin K epoxide reductase; Misenheimer *et al*. 1994). Thus, there is the precedent of a variety of rodent species developing genetic resistance to at least some vertebrate pesticides. Genetic resistance to DDT has also been identified in Japanese quail *Coturnix coturnix japonica* (Poonacha, Wentworth & Chapman 1973).

Fluoroacetate-bearing vegetation (shrubs) also occurs in the areas where our rabbit populations exist, and native animals that coexist with such vegetation have developed varying degrees of tolerance to 1080 (Twigg & King 1991). However, for the following reasons, it seems unlikely that the presence of these plants has had any impact on the sensitivity of WA rabbits to 1080. Rabbits are a grazing, rather than browsing, species with a preference for domesticated pasture species (Wheeler & Hart 1979). In most agricultural districts, fluoroacetate-bearing plants are confined to areas of bush remnants where rabbits are less likely to feed. Wheeler & Hart (1979) also determined the sensitivity of rabbits with and without past potential exposure to fluoroacetate-bearing plants, and they found no differences in sensitivity to 1080 of rabbits from either habitat type.

PLASMA CITRATE TECHNIQUE

This procedure has been used to compare the tolerance to 1080 of geographically separated populations of Australian native animals, particularly those with and without exposure to fluoroacetate-bearing plants where large differences in sensitivities can occur (Twigg & King 1991). It has never been proposed that this technique would be suitable for establishing very small differences/increases in the tolerance of individuals such as those seen in our rabbits. Nevertheless, given the concern regarding the use of animal experimentation, the suitability of this technique for assessing the sensitivity of different rabbit populations was worthy of investigation. However, as predicted, the plasma citrate procedure lacked the necessary level of sensitivity to detect the increases in the tolerance to 1080 in our rabbit populations. Furthermore, the citrate response of dosed rabbits was more variable than that often seen in the native species tested. This variation has also been recorded in the plasma of New Zealand large white rabbits administered with approximately 0·8 mg 1080 kg^{-1} and sampled at regular intervals over a 7·5-h period, although there was a dose–response in these rabbits (Gooneratne *et al*. 1994). This suggests that a large number of rabbits would be required in order to detect very small differences in their sensitivity to 1080 using the plasma citrate technique. These numbers would need to be far in excess of those required for the modified LD_{50} procedure used in our trials.

MANAGEMENT IMPLICATIONS

The increased tolerance to 1080 by rabbits has potentially serious implications for both agricultural production and wildlife management. Increasing the amount of 1080 in the poisoned one-shot oats may overcome the problem in the medium term, but this approach could also increase the potential risks to non-target species. Such an approach may also create a 'catch 22' with respect to these rabbits, their level of 1080 tolerance, and associated control programmes. Assuming 1080-tolerant rabbits do not become bait- or poison-shy, then employing a strategy where the use of one-shot oats and conventional oats in rabbit control programmes is alternated each year may help to overcome the problem. As all oat grains in conventional oat bait contain 1080, all rabbits feeding on the bait trail should, in theory, ultimately ingest a lethal dose of 1080. Although more expensive, and with potentially greater hazards to non-target species, the occasional inclusion of the anticoagulant pindone (Oliver, Wheeler & Gooding 1982; Twigg *et al*. 2001) into broadacre control programmes may also be beneficial. Provided that 1080-resistant rabbits do not display any bait shyness to pindone bait, then this approach should help to reduce the number of 1080-resistant rabbits in a given population (i.e. decrease the size of any 'resistance' gene pool). Another option would be to examine potential alternatives to using oats as the bait material in rabbit control programmes. Barley is a likely candidate but there is some concern that it would not be possible to impregnate this grain with a sufficient amount of 1080 for it to be lethal to all rabbits (A. Eastman, personal communication). The strategic (re)introduction of RHD or more lethal strains of the myxoma virus may also help to reduce any impact of genetic resistance to 1080 in Australian rabbit populations (see below).

