

Secondary poisoning risk for encapsulated sodium nitrite, a new tool for possum control

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Abstract: Brushtail possums (*Trichosurus vulpecula*) present an ongoing threat to New Zealand's environment and economy. Research into additional control techniques is vital to ensure that a variety of efficient tools are available to help achieve population suppression. Encapsulated sodium nitrite (NaNO₂) has been developed in New Zealand as a new toxin for possum and feral pig (*Sus scrofa*) control. Its toxic effects at high doses are mediated through the induction of methaemoglobinaemia, a condition in which the carrying capacity of oxygen in red blood cells is reduced. This study investigated the potential secondary poisoning risks associated with NaNO₂. Secondary poisoning risks were assessed for dogs, cats and chickens in small-scale trials. Trial groups for each species consisted of two treatment groups with four individuals per group and one non-treatment group with two individuals. For 6 consecutive days, the treatment groups of dogs, cats and chickens were fed entire or partial carcasses from possums lethally poisoned with paste bait containing encapsulated NaNO₂. Individuals in each group were observed continuously for 3 hours following each daily feeding and blood samples were taken from dogs and cats. Individuals were observed for obvious physiological signs of NaNO₂ poisoning and symptoms of methaemoglobinaemia specific to dogs, cats and chickens. None of the dogs, cats or chickens displayed any obvious physiological signs of poisoning or symptoms of methaemoglobinaemia. Blood chemistry and haematology parameters measured for dogs and cats were either within the range considered normal or when outside this range comparable levels were also recorded in the control group. No changes in histology relating to NaNO₂ intoxication were observed in dogs or cats after being fed carcasses, minced meat, vital organs or stomachs of possums poisoned with NaNO₂. Therefore, the secondary poisoning risk appears to be minimal.

Keywords: encapsulated sodium nitrite; methaemoglobinaemia; NaNO₂, non-targets; secondary poisoning; *Trichosurus vulpecula*

Introduction

In New Zealand, the brushtail possum (*Trichosurus vulpecula*) is an ongoing threat to native flora and fauna, and threatens the primary sector as a vector of bovine tuberculosis (Coleman & Caley 2000; Cowan 2005). As a consequence, research continues to focus on potential new tools to assist with possum control strategies. Encapsulated sodium nitrite (NaNO₂) in a paste bait was registered in New Zealand in 2013 by the Agricultural Compounds and Veterinary Medicines (ACVM) group for the control of brushtail possums and feral pigs (*Sus scrofa*). It has provided an effective addition to current control techniques for these two pest species (Shapiro et al. 2015, 2016).

NaNO₂ is commonly used at low concentrations as a colour fixative and preservative in meats and fish (Binker & Kolari 1975; Epley et al. 1992). However, at higher doses NaNO₂ can become toxic because of the effect it exerts on red blood cells. NaNO₂ has the same mode of action as para-aminopropiophenone (PAPP), a toxin registered for stoat and feral cat control in New Zealand in 2011 (Eason et al. 2014). Both compounds induce methaemoglobinaemia, a condition in which the carrying capacity of oxygen in red blood cells is reduced (Lee et al. 2013). In mammals, methaemoglobin (MtHb) levels in the blood are normally below 2% (Fan et al. 1987). Levels of MtHb in the blood above 70% are usually

fatal, creating a lethal deficit of oxygen in cardiac muscle and the brain, and the resulting rapid lack of oxygen to the brain and other vital organs quickly leads to death from respiratory failure (Wright et al. 1999; Eason et al. 2010). For mammals the common signs of severe methaemoglobinaemia include shortness of breath, cyanosis, lethargy, loss of consciousness and bluish colouring of the skin especially in areas of high blood supply like lips, gums, hands/paws and nose (Kennedy et al. 1997; Wright et al. 1999; Eason et al. 2014; Shapiro et al. 2015, 2016). In possums, death after a lethal dose usually occurs within 2 hours after bait has been consumed, with clinical signs first appearing within 15 to 35 minutes (Shapiro et al. 2016). Animals become lethargic and sleepy before they die and, at high doses, NaNO₂ induces a relatively fast time to unconsciousness compared to most currently registered toxins (Shapiro et al. 2016). Encapsulated NaNO₂ also has the benefit of an antidote (methylene blue).

Trials on possums and feral pigs with baits containing encapsulated NaNO₂ have demonstrated high efficacy (>80%) (Shapiro et al. 2015, 2016). The effects from a lethal dose of bait are comparable to those previously observed on stoats, cats and foxes dosed with PAPP, a toxin that is also considered to be relatively more humane than currently registered toxins used for these species such as 1080 (Marks et al. 2004; Beausoleil et al. 2010; Eason et al. 2010, 2014; Murphy et al.

