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A simplified protocol for detecting two systemic bait markers (Rhodamine B and iophenoxic acid) in small mammals

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Abstract We developed a method of quantifying levels of fluorescence in the whiskers of wild stoats (*Mustela erminea*) using fluorescence microscopy and Axiovision 3.0.6.1 software. The method allows for discrimination between natural fluorescence present in or on a whisker, and the fluorescence resulting from the ingestion of the systemic marker Rhodamine B (RB), although some visual judgement is still required. We also developed a new high performance liquid chromatography (HPLC) protocol for detecting the systemic marker iophenoxic acid (IPA) in the blood of laboratory rats (*Rattus norvegicus*) and wild stoats. With this method, the blood of an animal that has consumed IPA can be tested for the presence of the foreign IPA compound itself. This is a more reliable test than the previous method, which measured the raised level of natural blood protein-bound iodine correlated with IPA absorption. The quantity of blood required from animal subjects is very small (10 µl), so the testing is less intrusive and the method can be extended to smaller species. The extraction technique uses methanol, rather than acids and heavy metal salts, thereby simplifying the procedure. Recovery of IPA is quantitative, giving a highly reliable reading. In experiments on captive rats the IPA method proved successful. Of 12 positively marked carcasses, two that had not been frozen for the 24 h before blood samples were taken showed relatively lower IPA levels. The same IPA detection method, as well as the whisker analysis for RB, was applied successfully to a population of wild stoats to which both Rhodamine B and IPA were made available at bait stations. The presence of both bait markers was detectable in rats for at least 21 days and in stoats for at least 27 days.

Keywords stoat; *Mustela erminea*; rat; *Rattus norvegicus*; bait markers; systemic markers; iophenoxic acid; Rhodamine B; high performance liquid chromatography (HPLC)

INTRODUCTION

In wildlife management programmes there is frequently a need to determine whether an animal has consumed a bait, or to trace the movements and activities of resident individuals. The purpose may be, for example, to determine what proportion of a population will be reached by the distribution of a bait that will, in the future, be laced with a poison or vaccine (Hadidian et al. 1989), or to determine the degree of acceptance of a new bait (Morgan 1981).

Many agents have been developed for marking and identifying animals, and some have proven very useful and practical and are now widely used in wildlife studies (Savarie et al. 1992). In New Zealand, the two systemic markers iophenoxic acid (IPA) and Rhodamine B (RB) have been tested on ferrets (Ogilvie & Eason 1998) and stoats (Spurr 2002a,b).

This paper reports on a simple test for detecting presence or absence of IPA in blood samples of small mammals using high performance liquid chromatography (HPLC), and a simplified procedure

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for detecting RB in their whiskers. Both procedures overcome some of the practical disadvantages typically associated with the use of these bait markers. Laboratory rats were used in preliminary trials, because captive stoats are hard to obtain, and the most obvious alternative model carnivores, ferrets, have been tested before (Ogilvie & Eason 1998). We then used the results to design a field trial on stoats.

Rhodamine B

Rhodamine B (RB) is a non-toxic dye that has been used for the coloration of cosmetic, pharmaceutical, and agricultural spray products. Its application to staining biological tissue has been extended for use as a systemic marker in a variety of animals, because it often shows a high degree of persistence (Fisher 1998).

Persistence appears to vary in different species, although the tissues examined and mode of administration are not always consistent among studies. Fichet-Calvet (1999), working with coypu (*Myocaster coypus*), found under-fur hairs to retain visible traces of RB for up to 225 days after feeding them for 3 days with carrots containing RB at 0.5 g/kg of carrots. Lindsey (1983) found the hair of mountain beavers (*Aplodontia rufa*) to remain visibly marked for up to 196 days after 15–35 mg/kg was delivered by oral gavage, whereas the blood plasma, analysed with a fluorometer (Turner Model 111), remained marked for only 7 days. In the same study, beavers fed apple bait coated with 0.1–0.34% RB remained stained around the urogenital area for only 14 days. Spurr (2002b) found whisker marking in stoats to last for at least 19 weeks with a dose of 83 mg/kg, though the whisker that remained marked for longest was believed to have ceased growing after administration.