The development of bait/poison shyness has been reported in New Zealand rabbits (Bell 1975), but this phenomenon is rarely considered to be important to rabbit control programmes in Australia. It has been suggested that neophobia (in the strict formal sense, avoidance of an unfamiliar object in a familiar place; Barnett 1958) may be a partial cause of the variation in the kill rates sometimes observed with 1080-baiting programmes (Oliver, Wheeler & Gooding 1982). The relatively lengthy presence of the myxoma virus in Australia but not in New Zealand (Fenner & Ratcliffe 1965) may well explain this difference. If the myxoma virus imposes a level of mortality that is independent of the development of increased tolerance to 1080 by rabbits (a reasonable assumption), then the mortality inflicted by this virus would be removing both 1080-resistant and 1080-susceptible individuals alike. This would not happen in New Zealand rabbit populations because, until very recently, there have been no similar diseases that impart wide-scale mortality on rabbits. Now that RHD is present in New Zealand, it will be interesting to see what happens to the prevalence of bait/poison shyness to 1080 in New Zealand rabbit populations.

Oliver, Wheeler & Gooding (1982) found that there was a decline in the kill rates of 1080-baiting campaigns in WA from 1958–62 to 1971–75, and suggested neophobia by rabbits towards 1080 oat bait trails as a possible cause of this decline. However, there were several confounding factors during their study. While the mean change in rabbit numbers on their untreated 'null' sites was –2·6%, the 95% CLs for these data were approximately ±16% (Oliver, Wheeler & Gooding 1982). As the percentage reductions they reported for treated sites were not adjusted relative to those changes that occurred on the corresponding untreated null sites, we suggest there could be as much as a 15% variation in their reported reductions from the 'true' reduction that was actually achieved. Their data were, however, treated similarly for both time periods examined. Many of the rabbit populations studied by Oliver, Wheeler & Gooding (1982) are highly likely to have had previous exposure to oats, as most of these populations would have had some prior exposure to cereal crops, given that many populations were in the cereal-growing areas of WA. Furthermore, as mentioned above, the myxoma virus should have been acting as an independent mortality factor at this time, and this should have overcome any selection advantage neophobic rabbits may have had. Other forms of mortality, such as predation, are also likely to be acting independent of any 1080-baiting induced neophobia. However, we are not saying that neophobia towards 1080 bait cannot, or does not, occur in some rabbit populations (Rowley 1957; Poole 1963; Bell 1975), but rather that the Oliver, Wheeler & Gooding (1982) study could not totally discount the possibility that genetic resistance to 1080 had developed in their rabbit populations. The effect of 1080 leaching from baits, and the dehusking of oat bait by rabbits, could have also been contributing factors to the results of their trials. Dehusking of oat bait by rabbits really only became a potential problem with the use of the one-shot baiting technique in WA (M. Robinson, personal communication). Clearly, the best strategy for reducing the detrimental impact of any pest on a particular ecosystem is one that integrates all available control techniques. The type and quality of the bait material used, and the techniques employed, must maximize the number of pest animals exposed to the poison bait. With rabbits, this means maximizing the impact of myxomatosis and RHD, and incorporating the impact of these agents with well-planned baiting programmes at a regional level. It is also important to ensure that the concentration of the active ingredient in bait is as close as possible to the nominated amount.

Based upon our results, together with the known development of tolerance to fluoroacetate (1080) in animal populations with natural exposure to fluoroacetate (Twigg & King 1991), and the demonstration that resistance to 1080 can be selected for in rats (Howard, Marsh & Palmateer 1973) and flies (Tahori 1963) in the

laboratory, the development of resistance to 1080 may well be occurring in free-ranging populations of pest species other than rabbits. For example, the use of 1080 to control introduced predators such as the fox *Vulpes vulpes* in Australia, and stoats *Mustela erminea* in New Zealand, has increased considerably in recent times (Seawright & Eason 1994; Saunders *et al.* 1995). In New Zealand, 1080 baits are also used to reduce the impact of the introduced brush-tail possum *Trichosurus vulpecula* on both agricultural production (e.g. reducing the spread of tuberculosis) and the environment (Seawright & Eason 1994). It is highly likely that some resistance to 1080 is developing in at least some populations of these species. However, the development of such resistance is likely to be more rapid in *r*-adapted species like rodents and rabbits, which can have a relatively rapid population turnover, than in species with *k*-adapted life histories, such as the canids and mustelids. Nevertheless, the development of any such resistance that results in a significant decrease in the efficacy of 1080-based pest control operations will have obvious implications for wildlife conservation and agricultural production in those countries that rely heavily on the use of 1080 to mitigate the damage caused by vertebrate pests, and further research into this phenomenon is urgently required.

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