

Sensitivity of The Stripe-Faced Dunnart, *Sminthopsis Macroura* (Gould 1845), To The Phenyl Pyrazole Insecticide, Fipronil, Toxicological Signs And Implications For Pesticide Risk Assessments In Australia

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Research Article

Keywords: Dunnart, acute oral toxicity, fipronil, median lethal dose, *Sminthopsis macroura*, pesticide risk assessment

Posted Date: September 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-890972/v1>

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Abstract

A lack of toxicity data quantifying responses of Australian native mammals to agricultural pesticides prompted an investigation into the sensitivity of the stripe-faced dunnart, *Sminthopsis macroura* (Gould 1845) to the insecticide, fipronil (5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-trifluoromethylsulfinyl pyrazole, CAS No. 120068-37-3). Using the Up-And-Down method for determining acute oral toxicity in mammals, derived by the Organisation for Economic Cooperation and Development (OECD), median lethal dose estimates of 990 mg kg^{-1} (95% confidence interval (CI) = $580.7\text{--}4770.0 \text{ mg kg}^{-1}$) and 270.4 mg kg^{-1} (95% CI = $0.0\text{--}>20000.0 \text{ mg kg}^{-1}$) were resolved for male and female *S. macroura* respectively. The difference between median lethal dose estimates for males and females may have been influenced by the increased age of two female dunnarts. Further modelling of female responses to fipronil doses used the following assumptions: (a) death at 2000 mg kg^{-1} , (b) survival at 500 mg kg^{-1} and (c) a differential response (both survival and death) at 990 mg kg^{-1} . This modelling revealed median lethal dose estimates for female *S. macroura* of 669.1 mg kg^{-1} (95% CI = $550\text{--}990 \text{ mg kg}^{-1}$; assuming death at 990 mg kg^{-1}) and 990 mg kg^{-1} (95% CI = $544.7\text{--}1470 \text{ mg kg}^{-1}$; assuming survival at 990 mg kg^{-1}). These median lethal dose estimates are 3–10-fold higher than the only available LD50 value for a similarly sized eutherian mammal, *Mus musculus* (L. 1758; 94 mg kg^{-1}) and that available for *Rattus norvegicus* (Birkenhout 1769; 97 mg kg^{-1}). Implications for pesticide risk assessments in Australia are discussed.

Introduction

Fipronil (5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-trifluoromethylsulfinyl pyrazole, CAS No. 120068-37-3), a phenyl-pyrazole compound, is a broad spectrum, low dose chemical registered for use in many countries including Russia, South Africa and Australia (Balanca and de Visscher 1997; Bobe et al. 1998) and in 2013 was banned from use on corn and sunflower crops by the European Union based on its role in “colony collapse” in bee populations in France (Bijleveld van Lexmond et al. 2014; Chagnon et al. 2014; Holder et al. 2018). This pesticide is an extremely active neurotoxicant and is a potent disrupter of insect central nervous systems where it works by interfering with the passage of chloride ions through the chloride-gated channel regulated by gamma-aminobutyric acid (GABA) receptors (Hainzl and Casida 1996; Hainzl et al. 1998; Kitulagodage et al. 2008; Kitulagodage et al. 2011a; Kitulagodage et al. 2011b; Smith et al. 2010) and acts via both direct contact and via-stomach action (Story et al. 2005).

Although fipronil is used throughout the world as a crop protection agent, little information exists concerning either its toxicological impacts on vertebrates, or what the ecological and population-level consequences of exposure might be (Smith et al. 2010). This data gap is problematic for the assessment of environmental risk associated with the use of fipronil for locust control where low-volume, oil-based insecticide formulations are used over arid and semi-arid native grasslands to control acridid (grasshopper and locust) populations in several countries including Australia (Story et al. 2005; Walker et

al. 2016). The use of fipronil for acridid control in Africa has been discontinued largely due to its environmental impacts (Peveling et al. 1999; Peveling et al. 2003; Steinbauer and Peveling 2011).

Mammalian risk assessments undertaken in Australia for fipronil cite only two LD₅₀ values, making the development of species sensitivity distributions for a complete probabilistic risk assessment impossible (Posthuma et al. 2002). Furthermore, both estimates of acute oral toxicity are contained within industry reports listed as “Commercial in Confidence” and so the complete details of the study parameters and results are not available through the established scientific literature. Rather, summaries by the United States Environmental Protection Agency (USEPA) need to be relied upon to gauge the potential mammalian responses to fipronil exposure (Food and Agriculture Organisation of the United Nations 1997). In one study, an LD₅₀ estimate of 97 mg kg⁻¹ has been reported for an unspecified rat species with abnormal gait and posture, piloerection, lethargy tremors and convulsions all reported as signs of intoxication in the study (Environment Australia 1998). A second estimate of acute oral toxicity has been reported as 94 mg kg⁻¹ for *Mus musculus* (L. 1758). To date, there are no studies quantifying the acute oral toxicity of fipronil for Australian endemic mammalian fauna. A further estimate of 95 mg kg⁻¹ has been reported for *M. musculus* (Tomlin 2006).

Recent research has shown that two dunnart species (the fat-tailed dunnart, *Sminthopsis crassicaudata* (Gould 1844); and stripe-faced dunnart, *S. macroura*), were 10 - 14 times more sensitive to the organophosphorus insecticide, fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenyl)-phosphorothioate), another locusticide (Story et al. 2011) compared to eutherian mammals. The inclusion of these median lethal dose estimates into species sensitivity distributions (SSD) approximately halved the allowable residue values derived at the 5% protection threshold (HD₀₅) from 177 mg kg⁻¹ to 93.5 mg kg⁻¹ (Story et al. 2011). There is often a need to extrapolate from a narrow range of organisms tested under standard laboratory conditions to free-living populations or ecosystems during pesticide risk assessments (Barnthouse et al. 2008; van Straalen 2002). However, significant differences in the estimated hazard thresholds for fenitrothion acute oral toxicity values for dunnarts (Story et al. 2011), highlight the need to evaluate effects of pesticides on non-target species, particularly when these species are phylogenetically distinct from those originating in North America or the European Union (Story et al. 2011).

Previous research into the impacts of fipronil exposure on avian species has shed light on the importance of toxicological testing on a broader range of species than those currently presented in pesticide registration evaluations (Smith et al. 2010). Previously, acute fipronil toxicity was only considered of concern in the Galliformes (Tingle et al. 2000). More recently, fipronil's avian acute oral toxicity has been shown to group phylogenetically when additional species are tested (Kitulagodage 2011). Moreover, it has been shown that pesticide adjuvants add synergistically to the overall toxicity of formulations (Kitulagodage et al. 2008), the metabolic fate of fipronil closely resembles that of organochlorine insecticides OCs (Kitulagodage et al. 2011b) and that it can be maternally transferred resulting in developmental abnormalities in hatchlings (Kitulagodage et al. 2011a).

The Up-And-Down protocol (UDP), devised and recommended by the Organisation for Economic Cooperation and Development (OECD) for resolving estimates of acute oral toxicity, is a useful alternative to conventional LD₅₀ testing (Bruce 1985; Story et al. 2011). The UDP technique enables a median lethal dose estimate to be quantified for a toxicant that is comparable to one achieved from conventional toxicity testing, but requires far fewer animals (Lipnick et al. 1995). Moreover, the LD₅₀ values derived using the UDP method are comparable to other acute toxicity testing classification systems, thus allowing a comparison of pesticide sensitivity of Australian marsupial fauna derived here with non-native eutherian mammals tested elsewhere (Story et al. 2011).