The dye is incorporated into the hairs of animals as they grow during the few days following ingestion of RB, and can be detected in the hair shafts under ultraviolet (UV) light. Whiskers are useful because, unlike body hair, they grow all year round, so RB can be used and detected in any season. RB is typically expressed as a bright band at some point along the shaft length (Johns & Pan 1981; Fisher 1998) or as a series of bands depending on the number and frequency of doses taken (Fisher 1998; Spurr 2002b).

The standard method of detection is relatively unobtrusive. Only the whiskers of live-trapped animals need be collected, rather than blood or other tissue samples that may require traumatic surgical procedure or destruction of the animal. However, not all hairs are growing at any given time, and if too many non-growing hairs and whiskers are included in the sample, the result may be a false negative. One way to overcome this problem is to take a large sample from each animal, which is easy to do from carcasses, but there may be a strict limit on the number of whiskers that can be safely or ethically taken from live animals. A further problem is natural fluorescence, which is sometimes evident on whiskers and can be difficult to distinguish from RB induced staining (Fisher 1998).

Iophenoxic acid (IPA)

IPA (α-ethyl-3-hydroxy-2, 4, 6-triiodohydrocinnamic acid 98%) has been used in many wildlife management programmes requiring systemic marking. It acts to raise the level of serum or plasma protein-bound iodine (PBI) in the blood above the natural background level. The highly persistent nature of IPA makes it a very functional systemic marker. Raised PBI levels from a dose of 10–20 mg/kg were still detectable after up to 52 weeks in the domestic dog (*Canis familiaris*) (Baer et al. 1985), and after more than 8 weeks in badgers given a 5 mg/kg dose (Larson et al. 1981). However, this persistence is not found in all species; IPA lasted only a week in wild swine given a 20 mg/kg dose (Fletcher et al. 1990). Stoats also retain raised PBI levels for a relatively short period, about 14 days after a dose of up to 19 mg/kg (Spurr 2002a).

The use of IPA as a bait marker requires prior estimation of the expected natural background level of PBI. An animal can be deduced to have eaten an IPA-laden bait only if the level of iodine concentration in the sample is higher than the value typical of the species and location. Samples of blood are required from untreated animals to establish this preliminary base level and its variance, and further samples from treated animals to test for the expected comparative increase after IPA consumption. Blood sampling is difficult, intrusive and stressful for live, wild animals. It is particularly intrusive in small animals, where anaesthesia and cardiac puncture is required to obtain a volume of blood sufficient to analyse by the methods described by Hadidian et al. (1989). If the animal has consumed only a small amount of IPA-laced bait, the iodine level recorded may not exceed the confidence limits around the average base level, which would require the individual to be falsely declared negative for IPA. Furthermore, there is a considerable cost involved in testing for serum or plasma iodine levels, which

in New Zealand in 2001 was in the range of NZ$90–100 per sample (G. Wright, Landcare Research, Lincoln, pers. comm.), and would have made the cost of testing our 63 samples about $6,000.

Jones (1994) described a method for detecting IPA in wild foxes (*Vulpes vulpes*) in Britain using HPLC. Each animal had been fed with meat containing 40 mg of IPA per dose. As in our trial, establishing a base level of serum or plasma protein-bound iodine from the wild fox population was not possible, so Jones (1994) developed a method of detecting the presence of the IPA compound directly. We independently discovered and developed a method for stoats similar to that used by Jones, but with some differences as described below.

