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Evaluation of a novel rodenticide: acute sub-lethal effects of a methaemoglobin-inducing agent

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Abstract

In a series of experiments the welfare of para*-aminovalerophenone (PAVP) sub-lethally poisoned rats (*Rattus norvegicus*) was assessed. The experiments: (i) examined the acute methaemoglobin (MetHb) profile over time; (ii) refined the LD50 estimate for PAVP in adult female rats; (iii) developed and validated three neurological tests; and (iv) assessed rats for neurological deficit following prolonged methaemoglobinaemia. The results from the first three experiments were used to refine the sub-lethal study. In the sub-lethal experiment 20 rats were gavaged with a single dose of 40 mg kg^{-1} PAVP (based on an LD50 estimate of 43.3 mg kg^{-1}). Control rats (n = 10) were treated with the carrier only. Eleven (surviving) PAVP-treated rats and controls were assessed over a two-week period. Rats were tested for forelimb grip strength, stability on an inclined plane and the ability to remove tape wrapped around a forepaw in order to determine deficits in motor functions and sensorimotor integration. Signs of recovery began 3–6 h post-dosing, with all animals showing no outward signs of poisoning within 48 h, and over the 14-day post-treatment monitoring period they gained weight and increased their food consumption. There was no significant overall difference in performance between PAVP-treated and control rats in any of the three neurological tests. In the inclined plane test, performance of sub-lethally PAVP-poisoned rats appeared to be temporarily impaired with treated animals slipping at a lower angle than controls on day two. During the tape removal test, four PAVP-treated rats failed to remove the tape within the 3-min time limit on one occasion each (4/77 occasions) up to seven days post-dosing. The severity and duration of signs following acute sub-lethal PAVP poisoning appeared to be lower than those reported for existing rodenticides. It is likely that the results presented in this study extend to other MetHb-inducers.*

Keywords: *animal welfare, hypoxaemia, methaemoglobin (MetHb), methaemoglobinaemia, rat, sub-lethal*

Introduction

Vertebrate pesticides are used to control pests, such as rats (*Rattus* spp), mice (*Mus musculus*) and other non-native animals in order to reduce damage to crops and property, prevent the spread of disease, reduce public nuisance and conserve native species. For rodent control, poison baiting is often preferred to other methods, such as trapping, on grounds of cost-effectiveness, but many of the poisons currently on the market are under frequent scrutiny due to concerns about humaneness and non-target effects. For example, the nature and duration of signs of poisoning and the time to death caused by anticoagulant poisons and the non-anticoagulant calciferol (cholecalciferol), have led to these pesticides being considered markedly inhumane (PSD 1997; Littin *et al* 2002; Mason & Littin 2003; Fisher *et al* 2010). A commonly used alternative, zinc phosphide, kills quicker and thus could be considered relatively more humane (PSD 1997; Mason & Littin 2003; Ross & Henderson 2006; Eason *et al* 2013). However, the withdrawal of non-anticoagulant rodenticides, including zinc phosphide and calciferol, from some markets has led to even more reliance upon the use of anticoagulants. With the aim of improving humaneness and minimising the risk to non-target species without compromising efficacy, new bait formulations and novel vertebrate pesticides are being developed (Eason *et al* 2008).

One group of novel pesticides targets red blood cells in mammalian pests and induces the formation of methaemoglobin (MetHb), which at high concentrations leads to rapid and lethal hypoxia in the brain and heart, resulting in animals becoming lethargic and unconscious prior to death (Vandenbelt *et al* 1944). The potential of one particular MetHb-inducing chemical, *para*-aminopropiophenone (PAPP), as a vertebrate control agent has been investigated (Saverie *et al* 1983) and more recent research has demonstrated the efficacy of PAPP against non-native cats (*felis catus silvestris*), foxes (*Vulpes* spp) and stoats

(*Mustela erminea*) in Australia and New Zealand (Marks *et al* 2004; Fisher *et al* 2005; Murphy *et al* 2011). While PAPP appears to be relatively toxic to carnivores, it is less toxic to rodents: Scawin *et al* (1984) reported oral LD50 values of 474 and 223 mg kg^{-1} to male and female laboratory (Porton-Wistar strain) rats, respectively. Durie and Doull (1968) found similar intraperitoneal LD50 values for males and females but marked differences between strains (273 and 85 mg kg^{-1} for Holtzman and Charles River strains, respectively). For practical rodent control, poisons that fail to kill at 100 mg kg^{-1} are unlikely to make effective rodenticides (EPPO 1982). However, research reviewed by Baskin and Fricke (1992) investigated the use of PAPP as a cyanide antidote and also examined structural analogues to find compounds that had a greater half-life for the formation of MetHb in blood and therefore prolonged protection against cyanide. One particular analogue, PAVP (*para*-aminovalerylphenone, also referred to as *para*-aminovalerophenone) appeared to be able to maintain MetHb levels in mouse blood longer than PAPP and thus might make a more effective acute rodenticide. Pan *et al* (1983) tested PAPP and four analogues and found an inverse relationship between the length of the alkyl chain and oral LD50 values in laboratory rats and mice: using propylene glycol as a carrier, PAVP LD50 in Sprague-Dawley male rats was 84 mg kg^{-1} compared with 221 mg kg^{-1} for PAPP. More recently, Rennison *et al* (2013) reported similar LD50 values of 85 mg kg^{-1} in rats. While the toxicity of PAVP appears to be relatively low compared with other established acute rodenticides, the prospect of an improvement in the welfare of target animals during poisoning makes further consideration worthwhile.