2011). However, possum control is often undertaken in close proximity to areas where domestic non-target species are present. An important attribute for toxins used in these areas is that they present a low risk of causing secondary poisoning. Therefore, this research investigated the associated risk of secondary poisoning to non-target species fed with carcasses of possums that had consumed a lethal dose of paste bait containing encapsulated NaNO_2 .

Secondary poisoning, as defined by Ward (2008), occurs when a predator or scavenger consumes a prey item which has consumed a toxicant, thereby ingesting the toxicant and/or any toxic metabolites or residues. In monogastric animals like possums and pigs, most NaNO_2 is absorbed in the upper part of the gastrointestinal tract and transferred to the blood (European Food Safety Authority 2009; Crowell et al. 2013). Nitrites are seldom found at detectable levels in tissues and bodily fluids following consumption and are unlikely to accumulate in tissues; it is hypothesised that this is due to the rapid oxidation of nitrite to nitrate and the rapid and extensive excretion of nitrite (Walker 1996; European Food Safety Authority 2009). The scientific literature on the pharmacokinetics (depletion rates) demonstrates that NaNO_2 is rapidly excreted, with plasma elimination half-life values under 1 hour for numerous species of mammals (Schneider & Yeary 1975; Kohn et al. 2002). This characteristic, coupled with NaNO_2 being less toxic than most other vertebrate pesticides, could be viewed as being indicative of a low risk of secondary poisoning (Boink & Speijers 2001). However, sodium fluoroacetate (1080) is also excreted rapidly when compared to anticoagulants and yet has considerable secondary poisoning risk associated with its persistence in carcasses (Meenken & Booth 1997; Eason et al. 2012).

While there is a great deal of data on the acute toxicity of NaNO_2 (Winks et al. 1950; London et al. 1967; Sullivan 1985; Cowled et al. 2008; Eason et al. 2009; Lapidge & Eason 2010; Shapiro et al. 2015, 2016), very little data on the risk of secondary poisoning exists. Therefore, it is important to understand the potential risks, from carcasses of possums poisoned with NaNO_2 , to working farm dogs, livestock, pets and other non-target animals.

The purpose of this research was to determine the secondary poisoning risk for encapsulated NaNO_2 , by feeding carcasses of possums poisoned with baits containing NaNO_2 , to cats (*Felis catus*), dogs (*Canis lupus familiaris*) and chickens (*Gallus gallus domesticus*) for 6 days. Similar secondary poisoning studies have been undertaken during the evaluation of the suitability of other possum control tools, such as cholecalciferol and microencapsulated zinc phosphide (Henderson et al. 2003; Eason et al. 2013). Chickens were included in our study as a proxy for weka (*Gallirallus australis*), a flightless native bird known to scavenge carcasses and to have experienced varying degrees of mortality in previous 1080 operations (van Klink 2008). The similar body size, food preference and potential consumption rates of food make chickens an appropriate proxy for weka, which is why they have been routinely included in previous studies evaluating primary and secondary poisoning risk instead of native bird species (Eason et al. 2013). Dogs, cats and chickens fed these carcasses were exposed to concentrations of residues that would realistically be encountered by scavengers after poisoning operations.

Materials and methods

Poisoning possums with NaNO_2

Fifty-six brushtail possums were captured using live capture cage traps in Hororata, Canterbury, New Zealand. No vertebrate toxic agents had been used at this site for at least 6 months prior to possums being captured. Possums were transported to the Lincoln University Animal Facility, Lincoln, and housed individually in outdoor enclosures constructed of plywood and wire mesh and measuring 200 cm × 150 cm × 150 cm. Each cage had a hessian sack and wooden box for possums to use as a den. Possums were fed a selection of grains and fresh vegetables with water available *ad libitum*. Possums were acclimatised for 10 days and on three occasions in that period they were each pre-fed with approximately 50 g of a non-toxic paste bait consisting predominantly of grains and peanut butter. This formulation was the same as the toxic NaNO_2 paste bait (but without the NaNO_2) and this pre-feeding regime replicated common practice in control operations as well as the label recommendation for Bait-Rite paste (encapsulated NaNO_2).

Once acclimatised, possums were each presented with approximately 50 g of the paste bait, this time containing 10% NaNO_2 . This amount was selected due to its use in previous efficacy trials on possums outlined by Shapiro et al. (2016). The 50 g of paste bait consisted of 5 g (10%) of NaNO_2 , 0.26 g (0.5%) of encapsulant and 44.74 g (89.5%) of non-toxic paste. Groups of possums were poisoned in the week prior to the specific trial in which they were to be used. The paste bait used for killing possums was loaded with the same concentration of NaNO_2 (10%) proven to kill possums in previous cage trials (Shapiro et al. 2016) and the same formulation that was registered for possum and feral pig control in New Zealand in 2013. Two samples of the encapsulated NaNO_2 active (supplied by Connovation Ltd) were analysed by Flinders Cook Ltd (Auckland) to confirm the concentration of NaNO_2 active prior to the trial – the two samples were found to contain 95% w/w NaNO_2 active. The method of analysis was based on an internationally recognised analytical method described in Vogel (1979).