METHODS

Captive trials with rats

Administration of bait markers

Sixteen female laboratory rats, housed in standard cages, were split into 4 groups of 4 individuals. Dosage rates for the 16 animals were based on their average weight of 330 g. The rate for RB (BASF Australia Ltd, Victoria, Australia) was set at 30 mg/kg, i.e., 10 mg per rat (Eason et al. 1994; Ogilvie & Eason 1998). A dosage of 5 mg/kg was chosen for iophenoxic acid (IPA; Sigma-Aldrich, Milwawkee WI 5323, USA), i.e., 1.25 mg per rat. The two markers were mixed together into a vehicle of 20% ethanol (1 mg RB and 0.5 mg IPA/ml ethanol), which was administered in 1-ml doses via oral gavage. Groups 1, 2, and 3 received the markers on the same day, while Group 4, as a control, received none. Group 1 rats were euthanased after 7 days, Group 2 after 14 days, and Groups 3 and 4 after 21 days. All carcasses were stored in a –4°C freezer for 24 h prior to examination, except two (rats i and ii of Group 3) which were inadvertently left unfrozen for this time (Table 1).

Analyses

Whiskers (10 from each rat) and claws (all 18) as well as the stomach and intestine lining of all animals were examined for RB staining under both white light and also a hand-held long-wave (366 nm) UV lamp with the unaided eye.

Before freezing, about 1.5 ml of blood was retrieved from the opened thoracic and abdominal cavity and heart of the fresh carcasses with a transfer pipette, and spun at 14 000 rpm for 20 min. Approximately 300 µl of serum was obtained from each animal, and frozen at –20°C until the end of the 21-day trial. Therefore, Group 1 serum was frozen for 14 days, Group 2 for 7 days, and Groups 3 (except rats i and ii) and 4 for 1 day. Our HPLC equipment consisted of Shimadzu pumps, oven and auto-sampler, and a photodiode array detector (PDA) operated at 227 nm. A Prodigy 3µ ODS (3) 100Å, 100 × 2.00 mm column was used. PDA data were analysed with Class VP5.032 software.

Extraction

To extract the IPA compound from the blood sample, we used a process similar to that described by Dawidowicz & Fijalkowska (1995): 100 µl of serum was precipitated in 900 µl methanol (CH_3OH). Where 100 µl of serum was not available, the quantity of methanol was reduced to maintain the same proportions (1:10) in the mixture. This solution was then mixed by vortex for 10 s and centrifuged at 14 000 rpm for 10 min. The supernatant was then transferred directly into HPLC vials, and 10 µl was injected into the column for analysis. We calculated calibration curves to check that recovery of IPA, both in methanol and in precipitated blood spiked with the same concentrations as the standard, is 100% quantitative as described by Dawidowicz & Fijalkowska (1995).

The mobile phase consisted of 60% acetonitrile, and the pH was brought to 4.63 with acetic acid. With a flow rate of 0.2 ml/min and a temperature of 35°C, the retention time for IPA was 3.52 min.

Field trials with stoats

Subject animals

The 63 subjects of this experiment were captured between 17 and 28 January 2001 in Fiordland National Park, southern New Zealand. They were collected during a field trial designed to test a new bait delivery system, the Scentinel® (Purdey 2002). In two sets of identical tunnels, the Scentinel® and the current industry standard Fenn trap (locked open) were set over 12 days (1–13 January) to offer different baits (a semi-liquid egg mixture containing RB delivered on demand by the Scentinel®, and eggs containing IPA as bait in the Fenn traps). Many of the animals, caught when the Fenn traps were set to kill after the end of the trial, had been exposed to the baits and bait markers delivered by the two systems.

The average mass of stoats in this area was expected to be 250 g, including both newly independent young and adults (King & Moody 1982).

RB

A dose rate of 60 mg/kg (15 mg per 250 g stoat) was considered an appropriate quantity to deliver to stoats in the wild. This dose was based on (1) results obtained from the above captive trials with rats using 30 mg/kg (10 mg per rat), and (2) consultation with P. Fisher and D. Fairbridge (Vertebrate Pest Research Department, Victorian Institute of Animal Science, P.O. Box 48, Frankston, Vic 3199, Australia). The higher level of 15 mg per dose was chosen for the field trial in case any wild stoat took only a partial bait from the Scentinel® system. RB was added to the egg bait installed into the Scentinel® at the rate of 5 mg/g of bait. Bait was delivered in measured quantities of 3 g at a time, i.e., 15 mg per dose.