Of the numerous mammal species in Australia, members of the Dasyuridae are the most likely to be affected by pesticide exposure resulting from locust spray operations (Story et al. 2016). A significant overlap in habitat preferences between the Australian plague locust (*Chortoicetes terminifera* Walker 1870), the species most commonly the focus of control operations, and *S. macroura*, the combination of dunnart's small body mass, some as low as 7 g (van Dyck and Strahan 2008) and high metabolic requirements, their primarily insectivorous diet, and their ability to gorge feed on intoxicated locusts make these species particularly vulnerable to the effects of chemically based locust control (Story 2015).

This study quantifies the acute oral toxicity of fipronil to the endemic Australian marsupial, *S. macroura* and compares the values obtained with the very limited amount of data available for mammals more broadly. Pesticide residue levels of the parent compound, fipronil and its metabolites in plasma, brain, liver, kidney and caudal and subcutaneous adipose tissues were also quantified from dunnart tissue to serve as a pilot investigation for a subsequent study into the comparative metabolic fate of fipronil in two similar-sized but systematically divergent species, *M. musculus* (eutherian) and *S. macroura* (metatherian). Implications for pesticide risk assessments in Australia are discussed.

Materials And Methods

Animal housing.

Dunnarts used in the trial were sourced from a breeding colony kept at Commonwealth Scientific and Industrial Research Organisation (CSIRO) Black Mountain Laboratories (Acton, Australian Capital Territory, Australia) made up of either field-collected animals (n = 9) or first-generation descendants of those individuals (n = 9). All dunnarts were sexually mature at the time of the experiment and were maintained in individual cages on a day:night cycle that reflected ambient Canberra conditions during May-August 2016 and kept at a constant temperature of 23⁰C. Dunnarts were fed low-fat minced beef, supplemented with calcium carbonate (25 g kg⁻¹) and 0.015% potassium iodide solution (43 mL per 12 kg lean beef mince) as used by previous authors to maintain *S. macroura* colonies (Selwood and Cui 2006). Water was available *ad libitum*. Dunnarts were fasted for 24 h before the administration of fipronil doses and then observed using video recording for 48 h after pesticide exposure (see below) and then daily without video recording for the following 12 d. Food was returned to the dunnarts' cages 24 h after dosing.

Determination of acute oral toxicity.

In total, 18 dunnarts (7 males and 11 females) were used to determine the acute oral toxicity of fipronil with doses administered according to the UDP dosing schedule. Each animal was weighed immediately prior to dosing and doses were made up using reference grade fipronil (ChemService Inc. West Chester, PA, USA; CAS number: 120068-37-3, Lot number: 3719000), dissolved in 20 µL of acetone made up to 0.2 mL using canola oil. Each dose was given oesophageally using a 23 gauge gavage needle attached to a 1 mL syringe.

We followed OECD Guideline 425 (Organisation for Economic Cooperation and Development 2001) to estimate the acute oral toxicity value, in this case a median lethal dose along with its corresponding confidence interval for each gender. We used the Main Test of this guideline, with an alpha value (α) of 0.25 and a starting dose of 175 mg kg⁻¹. The UDP protocol stipulates that where no estimate of the substance's lethality is available, dosing should be initiated at 175 mg kg⁻¹. In most cases, this dose is sublethal and therefore serves to reduce the level of pain and suffering experienced by animals used in the experiment.

The UDP dosing protocol consists of a single-ordered dose progression in which animals are dosed individually and then observed for a minimum of 48 h before a subsequent dose is administered to another animal. If a dunnart survived the dose given to it within this short-term interval, the next animal received a higher dose, but if an animal succumbed to dosing within this time period, the dose progression proceeded with a lower dose (see Tables 1 and 2) as prescribed in the Acute Oral Toxicity (AOT) software program (Organisation for Economic Cooperation and Development 2001) used for the analysis of dosing data. The long-term fate of dunnarts, defined here as the fate of animals at 14 d post-exposure surviving a given dose of fipronil, was also recorded. Dosing continued until one of the three standard stopping criteria was met:

- three consecutive animals survived at the upper bound of dosing,
- five reversals occurred in any six consecutive animals tested (when a reversal is created by a pair of responses in a situation in which a nonresponse is observed at a particular dose and a response is observed at the next dose tested, or *vice versa*),
- or at least four animals have followed the first reversal and the specific likelihood ratios exceed the critical value as determined by the AOT software.

After the stopping criteria had been reached, an estimate of the LD₅₀ value (calculated as the median lethal dose using maximum likelihood statistics) and the associated confidence limits were calculated using the AOT software Statistical Program version 1.0 (Organisation for Economic Cooperation and Development 2001).

The body mass of each dunnart was measured approximately 30 mins before pesticide exposure and then at daily intervals, up to 14 d thereafter for those dunnarts not incurring a lethal dose. Body mass

data was analysed using t-tests on data pooled by dose for males and females. Animals which became moribund were euthanased using isoflurane under oxygen and tissue samples collected and stored at -80°C until subsequent analysis (see below).

Quantification of tissue residue levels and determination of the purity of fipronil.

Dunnart liver, brain, plasma and fat tissue samples were weighed and homogenised in a Tissuelyser II homogeniser (Qiagen). Samples larger than 0.3 g (liver) were homogenised in a stainless steel 25 ml grinder (Retsch) with a 20 mm stainless steel ball and samples smaller than 0.3 g were homogenised in 2 ml disposable centrifuge tube with a 6 mm diameter stainless steel ball. For every 0.2 g of sample weight, 1 ml of acetonitrile (ACN) with 1% acetic acid (AA) was added. Liver, brain and plasma were homogenised for 3 minutes at 20 Hz and fat tissue for 9 min at 20 Hz, all at (or close to) -20°C .

Homogenised samples were transferred into disposable 15 ml centrifuge tubes to which 0.5 g of $\text{MgSO}_4/\text{NaOAC}$ (4:1 ratio) was added for every 1 ml of ACN +1% AA. Samples were vortexed for 1 min and centrifuged for 4 min at $4,000 \times g$. Supernatant (1 ml) was transferred into a 2 ml centrifuge tube with 0.3 g of QuEChERS Dispersive Solid Phase Extraction (1200 mg MgSO_4 , 400 mg primary secondary amine, 400 mg C18, 400 mg graphitized carbon black; LECO Cat. No.26222-248). The sample was then vortexed for 1 min and centrifuged for 4 min at $16000 \times g$. Supernatant (200 μl) was then transferred into a 2 ml glass vial with a 250 μl glass insert. Samples were kept at 4°C during assay.

Samples were analysed on an Agilent 6490 Triple Quad LCMS. Solvents A: H_2O + 5 mM ammonium formate + 0.2% formic acid. Solvent B: 90% methanol +10% H_2O + 5 mM ammonium formate + 0.2% formic acid. A Poroshel 120 EC C18 $2.7 \mu\text{m}$ ($2.1 \times 50 \text{ mm}$) column (InfinityLab) was used and analytes were eluted using a flow rate of 0.2 ml min^{-1} with the following gradient: 1 min at 70% B, 1-10 min 70 to 90% B, 10-11 min 90% B. The volume of injected sample was 1 μl . Fipronil desulfinyl (hereafter referred to as fip-desulfinyl, retention time (RT) 3.2 min), fipronil (RT 3.7 min), fipronil sulfide (hereafter referred to as fip-sulfide, RT 3.9 min) and fipronil sulfone (hereafter referred to as fip-sulfone, RT 4.5 min) residues were analysed in negative ion mode and were confirmed by their three most abundant product ions at optimised collision energies.