Once dead, possums were collected, placed in sacks and stored in a freezer at -10°C . Two possums were not frozen but instead were stored at room temperature for 2 days to enable tissue samples to be taken. A single sample of muscle, liver and stomach tissues was taken from both possums on days one and two, and these were sent to Flinders Cook Ltd (Auckland) to test for the presence of NaNO_2 residues. The first sample was taken on day one when the possums were found dead in their cages and the second taken the following day. Possum tissue samples were analysed by Flinders Cook Ltd (Auckland) by extracting them into distilled water, centrifuging, filtering and determining nitrite by measuring azo dye colour development.

Possum carcasses were defrosted for each trial, after which they were skinned and either retained whole or the meat (flesh removed from carcasses around the spine and the front and back legs), vital organs (heart, liver, lungs and kidneys) and gut (stomach and intestines) were carefully removed and placed into separate buckets. The stomach was included as it may have higher levels of residue due to the possibility of any undigested toxic bait. By feeding dogs, cats and chickens whole carcasses as well as specific organs and gut we were able to evaluate different scenarios of animals scavenging entire carcasses or only specific organs.

Animals used in the dog and cat trials were either fed whole carcasses or different combinations of minced possum meat,

vital organs and gut. For the trials where possum carcasses were retained whole, carcasses were defrosted 2 days prior to that specific trial (to allow them to thaw) and kept in sacks in a fridge at 2°C. For trials where possum meat, vital organs and gut were fed to cats and dogs, possum carcasses were defrosted 2 days prior to the trial. Meat, vital organs and gut were each minced separately in an industrial mincing machine to ensure no cross contamination of samples. The mincing machine was washed down between mincing meat, vital organs and gut. Once minced, the meat, vital organs and gut were weighed and equal amounts were allocated to individual trial animals. Allocated samples were then placed in individual snap-lock bags and kept in a fridge at 2°C until required during each trial.

Eighty-six additional brushtail possums, not exposed to toxic baits, were used as an experimental control. These possums were captured using Victor® leg-hold traps in Hororata, Canterbury, New Zealand. Possums were killed by professional trappers who harvested the fur before supplying the carcasses which were then stored in a freezer at -10°C. For each trial, possums were defrosted, skinned and then fed to dogs, cats and chickens. Whole possum carcasses or the minced meat, vital organs and gut were fed to dogs, cats and chickens to acclimatise them to possum meat prior to the trial. The minced non-toxic possums were also fed to the control group in the dog, cat and chicken trials.

Dog secondary poisoning trial

Ten dogs of various breeds, seven males and three females, scheduled for euthanasia by the Christchurch pound were transported to the Pest-Tech Ltd. animal facility, Leeston, Canterbury. Their average weight was 23.62 kg (range 19.75–30.40 kg). They were housed individually in cages constructed of wire mesh with a wooden kennel for shelter at one end and measured 250 cm x 150 cm x 80 cm. Dogs were fed, exercised and monitored daily with water available *ad libitum*. Dogs were fed a mixture of dog biscuits, sheep meat and dog roll – which is a commercially manufactured pet food product containing beef, chicken and lamb meat with vegetables and minerals. The animals were randomly divided into group one (n=4), group two (n=4) and group three (n=2). Groups one and two were both treatment groups, and group three acted as a control group. The feeding regime for the two treatment groups outlined below allowed us to evaluate the risk of secondary poisoning to dogs scavenging entire carcasses as well as specific organs. Group sizes were kept small in keeping with the ‘3Rs’ principles (Russell & Burch 1959), namely the second of the 3Rs, reduction, which aims to use as few animals in trials as necessary.

In the week before the trial commenced, animals in all three groups were fed non-toxic possum meat, to acclimatise them to the taste. Dogs in group one were each fed approximately 450 g of minced possum meat for days one and two; approximately 100 g of minced vital organs for days three and four; and approximately 375 g of minced gut for days five and six. Each dog received the equivalent of two whole possums. Dogs in group two were fed a single possum carcass on days one and two, and then two possum carcasses on day three. Dogs in group three were fed approximately 500 g made up of minced possum meat, vital organs and gut for six consecutive days; this was the equivalent of three whole possums per animal. Amounts fed to dogs were based on previous secondary poisoning studies in which possum carcasses were fed to dogs (Henderson et al. 2003; Henderson 2009).