IPA

A dosage rate of 8 mg/kg (2 mg per dose) was chosen after consulting the information reviewed by Ogilvie & Eason (1998) on dosage levels trialled on cats, ferrets, and brushtail possums, and from the above trial on laboratory rats. In the field, 2 mg of IPA powder was combined with ethanol to make up a volume of about 1 ml (20% solution). A small (1 mm diameter) hole was drilled into the larger end of an egg, and the laced fluid injected through the air bubble with a 0.5 mm needle to depths ranging from 10 to 30 mm, to ensure even distribution of IPA within the egg. The egg was then placed as bait between the two locked-open Fenn traps inside the tunnel.

Preparation and analysis of samples

RB

Facial whiskers were removed from all the dead stoats upon their retrieval from the Fenn traps. At least three whiskers from each animal were prepared and examined for evidence of RB-induced bands as described by Fisher (1998). Some animals were recorded as positive from the first three whiskers, while further whiskers were added to the sample for all negative animals to a maximum of 11 (Table 2).

Presence or absence of RB in the whiskers was determined by identification of the pink fluorescent bands using fluorescent microscopy methods resembling those of Fichet-Calvet (1999) and Fisher et al. (1999). A Ziess Axioskope 20 fluorescent microscope was fitted with a Plan-Neofluar 1.25×/0.04 objective. Whiskers were observed under green light ($\lambda = 546$ nm). Axiovision 3.0.6.1 software was used to quantify the brightness of samples. The colour mode of the camera window was set to black and white, gain 1, resolution 324 × 256 binned 4 × 4, exposure time 150 ms. The brightness levels of the samples were taken as the maximum reading of brightness for the whiskers, and a minimum threshold of brightness of five for positives was defined.

Table 1 Results from trials of two bait markers on captive rats. Rhodamine B analysis was carried out under white light (wt) and ultraviolet light (UV).

		Rhodamine B						
		Whiskers		Claws		Gut		IPA level
Group	Rat	wt	UV	wt	UV	wt	UV	(mg/ml)
1.	i	✓	✓	✓	✓	×	×	161.12
	ii	✓	✓	✓	✓	×	×	207.21
	iii	✓	✓	✓	✓	×	×	157.33
	iv	✓	✓	✓	✓	×	×	168.94
2.	i	✓	✓	✓	✓	×	×	222.56
	ii	✓	✓	✓	✓	×	×	141.98
	iii	✓	✓	✓	✓	×	×	130.47
	iv	✓	✓	✓	✓	×	×	176.52
3.	i*	✓	✓	✓	✓	×	×	7.67
	ii*	✓	✓	✓	✓	×	×	7.67
	iii	✓	✓	✓	✓	×	×	107.44
	iv	✓	✓	✓	✓	×	×	107.44
4.	i	×	×	×	×	×	×	0
	ii	×	×	×	×	×	×	0
	iii	×	×	×	×	×	×	0
	iv	×	×	×	×	×	×	0

*Sample not stored at –4°C for 24 h before examination.

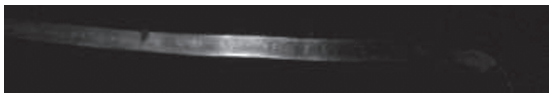

Fig. 1 Rat whisker marked with Rhodamine B displaying the bright band on the shaft.

In this system, the level of brightness depended on the position in the field of view of the whiskers' brightest section. By positioning this section of the whisker around all areas of the screen, the maximum value of each sample could be found. In the earliest stages of the examination, all samples were examined briefly to establish familiarity with the characteristics of positive and negative whiskers. Some time was required to identify false negative and positive whiskers, and to establish what visual judgement would be required to eliminate them.