All fipronil and fipronil derivatives standards were purchased from Sigma Aldrich. A calibration curve was produced using 0.001, 0.01, 0.1, 1 and 10 $\mu\text{g/ml}$. Standards were prepared fresh and read before, in the middle and at the end of the sample batch. A positive control containing 0.01 $\mu\text{g/ml}$ of fipronil and derivatives and a negative control (ACN +1% AA) was run every three injections to ensure no carry over from previous samples and consistency of quantification. Positive controls contained 0.01 $\mu\text{g/ml}$ of fipronil and derivatives, and negative controls (ACN +1% AA) were run every 3 samples.

Results

Determination of acute oral toxicity.

Estimates of the median lethal dose values calculated by the AOT (Organisation for Economic Cooperation and Development 2001) software for male and females *S. macroura* were 990 mg kg⁻¹ (95% CI = 580.7 – 4770 mg kg⁻¹) and 270.4 mg kg⁻¹ (95% CI = 0 - >20000 mg kg⁻¹) respectively. Concern over the difference between median lethal dose estimates for males and females potentially being influenced by the increased age of two female dunnarts (Table 2) resulted in further modeling of dunnart responses to fipronil using the assumptions;

(a) death at 2000 mg kg⁻¹,

(b) survival at 500 mg kg⁻¹, and

(c) a differential response (both survival and death) at 990 mg kg⁻¹.

This modeling revealed median lethal dose estimates for female *S. macroura* of 669.1 mg kg⁻¹ (95% CI = 550 – 990 mg kg⁻¹; assuming death at 990 mg kg⁻¹) and 990 mg kg⁻¹ (95% CI = 544.7 – 1470 mg kg⁻¹; assuming survival at 990 mg kg⁻¹).

Signs of intoxication.

Toxicological signs observed following pesticide exposure included piloerection, withdrawal, eye closure, shivering and, intermittently, a lack of response to disturbance. In dunnarts receiving higher doses (e.g. > 550 mg kg⁻¹), it was not until approximately 24 h after exposure that more severe signs typical of fipronil toxicity, such as tremors and convulsions were observed. The signs of intoxication displayed by each dunnart were video recorded and a full quantitative analysis will be presented in a subsequent publication.

Dunnart body mass.

Changes in dunnart body mass after exposure show high variability but no visually discernable pattern (Fig 2). No statistically significant change in body mass was detected for either males ($t_{0.05(2)3}; p = 0.283$) or females ($t_{0.05(2)8}; p = 0.035$) after pesticide exposure using pooled dose data for those dunnarts not receiving a lethal dose.

Time to death for dunnarts receiving a fatal dose.

As only 6 deaths (2 males and 4 females) occurred within the 48 h time limit placed on the determination of acute oral toxicity, across a range of dose levels from 99 mg kg⁻¹ – 2000 mg kg⁻¹ (Tables 1 and 2), insufficient data exists for a statistical examination of trends concerning the time to death for dunnarts receiving a lethal dose. From the limited data available, time to death tended to decline with increasing dose greater than 175 mg kg⁻¹.

Residues of fipronil and its metabolites in tissues.

Dunnarts given doses of either 990 mg kg⁻¹ or 2000 mg kg⁻¹ had higher tissue levels of both the parent, fipronil, and the oxidative metabolite, fip-sulfone, in subcutaneous and caudally stored fat samples, although no discernible pattern associating increased tissue residues with an increasing administered dose was evident (Fig 3). Fipronil and fip-sulfone residues were either very low or absent from liver, brain and plasma samples taken from dunnarts across all doses (Fig 3).

Dunnarts not surviving the administered dose had higher levels of the parent compound, fipronil, and the oxidative metabolite, fip-sulfone, in liver tissue but similar levels in brain tissue. These dunnarts showed higher levels of both fipronil and fip-sulfone in both the subcutaneous and tail fat, indicating that the fip-sulfone is being produced and rapidly (given the time course of the current study) stored in adipose tissues (Fig 4). Comparatively high levels of the fip-sulfide metabolite were also seen in the subcutaneous fat sampled from dunnarts not surviving a given dose (Fig 5). Brain, liver and plasma tissues from dunnarts surviving the dose contained very little, if any, fip-desulfinyl and fip-sulfide metabolite residues. However, the few that did not survive dosing contained relatively large amounts of these metabolites in subcutaneous fat (range = 5.91 - 6354.34 ug kg⁻¹), with smaller amounts stored in tail fat (range = 2.00 - 85.78 ug kg⁻¹) (Fig 5).

Mean fipronil and fip-sulfone tissue levels were similar in male and female dunnarts with maximal residues being detected in subcutaneous and caudally stored fat (Fig 6). Both male and female dunnarts demonstrated an equal propensity to store both fipronil and fip-sulfone in subcutaneous and tail fat reserves. Males had comparatively higher levels of fipronil in brain tissue than females, although sulfone in the brain and liver tissues sampled were similar (Fig 6). While male dunnarts showed fip-sulfide and fip-desulfinyl residues in subcutaneous and caudally stored fat, female residue levels were extremely low (Fig 7).

Discussion

Median lethal dose.

Both genders of *S. macroura* tested in the current study were significantly less sensitive to fipronil than the only other mammals tested, *M. musculus* (L. 1758; 94 mg kg⁻¹) and *Rattus norvegicus* (Birkenhout 1769; 97 mg kg⁻¹) (Food and Agriculture Organisation of the United Nations 1997) in the literature to date. This result directly contrasts with a 10 - 14 fold difference in acute oral toxicity for both dunnart species (*S. crassicaudata* = 129 mg kg⁻¹ CI = 74.2 - 159.0; *S. macroura* = 97 mg kg⁻¹ CI = 88.3 - 120.0) to the organophosphorous pesticide, fenitrothion, when compared to *M. musculus* (1100 - 1400 mg kg⁻¹), using the same technique for the resolution of median lethal dose estimates (Story et al. 2011). Whilst the two chemicals mentioned above exert their influence on different physiological pathways, the significant differences in patterns of acute oral toxicity compound the lack of acute oral vertebrate toxicological data thereby reducing the predictive value of pesticide risk assessments for endemic Australian vertebrates.

Current risk assessment frameworks for pesticides generally use, in part, the lowest median lethal dose for mammals to assess hazard of a chemical (Newman 2015). Increasingly, median lethal dose estimates, either LD₅₀ or LC₅₀ data, obtained from chemical exposure studies can be incorporated into species sensitivity distributions (SSDs) to comparatively assess toxicity and derive hazard threshold values (Posthuma et al. 2002). However, the generation of a distribution using three data points, while possible with the assistance of extrapolation factors (as outlined in (Posthuma et al. 2002)), is less likely to provide a robust representation of the desired risk thresholds (e.g. HD₀₅) rendering the estimation of safe residue levels problematic. Recent research has highlighted a similar problem in relation to the avian acute oral toxicity profile of fipronil. While previous risk assessments for this pesticide have cited a primarily bimodal toxicological profile with a highly sensitive species at one end (the northern bobwhite, *Colinus virginianus* L. 1758; LD₅₀ = 11.3 mg kg⁻¹) and an extremely insensitive species at the other (the mallard, *Anas platyrhynchos* L. 1758; LD₅₀ = 2150 mg kg⁻¹), Kitulagodage *et al.* demonstrated that, by testing other species, fipronil's acute oral toxicity fits a distribution similar to that of other pesticides, and, moreover, is grouped along avian orders (Kitulagodage 2011; Kitulagodage et al. 2011b).