Once the animals were well acclimatised to eating possum, then the trial commenced. The feeding regime and amounts fed for each of the three groups was identical to that used in the acclimatisation period the week prior to the trial. For groups one and two, the meat, vital organs, gut and whole possum carcasses were from possums poisoned with baits containing encapsulated NaNO₂. Group one dogs were each fed minced possum meat for days one and two, minced vital organs for days three and four and minced gut for days five and six; each dog received the equivalent of two whole possums. Group two dogs were each fed a single possum carcass on days one and two, dog biscuits and dog roll on days three, four and five and then two possum carcasses on day six. Group three were each fed a combination of minced possum meat, vital organs and gut for 6 consecutive days sourced from possums caught in leg-hold traps. This was the equivalent of three whole possums per dog. The amounts fed to dogs and the length of the trial was based on previous VTA secondary poisoning trials that involved dogs being fed possum carcasses, meat, vital organs and gut (Henderson et al. 2003; Henderson 2009).

After each feeding, dogs were observed continuously for 3 hours for signs of NaNO₂ poisoning including vomiting, excessive thirst, diarrhoea, heavy panting, the loss of co-ordination and methaemoglobinaemia (such as shortness of breath, cyanosis, lethargy, loss of consciousness, and bluish colouring of lips, gums, paws and nose). Each of the three trial groups was observed continuously by a single researcher standing within one metre of the wire cages and dogs within each group were observed simultaneously. The 3-hour observation period was chosen based on the known rapid absorption of NaNO₂ and its rapid excretion as evidenced in its plasma elimination half-life ($t_{1/2}$) of 30 minutes for dogs (Schneider & Yeary 1975).

Blood samples were taken on four occasions; an initial baseline sample prior to the trial, and three samples after each of the feeding sections of the trial on days one, two and six. Blood samples were taken between 30 to 60 minutes after individuals had finished feeding to ensure the best chance of detecting NaNO₂. This sampling regime was based on previous research that reported rats orally dosed with NaNO₂ experienced peak plasma concentrations 30 mins after dosing (MRI 2004).

Each blood sample was split into two collection vials. One standard vial was used to collect blood to be analysed for blood chemistry parameters. One heparinised vial was used to analyse haematology parameters including red blood cell counts and haemoglobin levels. Heparin prevents blood from coagulating and haematological measurements require whole uncoagulated blood (Lanning 2001). The baseline sample was taken 4 days prior to the toxic trial while dogs were being fed non-toxic possum meat to account for any effects of the possum meat on haematology and blood chemistry during the toxic trial.

All dogs were euthanised by a veterinarian (in accordance with the pound’s original euthanasia plans for these animals) on day seven of the trial. Five tissue samples (skeletal muscle, liver, kidney, heart and lung tissue) were taken from each dog post mortem. These samples were stored in a 10% formalin solution prior to preparation for histological analysis. The histological analysis enabled the identification of any tissue damage that may have resulted from the potential ingestion of any NaNO₂ residues. The analysis of blood samples included haematology and blood chemistry. Analysis of blood samples focused on several parameters that are key indicators of liver, kidney and muscle health; these were of particular interest as they would

highlight any potential tissue damage that may have been caused. Levels of bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are commonly measured as markers for liver health (Bush 1991). Toxic damage to the liver commonly results in elevated levels of these parameters (Bush 1991; Willard & Tvedten 2012). Abnormal levels of AST are also symptomatic of muscle tissue damage (Bush 1991; Willard & Tvedten 2012). Phosphate and creatinine are commonly measured as markers for kidney health and abnormal levels can indicate renal damage (Bush 1991; Willard & Tvedten 2012). A reference range of 'normal' values for each of these parameters, as well as key haematology parameters including red blood cell and haemoglobin levels, has been generated by Gribbles Veterinary Pathology New Zealand for diagnostic purposes and are supplied with diagnostic tests. The values of these key parameters recorded for animals in the trial were compared to the reference range to determine if there were any adverse effects from consuming carcasses of possums poisoned with encapsulated NaNO_2 .

Cat secondary poisoning trial

Ten feral cats were captured from the wild and transported to the Pest-Tech Ltd. animal facility. They were housed individually and cages contained a tray with sand to act as a latrine and a box with bedding for them to sleep in. Cages were constructed of steel mesh and measured 110 cm × 55 cm × 60 cm. Cats were fed daily with water available *ad libitum*. The average weight was 3.095 kg (range 2.150–4.128 kg). They were fed a mixture of cat biscuits and beef meat. Feral cats were randomly divided into three groups identical in sample size to those used for the dogs.