All whiskers were examined twice; first, without prior washing, and second, following a washing regime as described in Fisher et al. (1999). Each sample of whiskers was stored on a labelled slide without a mountant, which was not necessary to determine presence or absence of RB-induced bands. Instead, a cover slip was placed over the dry whiskers and secured by adhesive tape. This method allowed the whiskers to be removed from the slides or extra ones added.

IPA

Blood was collected from stoat carcasses in the field, as described above for laboratory rats. The blood of recently killed animals was abundant and easily collected (either by cardiac puncture, or by cutting the veins around the heart and syringing the blood out of the thoracic cavity), whereas most of the blood of those that had been dead for some hours had become congealed. This was not a great problem, however, as only a minute quantity was required for analysis. Samples were centrifuged at 14 000 rpm for 20 min, and the serum collected and frozen at –18°C for a maximum of 2 weeks in the field base deep freeze. The same method of analysis by HPLC developed for rats was applied.

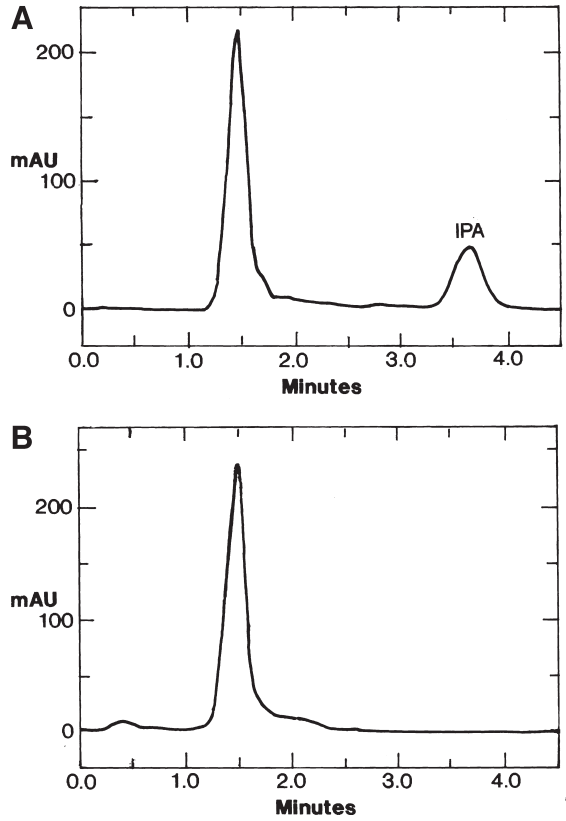

Fig. 2 **A**, A positive reading for IPA from rat 1(ii) with the newly developed HPLC method. The IPA peak shows at 3.52 min and is quantified at 227 nm. **B**, A blank plasma sample extracted with methanol. The peak at 1.5 min represents other substances extracted in methanol. There is no peak at 3.52 min that could interfere with the IPA reading.

RESULTS

Captive trials with rats

RB

Table 1 shows the results of the bait marker trial with laboratory rats. No RB staining was visible in the stomach or intestine wall of rats of any group under white or UV light. Claws of Groups 1, 2, and 3 rats appeared to be unstained when viewed under white light but were clearly fluorescent under UV light. Claws of Group 4 were unstained when observed under both UV and white light. Every rat in Groups 1, 2, and 3 showed whisker staining under white light, which was even clearer under UV light. No whiskers of Group 4 were stained.

Staining of whiskers appeared as a single pink band, positioned anywhere between the root and half way along the whisker shaft (Fig. 1). Of the total of 120 whiskers from the 12 rats in Groups 1–3, 50% were stained and the rest free of staining.

IPA

All rats in Groups 1, 2, and 3 measured positive for IPA (Table 1; Fig. 2A). The two dosed rats that were

Table 2 Whisker samples collected for Rhodamine B analysis taken from 63 kill-trapped animals between 17 and 28 January 2001. The number of bands present on whiskers represents the minimum number of occasions the animal fed from the Scentinel® experimental bait station.