The advantages of using the UDP protocol for the derivation of median lethal doses over the traditional LD₅₀ assessment techniques are well established (Newman 2013; Story et al. 2011). Specifically, a reduction in the number of individuals required to resolve an estimate of median lethal dose is desirable from an animal ethics perspective, particularly if the use of other chemical impact metrics (e.g. quantitative structure-activity relationships, QSARs) to assess the potential sensitivity of untested species to a pesticide are precluded due to a lack of data (Story et al. 2011). Additionally, the UDP method has been shown to produce a median lethal dose (LD₅₀) estimate similar to that achieved from conventional toxicity testing with the LD₅₀ values derived from this method being directly comparable to other acute toxicity testing classification systems, thus allowing a comparison of pesticide sensitivity of Australian marsupial fauna with non-native eutherian mammals (Story et al. 2011).

The assessment of agricultural and veterinary chemicals for registration in Australia is a process that is evolving over time as both the amount of data submitted to support registrations increases and assessment methodologies and detection levels improve (Hyman 1997). If the use of SSDs to assess protection thresholds in relation to Australian endemic species is to continue, then further sensitivity research will be required to circumvent the need to extrapolate from a narrow range of organisms tested under standard laboratory conditions to free-living populations or ecosystems. The results of the present study show the limitations of this approach and highlights the importance of evaluating the effects of pesticides on non-target species that are likely to be exposed, particularly when these species are phylogenetically distinct from those used in studies of pesticide sensitivity originating in North America or the European Union.

Fiprole (fipronil and metabolite) residues in tissues and body mass.

The use of the UDP methodology to quantify a median lethal dose unavoidably results in very small experimental groups, sometimes $n = 1$, thereby resulting in secondary data sets, such as residue loads from tissue samples, that are unable to be subjected to appropriate statistical analyses. Despite this limitation, the current study quantified fiprole residue levels in kidney, liver, plasma, brain and caudal and subcutaneous adipose tissue samples taken from individual dunnarts at either the time of death or at the end of the 14 day post-dose observation period. Obviously, these results need to be viewed with a great deal of circumspection. However, we report these results from the current study as a precursory dataset to maximise the amount of information derived and to better inform a subsequent study into the comparative metabolic fate of fipronil in two similar-sized, but systematically divergent species, *M. musculus* (eutherian) and *S. macroura* (metatherian) accepting the abovementioned limitations.

Studies investigating the biotransformation of fipronil in rats (Food and Agriculture Organisation of the United Nations 1997) have quantified 3 primary metabolites after hepatic transformation of the parent compound fipronil (Fig 1.). Of these metabolites, the fip-sulfone and fip-desulfinyl have been shown to be of toxicological concern in previous studies. The oxidative fip-sulfone metabolite has a six-fold higher binding affinity for the postsynaptic GABA receptor (Hainzl et al. 1998) and metabolism of the parent compound to this derivative has been shown to add synergistically to the overall toxicity of a fipronil-based formulation in pesticide-exposed birds (Kitulagodage et al. 2011b). Moreover, avian studies have demonstrated that inclusion of fip-sulfone residues in a regression analysis of post-exposure body mass loss provided a much better fit than regressions comparing loss of body mass with the parent compound, fipronil, alone in brain, liver and adipose tissues (Kitulagodage et al. 2011b). The overlap between symptoms of intoxication, the time course of fip-sulfone residues in brain, liver and adipose tissue, lack of post-dose feeding activity and subsequent weight loss in dosed birds provided insight into an observed increased selective toxicity to the three galliform species tested (Kitulagodage 2011; Kitulagodage et al. 2011b).

In the current study, fipronil and fip-sulfone residues were more prominent at the higher doses administered (e. g. 990 and 2000 mg kg⁻¹) with the residue load occurring in subcutaneous and caudally stored fat, liver and brain, in descending order of magnitude. Slightly higher levels of fipronil were present in male (versus female) brains at the time of analysis, but very little difference existed between either fipronil or fip-sulfone levels in either subcutaneous or tail fat and plasma. Dunnarts not surviving the administered dose showed higher fipronil and fip-sulfone levels across adipose tissues, liver and brain. However, as was the case with dunnarts surviving a given dose, very little, if any, plasma-bound residue bringing into question whether the use of fipronil residue in plasma is suitable as a biomarker of pesticide exposure in wildlife monitoring studies. While the detection of fip-sulfone in the liver and adipose tissues of males and females across the various administered doses indicates the metabolism of fipronil to the fip-sulfone metabolite, the levels detected, in addition to low levels of this metabolite finding its way to brain tissue and an absence of weight loss in dunnarts surviving the administered dose, is contrary to the findings in the abovementioned avian studies. Further research into the metabolic fate of this pesticide in marsupials is required to better elucidate the role of the fip-sulfone metabolite in

determining the overall toxicity of fipronil-based pesticide formulations, as seems to be the case in more sensitive avian orders.

Fip-desulfinyl is generally considered to be a photolytic breakdown product and not a metabolite as such.

In the current study, analysis detected generally low levels of this compound (range = 0 – 46.07 ng g⁻¹ with one male dunnart (dose = 99 mg kg⁻¹) returning an outlier value of 281.89 ng g⁻¹ in adipose tissue) and due to its toxicological significance, we have reported these results. Fip-desulfinyl is considered of high toxicity with an acute oral LD50 of 15 (males) – 18 (females) mg kg⁻¹ for *M. musculus* (Food and Agriculture Organisation of the United Nations 1997). When administered orally to mice, the fip-desulfinyl metabolite has been shown to decrease body weight at doses of 30 and 60 ppm, whereas a lower dose of 3 ppm was seen to increase motor activity, irritability and aggression with convulsions also observed (Food and Agriculture Organisation of the United Nations 1997). Although present in small quantities, presumably as a result of photolytic breakdown of the dosing formulation immediately after preparation, its acute toxicity would necessitate its inclusion in residue analysis for any future field based trial investigating *in situ* wildlife impacts. Higher levels of the fip-sulfide metabolite (range = 0 – 85.78 ng g⁻¹ with the same male dunnart as above (dose = 99 mg kg⁻¹) returning an outlier value of 6345.34 ng g⁻¹ in adipose tissue) were also found in adipose tissues of pesticide-exposed dunnarts. The higher LD50 values for this compound reported for mice (69 (males) and 100 (females) mg kg⁻¹ (Food and Agriculture Organisation of the United Nations 1997)) indicates a moderate toxicity for this species, with similar toxicological signs as those reported for the other breakdown products (fip-sulfone and fip-desulfinyl) as well as the parent (fipronil).