In the week before the trial commenced, all three groups were fed non-toxic possum meat to acclimatise them to the taste. Feeding in this stage of the trial was identical to that undertaken for the dog trial in terms of the feeding regime and amounts fed. There was one difference, group two were each fed a single possum carcass on days one, three and five and this was done due to the difference in size and consumption rates between cats and dogs.

Once all animals were well acclimatised to eating possums, the trial commenced. The feeding regime for all three groups was identical to that used in the acclimatisation period the week prior to the trial. The meat, vital organs and gut fed to group one and the whole possum carcasses fed to group two were from possums poisoned with baits containing encapsulated NaNO_2 . Group three, the control group, was fed non-toxic possum meat, vital organs and gut sourced from possums caught in leg-hold traps. On each occasion after cats were fed they were observed continuously for 3 hours for signs of NaNO_2 poisoning including vomiting, excessive thirst, diarrhoea, difficulty breathing, the loss of co-ordination and methaemoglobinaemia.

All cats were euthanised by a veterinarian on day seven of the trial and a blood sample and five tissue samples (skeletal muscle, liver, kidney, heart and lung tissue) were taken from each cat post mortem. Due to the difficulty in obtaining blood samples from feral cats this was the only sample taken and it was collected and analysed following the same procedure as those taken for dogs. The tissue samples were stored in a 10% formalin solution prior to preparation for histological analysis.

Bird secondary poisoning trial

Ten chickens (brown shavers) were purchased from commercial breeders and transported to the Johnston Memorial Laboratory,

Lincoln University. The average weight was 1.907 kg (range 1.652–2.368 kg). They were housed individually in outdoor enclosures constructed of plywood and wire mesh and measuring 200 cm × 150 cm × 150 cm. Chickens were fed daily and water was available *ad libitum*. Chickens were split into group one (n=8) and group two (n=2). In the week before the toxic trial commenced, chickens in both groups were fed non-toxic possum meat to acclimatise them to the taste. Group one was fed approximately 200 g of minced possum meat for days one and two, approximately 50 g of minced vital organs for days three and four and approximately 100 g of minced gut for days five and six. Each individual received the equivalent of one and a half whole possums. Group two was fed approximately 200 g each day made up of a combination of minced possum meat, vital organs and gut for 6 consecutive days; this was the equivalent of one and a half whole possums per chicken.

Once all animals were well acclimatised to eating possum, the trial commenced. Group one chickens were each fed possum meat, vital organs and gut from possums poisoned with baits containing NaNO_2 and group two chickens, the control group, were each fed non-toxic possum meat, vital organs and gut all combined and sourced from possums caught in leg-hold traps.

During the toxic section of the trial, the feeding regime and amounts fed were identical to the acclimatisation period and the trial ran for 7 days. On each occasion after chickens were fed they were observed continuously for 3 hours for signs of NaNO_2 poisoning including difficulty breathing, excessive thirst, diarrhoea, the loss of co-ordination and methaemoglobinaemia (such as shortness of breath, cyanosis, lethargy, loss of consciousness and bluish colouring inside the beak). Each of the trial groups was observed continuously by a single researcher standing within one metre of the enclosures and chickens within each group were observed simultaneously. On day seven all chickens were euthanised using CO_2 , but no blood or tissue was taken.

Veterinarians from Selwyn-Rakaia Veterinary Services took blood and tissue samples from cats and dogs in each trial and also euthanised all cats and dogs with pentobarbital at the conclusion of each trial. Analysis of blood samples was undertaken by Gribbles Veterinary Pathology, Dunedin, and tissue samples were analysed by Gribbles Veterinary Pathology, Christchurch.

All animal manipulations were approved by the Lincoln University Animal Ethics Committee (AEC approvals 236, 369, 370 and 371). Statistical analysis was done using Genstat version 15. The mean results of the blood chemistry and haematology for each treatment group of dogs were compared using repeated-measures ANOVA. The same analysis was carried out for each treatment group of cats using a one-way ANOVA.

Results

None of the dogs, cats or chickens involved in the three trials displayed any obvious physiological signs of methaemoglobinaemia as a result of eating carcasses, minced meat, vital organs or stomach of possums poisoned with encapsulated NaNO_2 . Additionally, in the blood samples taken from all dogs, there were no significant differences ($P > 0.2$) in the haematology (Figs. 1a & 1b) or blood chemistry (Figs. 1c, 1d, 1e, 1f & 1g) between treatment and control groups. No changes in histology relating to NaNO_2 intoxication were