The evaluation of compounds as potential rodenticides typically begins with an estimation of toxicity in the form of an LD50 test (Bentley 1958). Although the LD50 test is now outdated for standard toxicological testing, it is still the standard measure of toxicity used in the development and registration of pesticides around the world. The LD50 test allows comparisons to other toxicants that are designed to kill pest species. In a number of countries, including New Zealand, the UK and USA, the LD50 is a mandated test of toxicity when producing the dossiers for registration of new toxicant agents. Initially in toxicity testing, small numbers of animals are used so that compounds insufficiently toxic or regarded as inhumane can be quickly excluded. Thereafter, more animals may be tested and in addition to refining the toxicity estimate, further assessments of the humaneness of the poisoning process can be made. The nature of the test means that assessments can be carried out not only on animals that die but also on those that recover. The work reported here concerns the latter. Acute sub-lethal poisoning can range from largely asymptomatic for animals receiving very small doses to the development of severe signs in individuals ingesting a near-fatal dose. In the latter case, the humaneness of a poison may become questionable if the severity of the signs leads to a slow recovery or, at worst, permanent damage. As MetHb-inducers result in an oxygen deficit in the brain, the likelihood of temporary or permanent brain damage in survivors could be a concern. While damage resulting in paralysis or severe loss of co-ordination, for instance, would most likely be detected during initial screening of the compound, subtle changes in behaviour might be missed during casual observations of an animal. Apparent full recoveries from PAPP intoxication within 1–2 days post-dosing were reported in stoats, ferrets (*Mustela furo*), brushtail possums (*Trichosurus vulpecula*), tammar wallabies (*Macropus eugenii*), and mallards (*Anas platyrhynchos*) (Fisher *et al* 2005, 2008; Fisher & O'Connor 2007). However, no neurological tests were carried out in these cases and the degree of methaemoglobinaemia was not determined.

In humans, clinical cyanosis is seen at MetHb concentrations of 15–20%, but patients are usually asymptomatic; concentrations of 20–45% induce dyspnoea and lethargy and at 45–55% there is increasing depression in the level of consciousness and at 55–70% there are major hypoxic symptoms (associated with circulatory failure and cardiac arrhythmias) (Hall *et al* 1986). The consequences of sub-lethal poisoning on the long-term welfare of rats are thus expected to be seen, if they occur, at concentrations above 45%. Detecting neurological damage that may be non-specific and unpredictable is likely to require more than one test to monitor brain functions. A number of tests have been used to examine neurological deficits in foetal hypoxia and stroke-based rodent models, including grip strength, inclined plane and tape removal (eg Aronowski *et al* 1996; Reglödi *et al* 2003; Lubics *et al* 2005). Grip strength tests have been developed to assess the hind limb extensor response and forelimb grip strength for detecting reduced motor power (Meyer *et al* 1979). The inclined plane test determines a rat's ability to maintain its equilibrium as well as assessing strength in the limbs (Rivlin & Tator 1977). The tape removal test assesses deficits in sensorimotor integration (Albertsmeier *et al* 2007).

This study monitored the recovery of rats from acute sub-lethal poisoning by PAVP as part of a general assessment of the humaneness of this potential rodenticide. The first step was to determine a MetHb profile in rats dosed with PAVP so that any effects observed could be related to a peak MetHb level that exceeded 45%. The next step was to obtain a reliable LD50 estimate in order that the dose administered in the last stage would be high enough to achieve the minimum MetHb level, yet low enough to ensure sufficient survivors without using excessive numbers of animals. The last stage was to compare the responses of survivors during a series of neurological tests with those of untreated controls given the same tests. It is expected that the nature of the sub-lethal effects would be similar for other MetHb-inducing agents.

Materials and methods

A series of experiments were performed to examine the behaviour, duration and the nature of the effects of PAVP sub-lethal poisoning of rats. The experiments: (i) examined the acute MetHb profile over time; (ii) refined the LD50 estimate for PAVP in adult female rats; (iii) developed and validated three neurological tests; and (iv) assessed rats for neurological deficit following prolonged methaemoglobinaemia.

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Study animals

All tests were carried out using adult female Wistar rats (*Rattus norvegicus*) obtained from a commercial supplier. Although a full toxicity profile for PAVP in rats has not yet been established, there is some evidence that males might be less susceptible to *para*-aminophenones (Scawin *et al* 1984; Bright *et al* 1987). It was therefore expected that the female response would be less variable.

Animals were housed in plastic cages in groups of two to three and allowed to acclimatise to laboratory conditions (12:12 h; light:dark cycle) for at least one week before experimentation. Rats were offered a standard laboratory rodent diet *ad libitum* throughout the trial except prior to the day of dosing when all food was removed overnight and new bedding provided (15/30 of the rats in the LD50 experiment were not fasted); water was available *ad libitum*. All procedures were carried out under the provisions of the Animals (Scientific Procedures) Act 1986 and with the approval of the institute's Ethical Review Process.