The Australian arid zone is characterised by low productivity and highly variable rainfall (Stafford-Smith and Morton 1990). Species inhabiting these environments have evolved a range of adaptations which assist them in coping with the inconsistent, and often sparsely distributed resources - such as the ability for rapid, long-range movement enabling animals to access areas of recent rainfall and capitalize on the increase food resources (Dickman et al. 1995; Letnic and Dickman 2005). The Dasyuridae caudally store fat to provide an energy reserve that can be utilised during times of resource limitation (Morton and Dickman 2008a; Morton and Dickman 2008b). The ability for lipophilic xenobiotic compounds, such as agricultural pesticides and their toxic metabolites, to be stored along with these fat reserves has the potential to ensure that pesticide residues remain biologically available by being constantly metabolized as dunnarts utilise caudally stored fat to maintain the energetic resources necessary for sustaining daily life during times of drought. Conventional toxicity testing used for chemical risk assessments generally defines exposure times for the determination of median lethal dose values to quantify mortality (Newman 2015). The tendency for toxic substances to be stored in adipose tissue and later metabolized when animals are facing resource limitations, extends the exposure period for chemicals significantly beyond, for example, either the 48 hr acute oral toxicity test limit or the 30 d reproductive test limit more commonly used in pesticide risk assessments (Buttemer et al. 2008; Story et al. 2016).

Conclusions

The scarcity of information quantifying the responses of evolutionarily unique Australian endemic species to pesticides impedes the development of biologically relevant risk assessments for the registration of chemicals in Australia. The lack of sensitivity to fipronil displayed by *S. macroura*, as measured by acute oral toxicity, directly contrasts with the increased sensitivity (10 – 14 fold) of the same species to another locusticide, fenitrothion (Story et al. 2011), highlighting the need for a better understanding of the biochemical pathways responsible for any species susceptibility to xenobiotics and thereby increasing the predictive value of risk assessments. Additional studies are now required to better understand the metabolic fate and biochemical parameters responsible for pesticide metabolism in mammals, particularly when the active ingredient of pesticide formulations can produce toxic metabolites. Finally, while the relatively high median lethal dose values quantified here would suggest a minimal impact of pesticide exposure on the species tested, no information quantifying the pesticide exposure of *S. macrourain situ* exists. Clearly, more research into dietary and non-dietary pesticide exposure pathways and residue loads are required to better inform impacts assessments.

Declarations

Funding and conflict of interest. This study was funded by the Australian Plague Locust Commission and the Commonwealth Scientific and Industrial Research Organisation (CSIRO). We acknowledge that the listed authors are employees of the funding organisations but that this relationship had no influence the outcomes of the work reported in this paper. None of the authors are associated with the company responsible for manufacturing the chemical under investigation in the current study.

Acknowledgements. We thank Nikki van de Weyer, Megan Pratt, Clare Mulcahy, Genevieve Buckton and Sarah Hickman for assistance with dosing observations and animal husbandry.

Animal ethics approval. This research was undertaken under CSIRO Animal Ethics Approval AEC 15-09 and all animals used in this experiment were treated according to the National Health and Medical Research Council's *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (7th Edition).

Author contribution statement/consent to participate/consent for publication. All individuals and organisations involved in this work have been included among the list of authors. Story and Hinds conceived and designed the experiment and prepared the manuscript. Story collated and analysed the data. Warden and Dojchinov conducted the tissue residue analysis and Henry provided technical assistance with the experiments. Story, Henry and Hinds all contributed to animal husbandry and data collection. All authors consent to the publication of this study.

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Tables

Table 1. Dose progression for Up-And-Down protocol given with short-term (48 h) and long-term (14 d) fates of individual male *Sminthopsis macroura* dosed orally with fipronil and time to death for those dunnarts encountering a lethal dose.

Test animal	Dose (mg kg ⁻¹)	Short-term fate (48 h)	Long-term fate (14 d)	Time to death (hh:mm)
014	175	O ^a	O	
015	310	O	O	
023	550	O	O	
026	990	X ^b	X	00:23
033	550	O	O	
036	990	O	O	
038	2000	X	X	12:39

^aO = Survival at the given dose; ^bX = Death at the given dose

Table 2. Actual and modeled^a dose progressions for Up-And-Down protocol given with short-term (48 h) and long-term (14 d) fates of individual female *Sminthopsis macroura* dosed orally with fipronil

Test animal	Dose (mg kg ⁻¹)	Actual dose progression*			Modelled dose progression, excluding animals 016 and 001, with <i>HH</i> surviving dose of 990 mg kg ^{-1b}		Modelled dose progression, excluding animals 016 and 001, with <i>HH</i> dying at a dose of 990 mg kg ^{-1c}	
		Short-term fate (48 h)	Long-term fate (14 d)	Time to death (hh:mm)	Short-term fate (48 h)	Long-term fate (14 d)	Short-term fate (48 h)	Long-term fate (14 d)
016	175	X	X	37:00				
001	99	X	X	23:16				
032	55	O	O		O	O	O	O
031	99	O	O		O	O	O	O
035	175	O	O		O	O	O	O
040	310	O	O		O	O	O	O
045	550	O	O		O	O	O	O
046	990	O	X	105:37	O	X	O	X
051	2000	O	X	64:24	O	X	O	X
054	2000	X	X	01:35	X	X	X	X
060	990	X	X	39:45	X	X	X	X
<i>GG</i> ^c	550				O	O	O	O
<i>HH</i>	990				O	O	X	X

^aModelled dose progressions assume survival at 550 mg kg⁻¹, death at 2000 mg kg⁻¹ and develop 2 scenarios (see ^b and ^c above) reflecting the differential response of dunnarts at a dose of 990 mg kg⁻¹.

^bModelled dose progression, excluding the first two female dunnarts used and including additional hypothetical animals (*GG* and *HH*) with *HH* surviving the final dose of 990 mg kg⁻¹

^cModelled UDP dose progression, excluding the first two female dunnarts used and including additional hypothetical animals (*GG* and *HH*) with *HH* succumbing to the final dose of 990 mg kg⁻¹