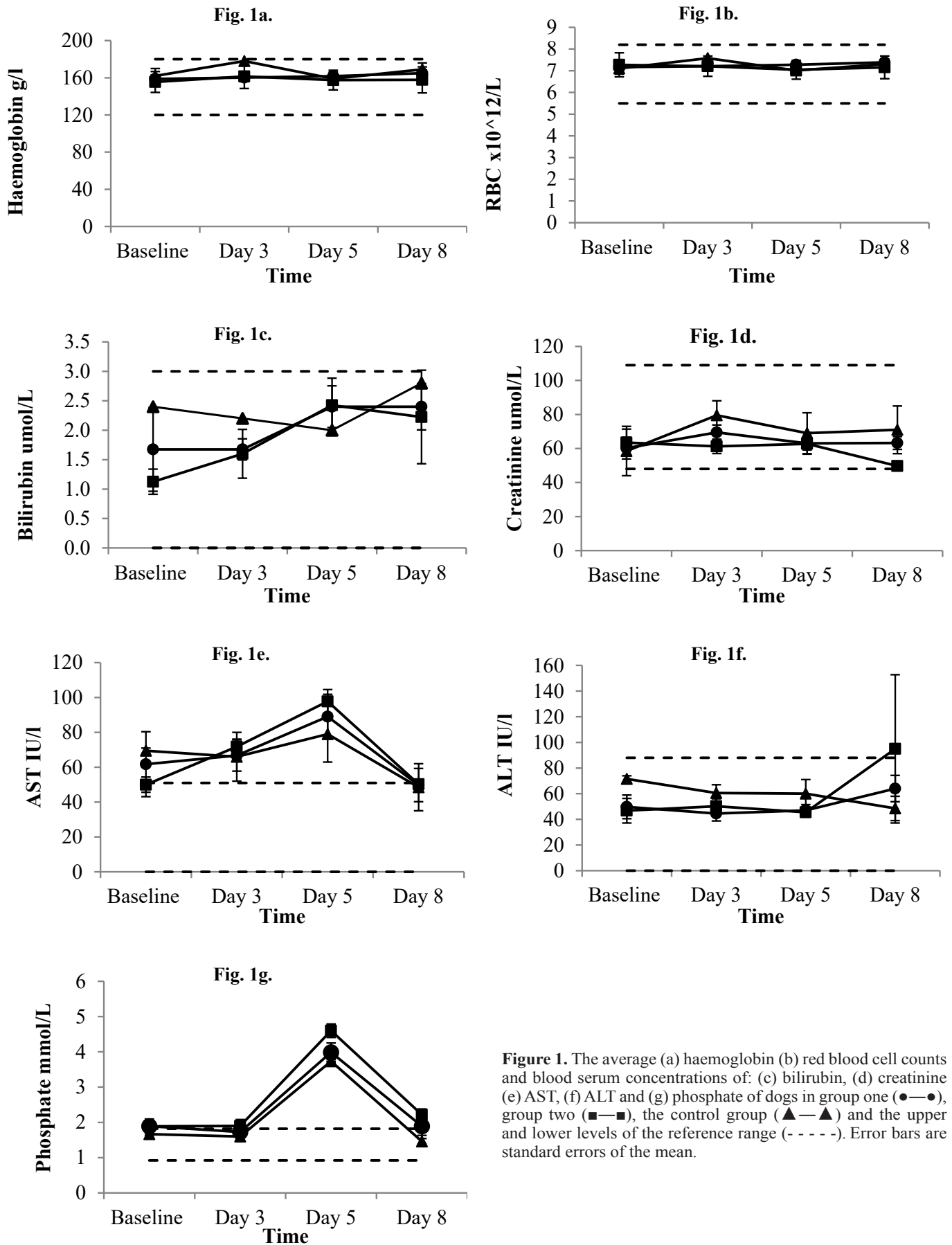


Figure 1. The average (a) haemoglobin (b) red blood cell counts and blood serum concentrations of: (c) bilirubin, (d) creatinine (e) AST, (f) ALT and (g) phosphate of dogs in group one (●-●), group two (■-■), the control group (▲-▲) and the upper and lower levels of the reference range (- - - -). Error bars are standard errors of the mean.

observed in dogs or cats after being fed carcasses, minced meat, vital organs or stomachs of possums poisoned with NaNO₂.

Analysis of tissue samples taken from two possums poisoned with paste bait containing encapsulated NaNO₂ detected very low levels of NaNO₂ residue ($\leq 5 \text{ mg kg}^{-1}$) present in the muscle and stomach samples from both possums on day one but no residues were detected from either possum on day two. No NaNO₂ residues were detected in liver samples from either possum on days one or two.

Dogs

No signs of methaemoglobinaemia were observed in any of the dogs over the course of the study. After dogs in group two were each fed two carcasses, dogs were observed as slightly subdued approximately 30 to 60 minutes after consuming the carcasses, although no obvious signs of methaemoglobinaemia (blue tongue or gums) were observed in any of the dogs. One dog, in group two, was observed regurgitating a small portion of possum carcass fed on days two and seven. On each occasion the dog proceeded to eat the regurgitated material without further untoward effects or any aversion.