Dosing

PAVP was synthesised at the University of Auckland, New Zealand. Purity (> 99%) was demonstrated by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR). A concentrate was prepared by dissolving PAVP in a 9:1 (by weight) mixture of polyethylene glycol 200 (PEG) and triethanolamine (TEA) at the rate of 100 mg ml^{-1}. Once dissolved, the solution was diluted with appropriate amounts of PEG according to the dose required. Solutions were prepared the day before dosing and stored at room temperature overnight prior to use. Animals were orally gavaged at a dose-volume rate of 5 ml kg^{-1} bodyweight. Dosing was carried out 3–4 h after the beginning of the light period.

LD50 test

A preliminary LD50 estimate (of 30–50 mg kg^{-1}) was obtained in adult female rats, and refined for a total of 30 rats by administration of four equally spaced doses (by oral gavage) between 20 and 50 mg kg^{-1}. The mean bodyweight of the rats was 235 g (range 210–274 g). Half of the 30 rats were fasted overnight before dosing. Rats were monitored continuously by direct observation under white lighting for the first 3 h and then at 30-min intervals up to 6 h post-dosing.

Methaemoglobin profile

To determine the acute profile of methaemoglobinaemia following PAVP administration, 17 fasted rats (mean bodyweight 220 g [range 203–250 g]) were dosed at 30 mg kg^{-1} (the lower preliminary LD50 estimate) and individuals were killed by cervical dislocation at 15, 30, 60, 90 and 240 min post-dosing. Post mortem blood samples were collected in pre-heparinised syringes (0.1 ml heparin, 500 IU ml^{-1} units) (Multiparin, Heparin Sodium, CP Pharmaceuticals Ltd, Wrexham, UK). Methaemoglobin concentrations were measured with a CO-oximeter (GEM OPL, Instrumentation Laboratory Ltd, Warrington, UK) and the mean of three readings was taken as the response variable. After dosing, rats were monitored continuously under white lighting.

Neurological tests

Before the tests were used on treated rats, preliminary trials were carried out to develop the protocol and to ensure that consistent results would be obtained on at least two separate occasions when applied to normal animals. For these trials, ten rats were used with a mean bodyweight of 219 g (range 204–236 g). Rats were subjected to each test over four consecutive days. The reproducibility of the results for each test was assessed by the calculation of an intra-class correlation coefficient (ICC) for a two-way design with 'rats' as a random variable and 'days' as a fixed variable. Intra-class correlation coefficients were calculated using SPSSv19 (IBM, Chicago, USA) and interpreted according to the methods of Shrout and Fleiss (1979) and McGraw and Wong (1996).

Grip strength

The apparatus used to measure grip strength consisted of a steel T-bar grasping device which was connected to a strain gauge (sampling rate of 1,000 Hz) that was mounted onto a weighted stand (BioSeb, Vitrolles, France). Rats were held around the body and the test measurement was made by allowing the animal to grasp the T-bar with its forepaws and then slowly pulling it away horizontally until its grip was broken. For consistency, a standard procedure was used, with a minimum of operator changeovers (operators were not changed within days). The response variable was the mean of three readings of the force (measured in g) at which release from the bar occurred. Intra-class correlation coefficients for single and average measures were 0.3 and 0.7, respectively.

Inclined plane

The apparatus consisted of an oblong transparent box (50 × 19.5 × 40 cm; length × width × height) with a floor that could be raised at one end to give an inclined plane with angles from 30–90°. The floor consisted of a Perspex™ sheet covered with the plastic film that the manufacturer used to protect the surface. Each rat was placed on the floor with its body axis parallel to the inclined plane. The floor was slowly raised from one end until the rat began to slip downwards — the angle at which this occurred was recorded. Between tests, the floor was wiped clean of urine and faeces in order to maintain the friction coefficient. The starting angle of the floor was 30° and the rat was placed ¾ of the way up the raised floor (Yonemori *et al* 1998), the endpoint was set at 70°. Three readings were taken and averaged to produce the response variable. The angle at which a comatose rat would slip (determined by testing a freshly dead rat) was found to be 22°. Intra-class correlation coefficients for single and average measures were 0.2 and 0.5, respectively.

Tape removal

Each rat was placed into a Perspex™ square arena (40 × 40 × 40 cm) with the back and sides covered with white paper to prevent distractions. Each animal was allowed a familiarisation period of 5 min or until it started grooming (whichever occurred first). The rat was then removed and a 15 × 10 mm (length × width) piece of adhesive surgical tape (Durapore™, 3M Health Care Ltd, Loughborough, UK) was wrapped around the toes of the left forepaw ensuring that the

edges of the tape did not stick together. It was then returned to the arena and the time (s) taken to remove the tape recorded. The results were consistent with those reported in the literature (eg Albertsmeier *et al* 2007) in that most rats removed the tape in about 20 s (the endpoint for the test was 180 s). Intra-class correlation coefficients for single and average measures were 0.7 and 0.9, respectively.