Stoat	Positive for RB	No. of whiskers collected	No. positive whiskers in sample	Max. no. of fluorescent bands	% positive whiskers from sample	Positive for IPA
1♀	–	9	0	0	0	–
2♀	–	8	0	0	0	–
3♀	✓	8	8	1	100	–
4♂	✓	3	2	2	67	✓
5♂	✓	10	5	1	50	–
6♂	✓	3	3	4	100	✓
7♂	✓	3	3	8	100	–
8♂	✓	3	1	1	33	✓
9♂	✓	9	0	2	0	✓
10♂	✓	3	1	1	33	✓
11♀	✓	3	3	5	100	✓
12♂	✓	3	3	4	100	✓
13♂	✓	3	1	1	33	✓
14♂	–	9	0	0	0	–
15♀	–	10	0	0	0	–
16♀	✓	9	1	6	11	–
17♀	✓	4	4	1	100	–
18♂	✓	11	5	1	45	✓
19♀	✓	8	3	2	38	✓
20♂	✓	3	3	1	100	–
21♀	–	7	0	0	0	–
22♂	✓	3	3	6	100	–
23♂	✓	3	3	1	100	–
24♀	–	9	0	0	0	–
25♂	✓	3	3	1	100	–
26♂	–	8	0	0	0	–
27♂	✓	4	2	1	50	–
28♂	✓	4	3	1	75	–
29♂	–	9	0	0	0	–
30♀	–	8	0	0	0	–
31♀	–	8	0	0	0	–
32♂	–	8	0	0	0	✓
33♂	✓	3	2	1	67	✓
34♂	–	9	0	0	0	–
35♀	✓	3	2	2	67	–
36♀	–	9	0	0	0	–
37♀	✓	8	8	2	100	✓
38♀	–	9	0	0	0	–
39♂	–	6	0	0	0	–
40♀	–	8	0	0	0	✓
41♂	✓	3	2	1	67	✓
42♂	–	8	0	0	0	✓
43♀	–	9	0	0	0	–
44♂	✓	4	2	4	50	–
45♂	–	8	0	0	0	–
46♀	–	8	0	0	0	–
47♂	✓	4	4	4	100	✓
48♀	–	10	0	0	0	–
49♀	✓	9	1	2	11	–
50♂	–	7	0	0	0	✓
51♀	✓	3	1	8	33	✓

(*continued*)

Table 2 (*continued*)

Stoat	Positive for RB	No. of whiskers collected	No. positive whiskers in sample	Max. no. of fluorescent bands	% positive whiskers from sample	Positive for IPA
52♂	–	8	0	0	0	–
53♀	–	9	0	0	0	–
54♂	–	8	0	0	0	–
55♂	–	6	0	0	0	–
56♀	–	8	0	0	0	–
57♀	–	7	0	0	0	✓
58♂	–	8	0	0	0	–
59♀	✓	3	1	1	33	–
60♂	–	7	0	0	0	–
61♀	–	8	0	0	0	–
62♀	–	8	0	0	0	–
63♂	–	8	0	0	0	✓
Total	30	412	83	76	–	21
%	47.6	–	20.1 ± 1.8	–	–	33.3