*X = Death, O = Survival, at the given dose

Figures

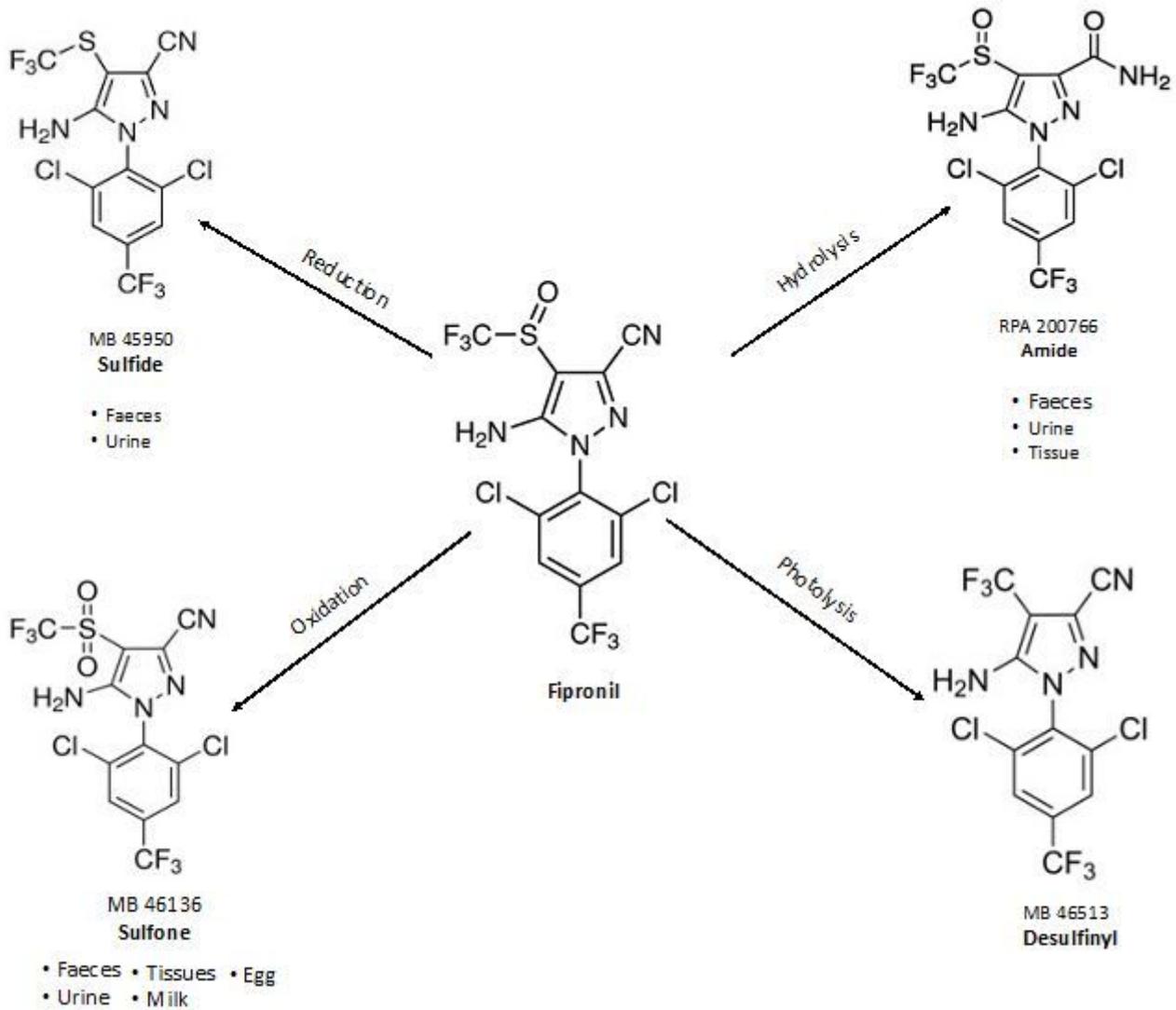


Figure 1

Metabolism of fipronil in vertebrates with the addition of the photolysis degradation pathway (Adapted from (Tingle et al. 2000) and (Kitulagodage 2011))

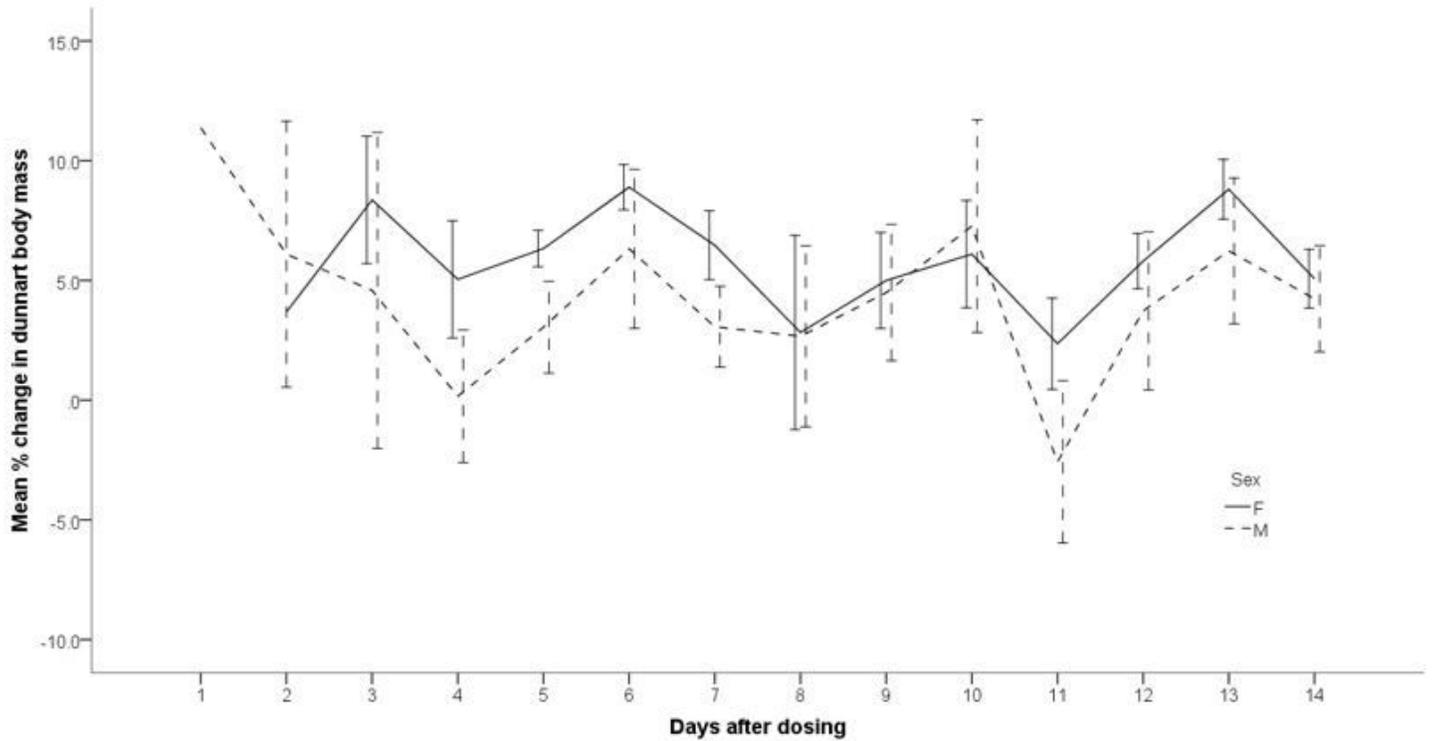


Figure 2

Mean percentage change in dunnart body mass after exposure to fipronil by gavage for all dose levels in those dunnarts not receiving a lethal dose. Error bars represent ± 1 standard error and are offset for clarity.