Neurological tests on survivors

To determine sub-lethal effects, 20 rats were dosed by oral gavage with 40 mg kg^{-1} PAVP (based on the results of the LD50 test) and ten rats were dosed with the carrier only (PEG/TEA). On the day of dosing the rats weighed 175–217 g (mean 193 g). After dosing, the rats were monitored continuously by direct observation under white lighting for the first 3 h and then at 30-min intervals up to 6 h post-dosing. The behaviour of the rats was directly observed and recorded using the protocol as described in a companion paper (Gibson *et al* 2015; this issue). However, due to inaccuracies in the recording sequences, the data are presented as changes in generalised signs of methaemoglobinaemia for all treated rats.

Seven rats in the treated group died between 5 min and 6 h post-dosing and another two died overnight (ie $6 < x < 24$ h post-dosing). None of the control rats died. The eleven surviving rats in the treated group and the ten controls were subjected to the neurological tests on 1, 2, 4, 7, 9, 11 and 14 days post-treatment. The tests were performed as described in the validation trials above in the order: tape removal, grip strength, inclined board. To minimise a possible test-sequence effect, on each of the seven days that the tests were conducted, the order in which the rats were tested, by cage, was reversed. Additionally, a subjective (appearance and health) score was devised to assess the overall condition of each rat on each day the tests were carried out: '0' normal; '1' normal apart from a colour difference (ie bluish tinge to ears and feet, eye colour pale); '2' some abnormal/unusual behaviour (eg long periods of rest in between periods of activity); '3' obviously abnormal/unusual behaviour (ie mostly inactive, hunched appearance, staring coat, unsteady gait); '4' moribund or comatose. The assessment was carried out during the familiarisation period at the beginning of the tape removal test. Also, 24-h food consumption (by cage) was recorded pre-treatment (day 0) and on days 4 and 11 post-treatment. Individual bodyweights were recorded pre-treatment (day 0) and post-treatment on days 3, 10 and 14. Data were analysed using repeated measures mixed models (REML) in Genstat v16.1 (VSN International, Hemel Hempstead, UK) to examine the effect of time and treatment on bodyweight and food consumption. At the end of the trial (day 14), blood MetHb levels were measured with a CO-oximeter in three treated and two control rats, which were dispatched by neck dislocation.

For the tape removal test, the total time for each rat to remove the tape was split into two periods: the time (s) until the animal began to remove the tape (latency) and the actual time taken to remove it. If a rat failed to complete the task within the designated maximum period (3 min), a default value of 180 s was recorded (Albertsmeier *et al* 2007). Each period was analysed separately.

An analysis of the data from the neurological tests was made using linear mixed models for repeated measurements with time (post-treatment days) and treatment as fixed effects. The covariance structure that gave the lowest AIC value (Akaike's Information Criterion) was used. The Mann-Whitney *U* test was used to examine for differences in performance between treated and control rats on the inclined board test for each day. Analyses were carried out using SPSSv19 (IBM, Chicago, USA) or Genstat v16.1 (VSN International, Hemel Hempstead, UK).

Results

LD50 test

Based on 30 animals, the calculated LD50 was 43.3 mg kg^{-1} (95% fiducial limits 37.4–68.5). There was no anecdotal evidence of any difference in response, such as time to onset of signs of methaemoglobinaemia, between animals that were fasted overnight before test and those that were not. Based on these results, 40 mg kg^{-1} was used as the dose for the sub-lethal experiment with the expectation, from the MetHb profile trial results, that the MetHb level in surviving rats would exceed 45%. Subsequently, incorporating the additional data from the 20 rats dosed with 40 mg kg^{-1}, the revised LD50 estimate of 43.3 mg kg^{-1} was further refined to 42.2 mg kg^{-1} (95% fiducial limits 38.2–55.4 mg kg^{-1}). The LD98 was 69.9 mg kg^{-1} (54.1–387.6 mg kg^{-1}).

Methaemoglobin profile

At a dose of 30 mg kg^{-1}, MetHb levels peaked between 30 and 60 min post-dosing and remained high for at least another 3 h (Figure 1). In rats surviving to their designated time-point, the minimum level recorded was 55%. One rat in the 90-min group died after 71 min with 81% MetHb and a second in the 240-min group died at 105 min post-dosing (MetHb level not obtained). At the 90-min point, two rats with 56 and 59% MetHb, respectively, displayed signs of methaemoglobinaemia (cyanosis, lethargy) but were fully responsive to auditory (click noise of ≈79 dB) and visual stimuli (threat response of rushing hand to the face); two animals with the highest levels (76, 77%) were recumbent and mostly unresponsive. Of the two rats that survived 240 min post-dosing, one with 67% MetHb was fully responsive to stimuli, drinking normally and able to move around the cage, while the other with 76% MetHb was comatose (no righting response, no blinking response after touching the cornea, no tail pinch reflex).

Observations on sub-lethally poisoned rats

After dosing with PAVP the rats showed similar signs of MetHb intoxication as reported by Gibson *et al* (2015; this issue), with the first (acute) signs apparent within 5 min; this included pale ears, feet and tail and a darkening of the eyes, which were all easily discernible in the albino rat. The feet, in particular, quickly developed a bluish tinge as cyanosis progressed. The animals' movements then rapidly slowed and after 20–30 min the rats became recumbent with the head and body upright, but resting on the floor with the feet underneath (sternal recumbency); breathing became abnormal.