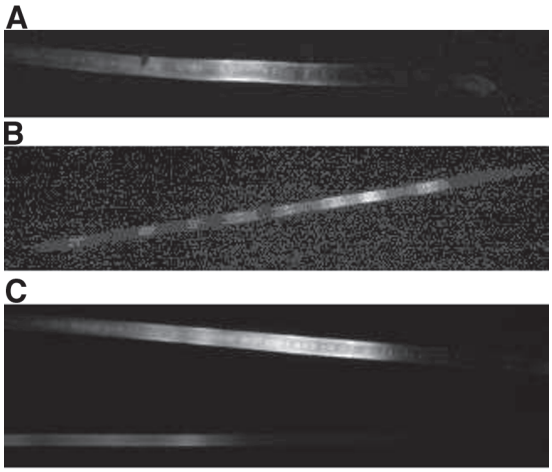

Fig. 3 Stoat whiskers marked with Rhodamine B. The single fluorescent band in **A** indicates a single dose of the bait marker; the multiple bands visible in **B** represent at least eight separate, well spaced feeding events; and **C**, a long stain which may result from several closely spaced feeding events, or from a single feeding event in a slowly growing hair.

left unfrozen before analysis showed the lowest levels, both 7.67 mg/ml, while the levels in the 10 dosed rats that had been frozen ranged from 107.44 to 222.56 mg/ml. IPA levels in the four rats of the control group (Group 4, all frozen) were 0 mg/ml (Fig. 2B). These results confirm that the HPLC method is able to detect IPA reliably in very small (10 µl) samples of blood serum.

Field trials with stoats

For full details of the distribution and capture locations of marked animals from the Scentinel® field trial within the Borland and Grebe valleys, refer to Purdey (2002).

RB

A total of 412 whiskers was collected from 63 stoats. At least one whisker from 30 of the 63 (47.6%) animals was found to be marked with one or more fluorescent bands due to RB (Table 2; Fig. 3A,B). Occasionally a single band appeared considerably longer in a whisker than other bands (Fig. 3C), but these were still counted as one single feeding event.

In 15 of 30 animals with marked whiskers, multiple (2–8) fluorescent bands were observed, indicating that half the animals had fed from the Scentinel® several times. One of the five stoats caught on the last day of Fenn-trapping was positive for RB (Fig. 4), showing that the marking must have lasted for at least 27 days.

Of the 412 whiskers collected from the 63 stoats, 75 (18.2% ± 1.7) were found to be positive for RB after examination without washing in iso-propyl alcohol, representing 29 animals (46% of the 63 stoats). Once all samples had been washed, a further eight whiskers, all taken from one animal, were determined positive. The final total was 83/412 marked whiskers (20.1% ± 1.8), representing 47.6% (30 of 63) of stoats examined, and 58% of the 143 whiskers collected from the 30 marked stoats.

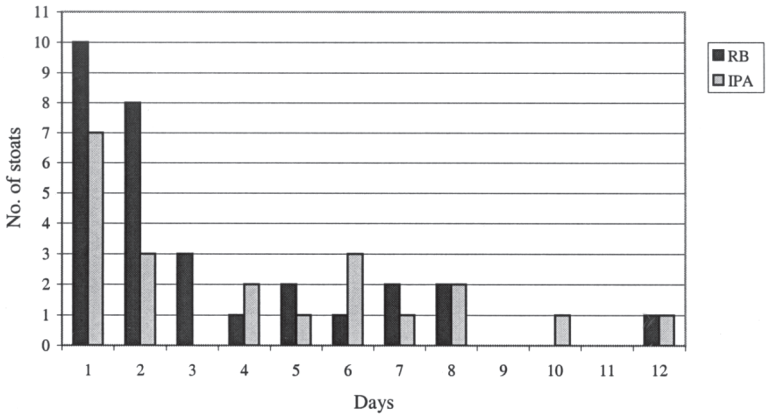

Fig. 4 Numbers of marked stoats captured per day during Phase 3 of the field trial. Days 1–12 represent 17–28 January 2001. Marked baits were available to the stoats during Phase 2 from 1–13 January 2001.

IPA

Of the 63 stoats, 21 (33.3%) were positive for IPA (Table 2), even though only 17 IPA-laced eggs were taken in the field trial. We assume that a few of the eggs could have been shared by more than one stoat, perhaps unwillingly if a dominant animal chased another from the egg. As with RB, one stoat caught on the last day of Fenn-trapping was positive for IPA (Fig. 4), showing that this bait marker also lasted for at least 27 days.