The haemoglobin (Figure 1a) and red blood cell (Figure 1b) levels were within the 'normal' reference range for all dogs for the four sets of blood samples taken. There was a significant change over time for levels of haemoglobin ($F_{(3,20)}=1.07$, $P = 0.013$). However, there was no significant time \times group interaction ($F_{(6,20)}=2.93$, $P = 0.081$). There was also no significant difference between treatment groups for haemoglobin ($F_{(2,7)}=0.46$, $P = 0.647$).

There was no significant change in the mean RBC over the length of the trial ($F_{(3,20)}=1.07$, $P = 0.372$). There was also no significant difference between treatment groups for the RBC ($F_{(2,7)}=0.03$, $P = 0.974$).

In the blood chemistry of all dogs in the trial there was no significant change over time for levels of bilirubin ($F_{(3,21)}=2.32$, $P = 0.137$) or for levels of creatinine ($F_{(3,21)}=4.05$, $P = 0.051$) and no significant difference between treatment groups for bilirubin ($F_{(2,7)}=0.53$, $P = 0.608$) or creatinine ($F_{(2,7)}=0.65$, $P = 0.551$).

There was a significant change over time for the levels of AST ($F_{(3,21)}=21.83$, $P < 0.001$). However, there was no significant time \times group interaction ($F_{(6,21)}=1.31$, $P = 0.314$) and no significant difference ($F_{(2,7)}=0.01$, $P = 0.993$) between treatment groups. A total of 40 blood samples were taken from the 10 dogs in the three treatment groups, only eight of these were within the reference range for AST. Only one of the eight blood samples taken from the two non-treatment dogs was within the reference range, AST levels were elevated for dogs in all three groups prior to and during the trial.

The levels of ALT were within the 'normal' reference range for all values except for group two that recorded an elevated mean level on day six (Fig. 1f). This was due to one of the dogs in that group recording an ALT level of 268 IU L^{-1} while the other three dogs recorded levels between 27 to 46 IU L^{-1} . There was no significant change over time for levels of ALT ($F_{(3,21)}=1.03$, $P = 0.346$). There was also no significant difference between treatment groups ($F_{(2,7)}=0.13$, $P = 0.883$).

There was a significant change in the mean levels of phosphate over the length of the trial ($F_{(3,21)}=290.45$, $P < 0.001$) and this was most likely due to the mildly elevated levels of phosphate in all three groups on day three. However, there was no significant time \times group interaction ($F_{(6,21)}=2.18$, $P = 0.153$) and no significant difference between treatment groups ($F_{(2,7)}=1.78$, $P = 0.237$).

The histology of heart, liver, lungs, kidney and skeletal muscle tissue from each dog reported no changes relating to NaNO₂ intoxication for any of the study dogs.

Of the blood chemistry and haematology parameters measured, only ALT, AST and phosphate recorded values outside the reference range. A power analysis carried out for each of these three parameters showed the following. For AST levels recorded in dogs on day three the variance between group means was 18.75 IU I^{-1} and the power was 0.338. To run a test with an appropriate level of power (i.e. $\beta=0.8$) we would need 14 dogs in each treatment group.

For ALT levels recorded in dogs on day six the variance between group means was 46.50 IU I^{-1} and the power was 0.157. To run a test with an appropriate level of power (i.e. $\beta=0.8$) we would need 41 dogs in each treatment group.

For phosphate levels recorded in dogs on day three the variance between group means was 0.86 mmol L^{-1} and the power was 0.954. To run a test with an appropriate level of power (i.e. $\beta=0.8$) we would need four dogs in each treatment group. This was the actual sample size and indicates that the statistical test had sufficient power to detect any differences.

Cats

No signs of methaemoglobinaemia were observed in any of the cats over the entire study; however, all four cats in group one refused to eat the minced possum gut over the 2 days these were presented. In group two, one cat refused to eat any of the whole possum carcasses presented to it, while another consumed two possum carcasses, and two cats consumed approximately 75% of a single possum carcass each.

Despite some differences between groups of cats for the levels of bilirubin and creatinine, they were within the 'normal' reference range for all cats for the blood samples taken at the conclusion of the trial. There was no significant difference between treatment groups for levels of bilirubin ($F_{(2,7)}=1.24$, $P = 0.346$). Levels of creatinine in individuals in group one were significantly higher ($F_{(2,7)}=9.43$, $P = 0.01$) than those of group two and the control group. However, the 2 days between blood collection and serum separation at the laboratory artificially elevated the phosphate, ALT and AST levels as well as the red blood cell and haemoglobin levels for all of the cats and so these parameters of the blood chemistry and haematology were not included in the formal analysis. The histology of heart, liver, lungs, kidney and skeletal muscle tissue showed no changes of significance for any of the study cats.