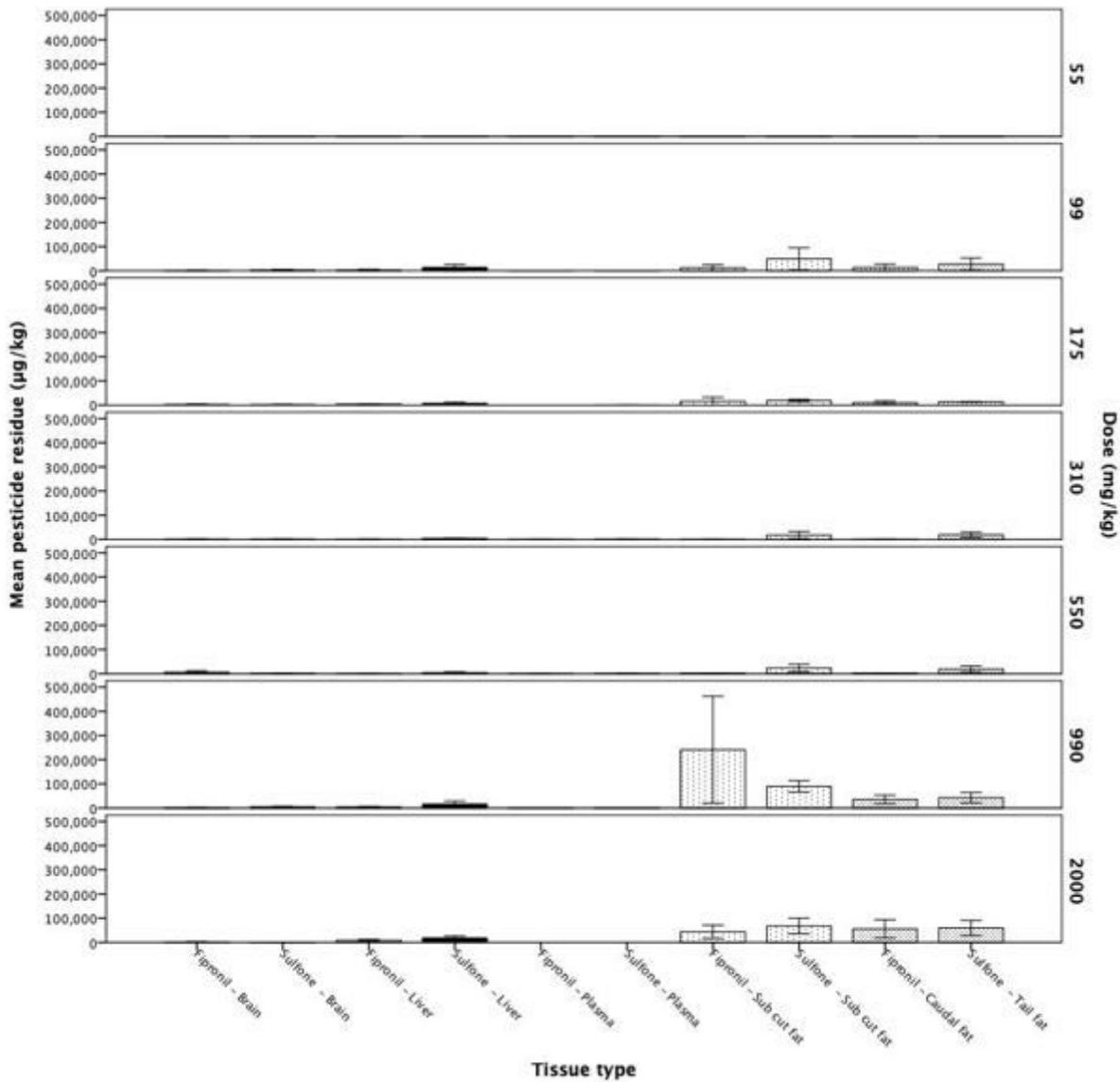


Figure 3

Fipronil and sulfone residues in *Sminthopsis macroura* (Gould 1844) brain, liver, plasma and subcutaneous and tail fat ($\mu\text{g kg}^{-1}$) tissue per administered dose (all animals $n = 18$). Error bars represent ± 1 standard error.

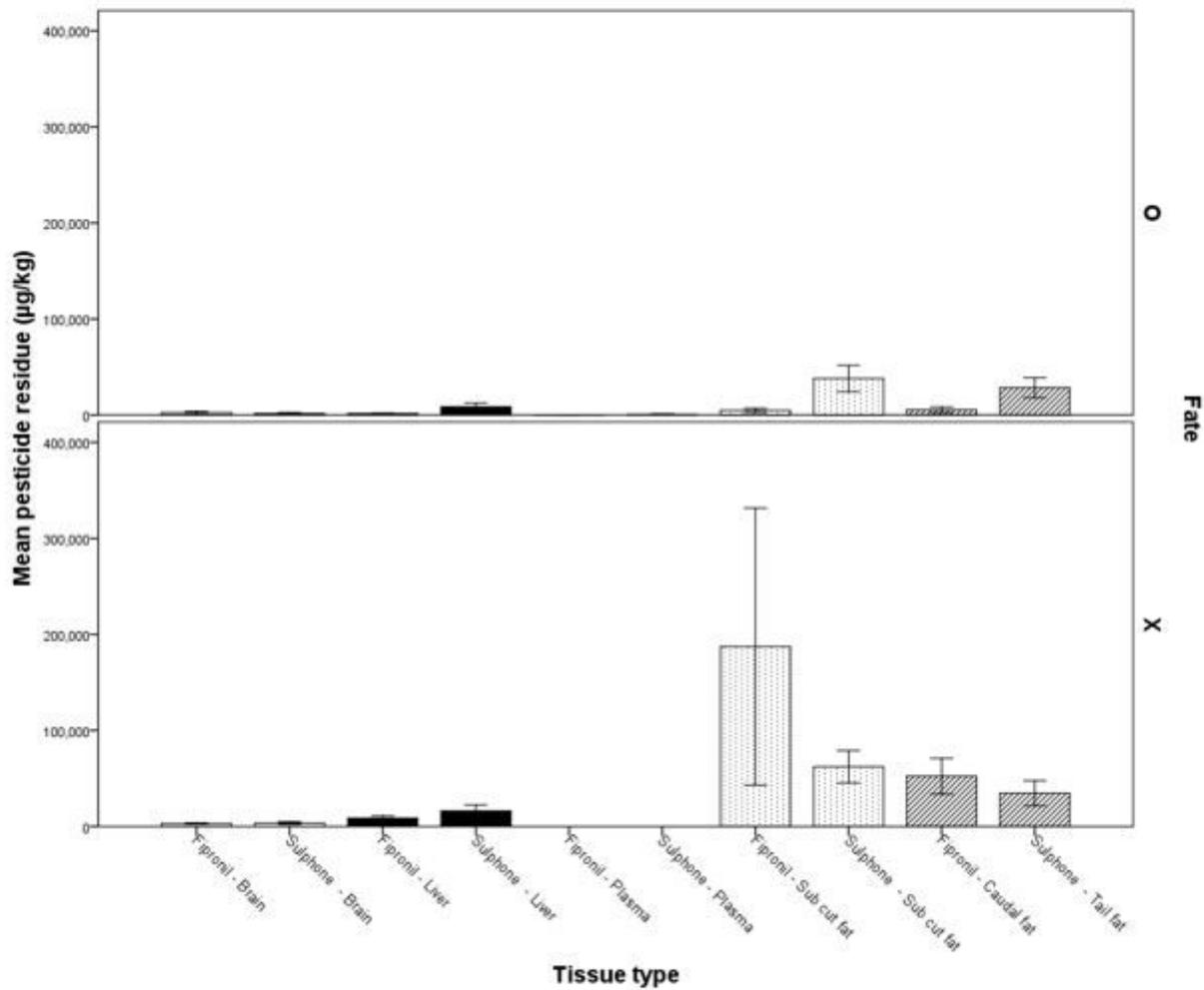


Figure 4

Mean fipronil and sulfone residue levels in *Sminthopsis macroura* (Gould 1844) brain, liver, plasma and subcutaneous and tail fat ($\mu\text{g kg}^{-1}$) in animals either surviving (Fate = 0, $n = 12$) or not surviving (Fate = X, $n = 6$) a given dose at 48 h post-exposure. Error bars represent ± 1 standard error.