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Figure 1

Time profile of methaemoglobin level in rats from 15 to 240 min (4 h) after dosing with 30 mg kg^{-1} PAVP. Each bar shows the value for an individual rat. One rat in the 90-min group died at 71 min with a MetHb level of 81% and another in the 240-min group at 105 min (level not obtained).

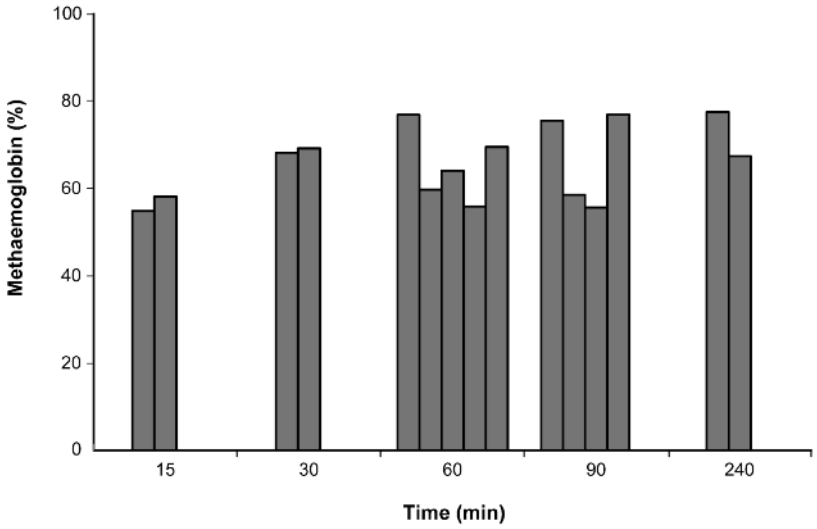
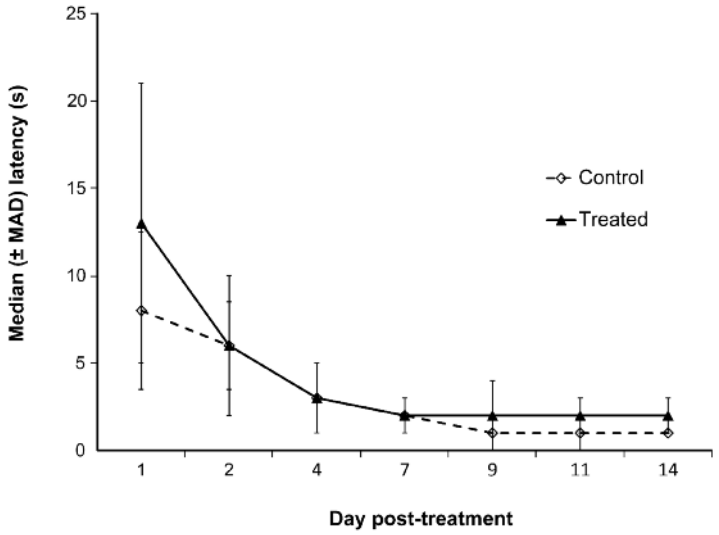


Figure 2

Median latency (± median absolute deviation [MAD]) (s). Latency was the time taken to begin to remove a piece of tape applied to the front paw.



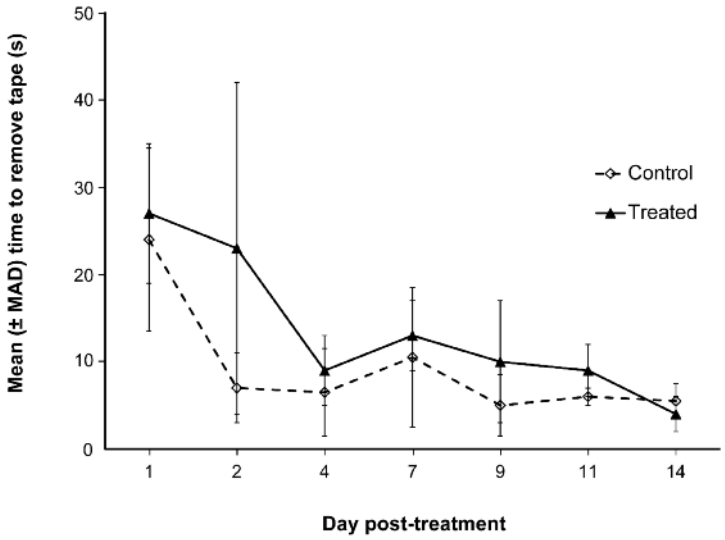
Thereafter, in the recording periods 3–6 h post-dosing, some animals were occasionally observed moving sluggishly around the cage or repositioned themselves on the same spot. The majority of rats were not seen eating or drinking 3–6 h post-dosing. The speed of recovery varied considerably with some individuals showing distinct signs after 3 h, such as resuming a normal posture with the head and body off the ground and resuming drinking and eating. Others did not show these signs for 6 h post-dosing and 4/11 surviving treated rats were noted as 'lethargic' 24 h post-dosing. However, within 48 h, all treated rats seemed normal in appearance and mobility. Using the subjective appearance and health scoring system, all control rats were rated as '0' (normal) on all days; for the treated group, on day one, one treated rat was rated '0', six rats were '1', two were '2' and two were '3'. From day two on, all treated rats were rated as '0 '. Blood MetHb levels on day 14 in the three treated rats and two control rats tested were ≤ 0.3 and 0%, respectively.