Birds

No signs of methaemoglobinaemia were observed in any of the chickens over the entire study. Chickens consumed all of the minced possum meat, vital organs and stomach fed to them (apart from two chickens that only consumed 50% of their minced possum gut on day six). These two chickens then resumed eating their standard grain feed on day seven when the trial had concluded.

Discussion

The results of this study indicate there is no, or at worst a very low, risk of secondary poisoning for dogs, cats and birds that eat the carcasses and/or vital organs of possums poisoned with baits containing 10% NaNO₂. The results indicating a lack of secondary poisoning risk are consistent with the relatively low

acute toxicity of NaNO_2 versus other vertebrate toxic agents and the rapid excretion of NaNO_2 (Kohn et al. 2002; MRI 2004). The LD_{50} for NaNO_2 in possums is approximately 121.6 mg kg^{-1} (95% CI $45.36\text{--}169.6 \text{ mg kg}^{-1}$) (Shapiro et al. 2016) compared to 1.2 mg kg^{-1} for 1080 in possums (Eason et al. 2011). As well as no obvious signs of methaemoglobinaemia being observed in dogs, cats or chickens, the blood chemistry parameters analysed for dogs and cats indicated that after they consumed possum carcasses their liver and renal function remained normal and there was no damage to muscle tissue.

The use of animals in research is strictly governed by ethics and the desire to keep group sizes as small as possible. The research reported here was no different; however, when the response is variable (within groups) statistical power can be compromised. For our analysis there is concern regarding recorded values outside of the reference range, namely for ALT, AST and phosphate.

For phosphate levels, the power analysis indicated that the sample size of four still provided high statistical power when analysing the results for day five. This analysis suggests that the elevated levels of phosphate at this time point were unlikely to be different to the control group.

For both ALT and AST, a power analysis indicated a low statistical power between the treatment and control groups with the sample sizes used. Certainly this warrants comment; however, only one dog in group two recorded an abnormally high level of ALT and this was on day six, all other dogs were within the reference range parameters at each time point. This dog had consumed two whole possum carcasses although it displayed no obvious signs of NaNO_2 poisoning or symptoms of methaemoglobinaemia. Also, on day six the haematology parameters and the other blood chemistry parameters for this dog were all within the 'normal' reference range. It was not the dog in group two that was observed regurgitating a small portion of possum carcass.

Additionally, the AST levels were elevated for all 10 dogs prior to and during the trial and to redo this experiment with acceptable statistical power we would need 14 dogs for each treatment group. Despite the low level of statistical power future trial work is not warranted given that there were no obvious signs of NaNO_2 poisoning and symptoms of methaemoglobinaemia observed in any of the dogs. Also, the histology of heart, liver, lungs, kidney and skeletal muscle tissue from each dog reported no changes relating to NaNO_2 intoxication.

NaNO_2 is rapidly eliminated from the blood of both rats and mice. In rats orally dosed with 80 mg kg^{-1} , peak plasma concentrations were achieved at 30 mins after dosing and these decreased to below the limit of detection after 8 hours (MRI 2004). In mice, peak plasma concentrations occurred after 10 minutes and NaNO_2 was undetectable in the blood after 4 hours. The pharmacokinetic data on NaNO_2 in such diverse species as mice, rats, sheep, dogs, horses and humans (Schneider & Yeary 1975; Kohn et al. 2002), coupled with information on the toxicodynamics of NaNO_2 , suggests that a considerable amount of the NaNO_2 ingested by a possum is likely to be excreted prior to death, even though death is comparatively rapid.

The toxicokinetics and toxicodynamics of NaNO_2 led us to feel reasonably confident that the secondary poisoning risk of NaNO_2 would be low and this has now been verified. The low risk of secondary poisoning for dogs, cats and birds that eat possum carcasses which contain NaNO_2 residues is comparable to cholecalciferol, which also has low secondary poisoning

risk for dogs and cats (Eason et al. 2000) and cyanide which has no secondary poisoning risk (Gregory et al. 1998). This outcome is in contrast with 1080, where the risk of secondary poisoning for dogs that eat possum carcasses poisoned with 1080 is high (Meenken & Booth 1997; Eason et al. 2014), and brodifacoum which can accumulate and cause secondary poisoning (Litten et al. 2002).

The low secondary poisoning risk from carcasses of possums poisoned with NaNO_2 , combined with an effective antidote for primary poisoning and its effectiveness for killing possums (Shapiro et al. 2016) and feral pigs (Shapiro et al. 2015) gave us confidence in pursuing registration of NaNO_2 baits for possum and feral pig control.

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