The cost of detecting IPA in a laboratory already equipped with an automatic HPLC system is minimal. All that is needed is the price of a single column (NZ$800) for all the samples plus the time required for the preparation and injection of each sample (approximately 5 min for each sample; 5.25 h for this study of 63 samples).

DISCUSSION

Both bait markers lasted at least for as long as we needed them, but we cannot tell how much longer they might have persisted after our trapping ended. More extended tests would help determine how long both RB and IPA remain detectable by our methods.

RB

Marked whiskers were commonly represented by single or multiple narrow fluorescent bands. Occasionally whiskers had a single long band on them, as in Fig. 3C, causing a short section of the shaft to appear fluorescent. This may be the result of either repeated multiple feeding events from the Scentinel®, manifested as one long stain, or a single event in a whisker of slow growth rate. In slow-growing whiskers the chemical may be absorbed by the follicle cells more gradually, so that the time between feeding events becomes blurred. Neither explanation could be proven, so we conservatively counted these long stains as a single feeding event from a Scentinel®. Where quantitative values are vital in bait acceptance fieldwork, this may become an important consideration and should be further investigated.

The difference in the proportion of positive records from washed and unwashed whiskers was small, and due only to a single animal. Before washing, the single sample of whiskers in question was classified as a negative, though with some uncertainty. It had a very faint band (brightness level 4) that registered only just below the minimum level of brightness we had previously defined for positives (brightness level 5). We traced this sample of whiskers to their source, and found that they came from the only animal to have displayed a pink, clearly RB-stained mouth area. We concluded that the marking on this animal's whiskers was attained through direct and very recent contact with Scentinel® bait, rather than by systemic expression through growth of the whisker.

Our results confirm that not all whiskers of stoats that have taken a dose of RB will be marked. Our total of 58% positives among whiskers from marked stoats sampled in the field is similar to the 56% positive whiskers from marked stoats dosed in a pen trial, reported by Spurr (2002b). The risk of declaring a false negative will clearly be higher if too few whiskers are sampled.

IPA

Our extraction process was developed independently of the one described by Jones (1994), which is difficult to carry out in the field as it involves the handling of sulphuric acid and toxic salts. IPA is highly soluble in methanol, and methanol can

efficiently precipitate the proteins from blood serum and release the bound compounds, including IPA. The actual extraction (mixing blood with methanol and shaking) is very simple and can be carried out on the spot. Centrifuging of precipitated proteins can be done at a later stage. The recovery is 100% quantitative, compared with up to 85% by Jones' method (Jones 1994), and so gives a more reliable reading.

The low levels of IPA found in rats (i) and (ii) of Group 3 might indicate an influence of freezing on this method of IPA analysis. The sample size was too small to be tested, but suggests a topic for a later trial. Investigation of this characteristic would be necessary if our method of analysis is to be used in a situation in which dead animals cannot be collected for more than 24 h, or serum cannot be frozen before analysis.

The HPLC method we developed for direct detection of IPA, combined with the simple methanol extraction procedure, can generate quick and easy assessments of serum marking in small mammals. It has several important advantages compared with previous methods. Firstly, it is much cheaper, and secondly, it is not necessary to establish a basal iodine level by species and location, because the indicator is the IPA compound itself, rather than a metabolised component of it. The IPA compound is not available naturally, so a sample must therefore be positive if any trace of the compound is detected. Thirdly, although blood is still required for testing, the samples can be much smaller (10 µl) than the 5–10 ml needed by methods testing for raised protein-bound iodine levels (Hadidian et al. 1989). This means that it may be possible to reduce intrusive sample collections, although in New Zealand bait markers are often used for assessing pest control programmes in which the death of the subjects is typically a consequence of the trial anyway. The small samples required may also make it possible to extend the method to smaller species of mammals or even birds. Lastly and perhaps most important, this method is much less vulnerable to false negatives than is standard serum testing.

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