Neurological tests

Tape removal

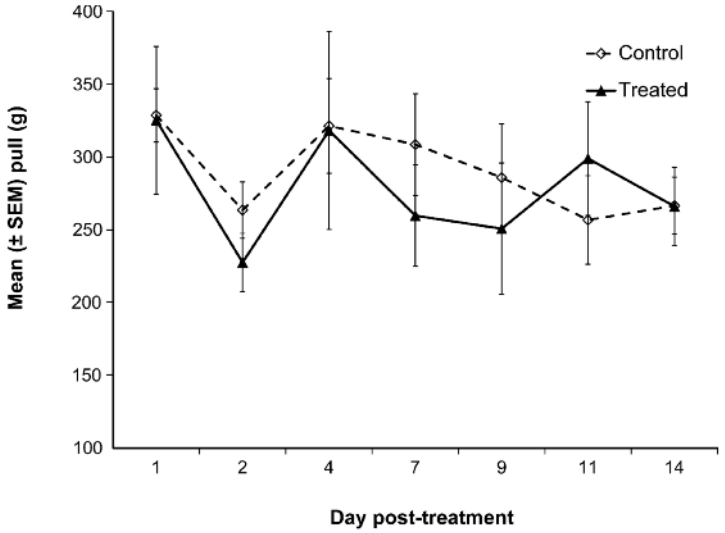
Results of the tape removal test are presented in Figures 2 and 3. All control rats removed the tape on all days. Four treated rats failed to remove the tape within the time limit; two on day one, one on day four and another on day seven. One of the treated rats that failed to remove the tape (on day one) did not attempt to remove the tape within 3 min; the other three attempted to remove the tape but did not complete the task within the time limit. No effect of treatment on latency to remove the tape (P = 0.329) (Figure 2) or tape removal time was (P = 0.111) (Figure 3) was detected. Over the 14 days of the trial, there was a significant reduction in latency to remove the tape (P < 0.001) and tape removal time (P < 0.001) but no day × treatment interaction for latency to remove the tape (P = 0.827) or tape removal time (P = 0.776).

Figure 3



Median time (± median absolute deviation [MAD]) (s) taken by rats to completely remove the tape attached to one forepaw.

Figure 4



The mean (± SEM) pull required to break the grip of rats holding with their forepaws onto a T-bar attached to a strain gauge.

Grip strength

There was no significant difference between treatment and control groups or evidence of trend in time. The pull required to break the grip of control rats varied between 145.1–553.7 g compared with 136.6–713.7 g for treated rats (Figure 4).

Inclined board

On day one, control rats slipped when the board was raised to a mean (± SEM) angle of 53.7 (± 1.4)° compared with 50.7 (± 2.2)° for treated rats. By day 14, these mean angles had increased to 60.3 (± 2.7) and 61.5 (± 1.2)° for control and treated animals, respectively (Figure 5). There was a trend over time for both groups of rats to hold at a steeper angle before slipping ($P < 0.001$), ie there was an improvement in performance of both groups over the 14 days. There was no overall difference due to treatment ($P = 0.5$). However, there was a near significant interaction between treatment and day of treatment ($P = 0.055$) suggesting that the performance of the treated group was lower than that of controls initially, but there was convergence in performance over time. The performance of treated rats was impaired relative to controls on day two ($P = 0.013$) but not on any other test days ($P > 0.10$).

Bodyweight and food consumption

Over the 14-day monitoring period, there was a significant increase in both bodyweight ($P < 0.001$) and food consumption ($P = 0.006$) but no difference between treated and control groups ($P = 0.301$ and $P = 0.076$ for bodyweight and food consumption, respectively). There was a significant interaction between time and treatment for food consumption ($P = 0.050$), indicating that the difference between treated and control groups changed over time; food consumption for treated rats appeared to be suppressed relative to controls on day four post-dosing, but then recovered by day 11 (Figure 6).

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Figure 5

Mean (± SEM) angle at which rats began to slip on a board that was raised at one end to form an inclined plane. The starting angle was ≥ 30°.

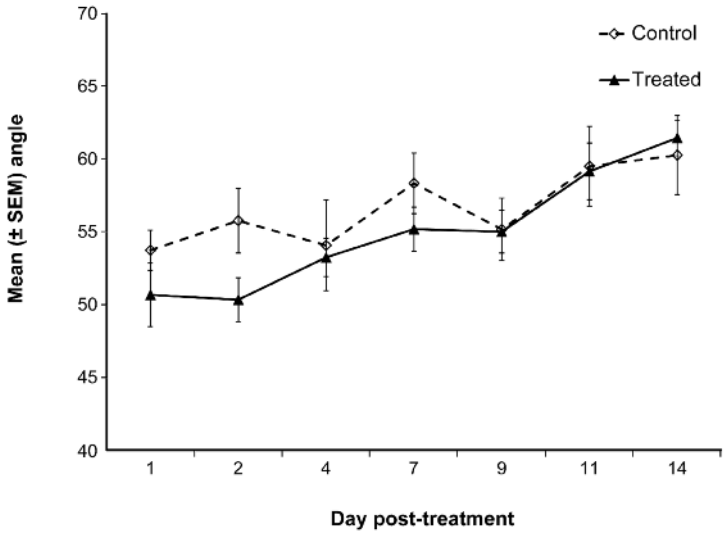
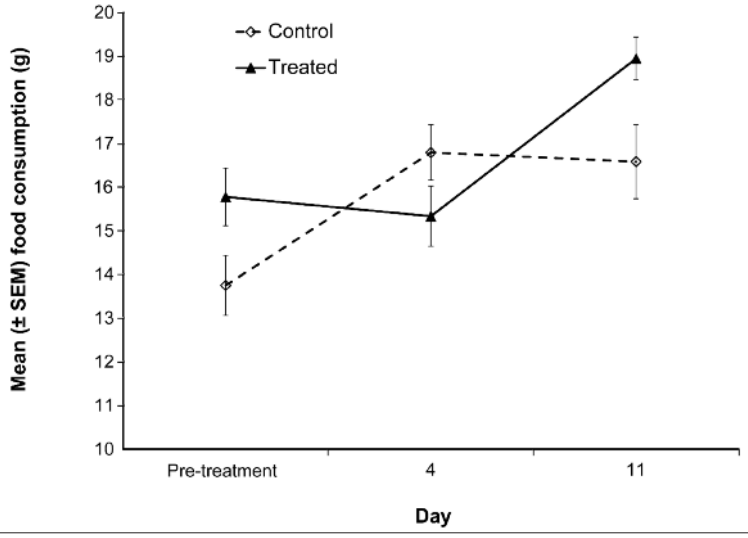


Figure 6

Mean (± SEM) food consumption (g) by adult female rats treated with 40 mg kg^{-1} PAVP relative to untreated controls.



Discussion

The effects of pesticide ingestion on the welfare of a vertebrate species depend on the number of animals affected, the duration of their suffering and the severity of that suffering. Presently, when a novel pesticide is submitted for registration, information on the suffering associated with sub-lethal intoxication in the target species is not explicitly required in the UK (CRD 2012), but it could be important if that suffering is protracted and common. From this background, in a series of experiments the behaviour of sub-lethally PAVP-poisoned rats and the duration and nature of effects was examined. Firstly, adult female rats were dosed with 30 mg kg^{-1} PAVP (based on a preliminary LD50 estimate of 30–50 mg kg^{-1}), and the concentration of circulating MetHb was measured in order to determine the acute MetHb profile over time. Secondly, a refined LD50 estimate for PAVP in adult female rats was obtained; this estimate was used for subsequent sub-lethal trials. Thirdly, three neurological tests (grip strength, inclined plane and tape removal) were developed and validated using untreated adult female rats. Finally, 20 adult female rats were dosed with 40 mg kg^{-1} PAVP (based on the revised LD50) (plus ten controls) and survivors were assessed using the three neurological tests to look for any impacts of sub-lethal PAVP poisoning on sensorimotor and neurological function.

The refined estimate of acute oral LD50 was 42.2 mg kg^{-1} for PAVP in female rats, which is similar to that of other acute rodenticides, such as zinc phosphide (Dieke & Richter 1946; Hood 1972). *Para*-aminovalerophenone therefore appears to be sufficiently toxic to be a candidate rodenticide, but as with other acute toxins, the rapid mode of action is an undesirable property. The onset of signs of toxicosis

was accompanied by a temporary suppression in food uptake; this effect would likely lead to reduced levels of bait consumption (discussed in more detail in Gibson *et al* 2015; this issue) and potential for sub-lethal poisoning. As with other acute (fast-acting) rodenticides, these issues could be at least partly mitigated by pre-baiting, or even microencapsulation to delay onset of toxicosis. Without further work (including feeding trials), it is not possible to estimate the number of animals likely to be affected by sub-lethal poisoning from use of PAVP as a rodenticide; in the field bait uptake depends on many factors including stability of the environment and the availability of alternative food (Quy *et al* 1992, 1994) and also the feeding behaviour of individual rats (Quy *et al* 2003). The current study therefore aimed to examine the duration and severity of the effects of sub-lethal PAVP poisoning.

Following dosing with PAVP, the onset of toxicosis was rapid, with cyanosis within 5 min and behavioural changes apparent within 30 min, following a similar sequence as reported by Gibson *et al* (2015; this issue). However, recovery of survivors following sub-lethal dosing was also relatively fast, and by day two all were rated as normal in subjective visual assessments. In the grip strength test, there was no evidence that the performance of sub-lethally poisoned rats was impaired relative to untreated controls, and there was no evidence of an improvement in performance over time for either group.

On the tape removal test, performance of sub-lethally poisoned and control rats improved over time, but there was no overall significant treatment effect. There was also no interaction between time and treatment group, suggesting that there was no significant difference in the change in response over time between groups; both groups therefore demonstrated a similar rate of learning in this task. However, four rats in the treated group failed to complete the task within the default time limit of 3 min (two on day one and one on days four and seven). These rats were assigned a default time (of 3 min) in the analyses, on the basis that all 'normal' rats should complete the task within this time limit (Albertsmeier *et al* 2007). Assigning a default value potentially biases the result, as would excluding these animals from the analyses. The number of rats assigned a default value was relatively small (4 of 147 data-points) and it is therefore unlikely that there was a significant bias. It is important to note that the lack of statistical significance does not necessarily demonstrate the lack of a subtle biological response. Furthermore, it is possible that more subtle differences would have been detected if tape had been applied to both paws to test for asymmetry in brain malfunctions (Schallert & Whishaw 1984). Some subtle differences between treated and control rats were also found during the inclined plane test (impaired performance of treated rats relative to controls on day two) indicating potential impairment in the functions of the frontal motor cortex. While the rats were always tested in the head-up orientation, testing left- or right-side up might have been more discriminatory (Yonemori *et al* 1998). However, intuitively, in sternally recumbent animals (which was the normal posture during acute methaemoglobinaemia) it would be expected that methaemoglobinaemia would lead to a global rather than a focal or lateral cerebral hypoxaemia and thus which paw was taped or the orientation of the rat was immaterial. In validations of the three neurological tests using untreated female animals, the reproducibility of the inclined plane and grip strength tests was lower (as demonstrated by the lower ICC for individual days compared to an average response measured over four days) than for the tape removal test, suggesting that the tape removal test was likely to give less variable (and hence potentially more reliable) results than the other two tests.

The authors could find no other similar empirical studies of the effects of sub-lethal rodenticide poisoning in rats by which the current results could be compared with. However, there have been several reviews comparing the relative humaneness of different vertebrate control techniques. These conclude that lethal doses of anticoagulant rodenticides are associated with protracted deaths, distress, disability and/or pain; they also concluded that animals sub-lethally poisoned with anticoagulant rodenticides are likely to experience haemorrhages and their sequelae (Mason & Littin 2003; Fisher *et al* 2010; Sharp & Saunders 2011). Very protracted effects of anticoagulant sub-lethal poisoning have been documented in humans, particularly for the most toxic (and persistent) anticoagulants such as brodifacoum and flocoumafen, with symptoms sometimes lasting several months (Hui *et al* 1996; Gunja *et al* 2011). Persistence of PAPP analogues appears to be much less than for anticoagulants; a half-life of 2.5–8.5 h in dogs has been reported (Baskin & Fricke 1992) compared to 220 days for flocoumafen in rat liver tissue (Huckle *et al* 1989). The duration of sub-lethal PAVP poisoning effects is therefore likely to be lower than that of anticoagulants. In the current study after sub-lethal poisoning with PAVP, outward visual signs of behavioural impairment lasted less than 48 h, and there was no evidence that neurological functions were impaired beyond seven days. Mason and Littin (2003) also concluded that the acute rodenticides zinc phosphide and calciferol are also generally inhumane; the former typically causing severe pain for several hours, and the latter pain and illness for several days. Morgan and Milne (2002) recorded a depression of appetite that lasted for 7–15 days in brushtail possums following sub-lethal poisoning with cholecalciferol, while Prescott *et al* (1992) reported a reduction in bodyweight over the first seven days of recovery following ingestion of cholecalciferol baits by laboratory rats. Following sub-lethal PAVP toxicosis there was no overall difference in food consumption and increase in bodyweight between treated and control rats over 14 days.

The evidence from the neurological tests suggests that the rats were able to survive relatively high levels of MetHb (55–67% for ≥ 4 h) in their blood and make a full recovery without any permanent impairment. The tests appeared sensitive enough to detect subtle changes in behaviour that would probably not be detected by casual observations of animals post-treatment. During the tests, the treated animals responded normally to handling with no apparent signs of pain in limbs or body. The findings from this study and the accompanying paper by

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Gibson *et al* (2015; this issue) indicates that the severity and duration of signs following acute lethal and sub-lethal PAVP poisoning appear to be substantially less than that caused by existing rodenticide compounds.

Many millions of rodents are killed each year as pests (Mason & Littin 2003) and use of rodenticides (particularly anticoagulants) is often cited as the most commonly used method of control (eg Witmer *et al* 2007; Laasko *et al* 2010). Given the potential numbers of animals involved, it seems slightly surprising that more data do not exist on the comparative humaneness of different rodenticide compounds, and that welfare considerations do not routinely form part of the initial phase of the rodenticide evaluation and registration process. Potentially, the methods presented here could form the framework for such future evaluations.

Animal welfare implications and conclusion

It is concluded that sub-lethal poisoning of rats by PAVP resulted in substantial changes in behaviour (reduced movement and responsiveness to external stimuli) lasting for between 24 and 48 h (all PAVP sub-lethally treated rats were rated as normal by day two). More prolonged, but subtle, differences were detected by behavioural tests; performance of treated rats on an inclined plane test was impaired relative to untreated controls on day two, and on four of 77 occasions (up to day seven), treated rats failed to remove tape applied to the front paw within a 3-min time limit. These results, combined with those of the accompanying paper (Gibson *et al* 2015; this issue), suggest that the effects of sub-lethal PAVP poisoning are less severe and prolonged than those reported for the anticoagulants.

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