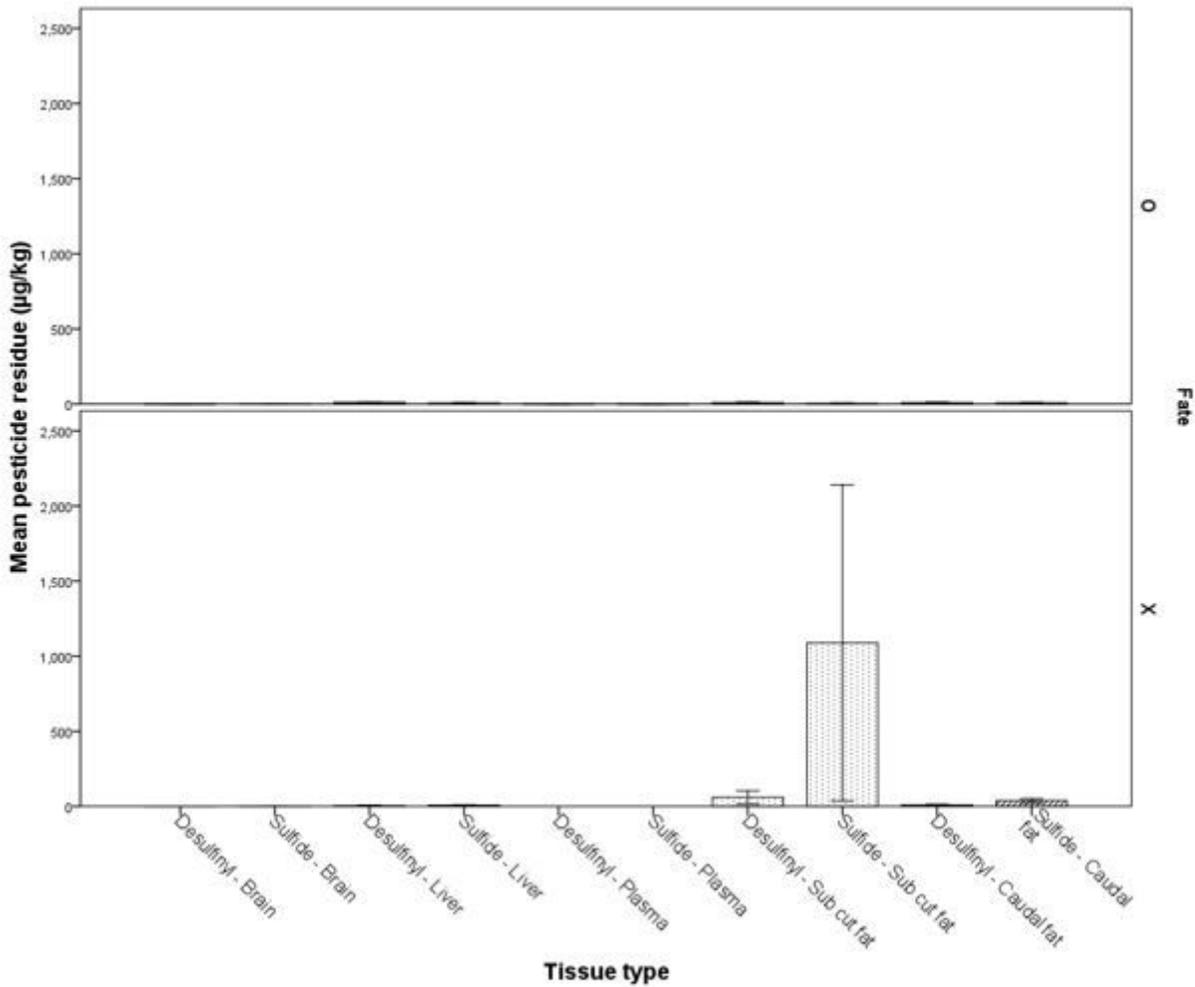


Figure 5

Mean desulfinyl and sulfide metabolite residue levels in *Sminthopsis macroura* (Goeld 1844) brain, liver, plasma and subcutaneous and tail fat ($\mu\text{g kg}^{-1}$) in animals either surviving (Fate = 0, n = 12) or not surviving (fate = X, n = 6) a given dose. Error bars represent ± 1 standard error.

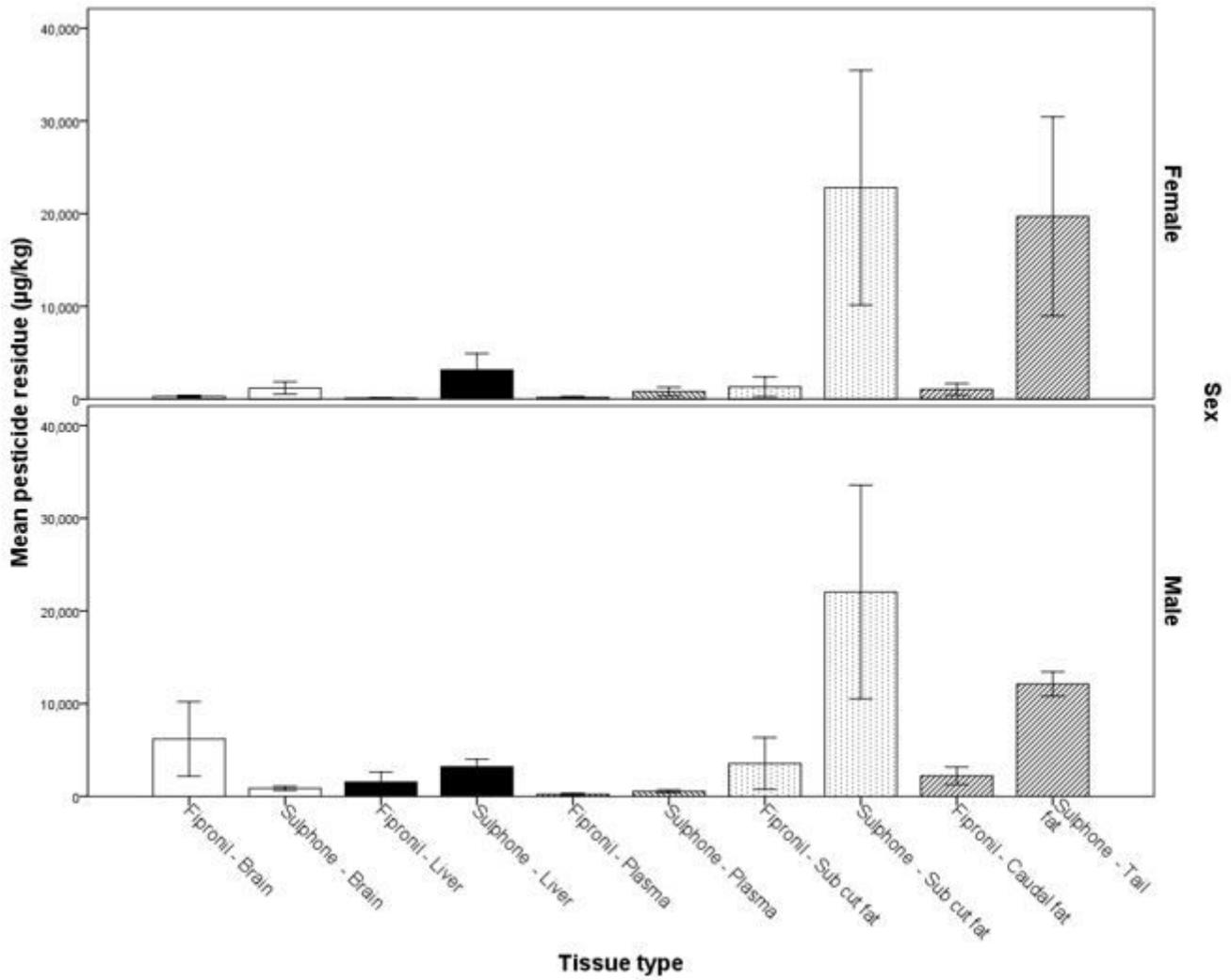


Figure 6

Mean fipronil and sulfone residues in male (n = 7) and female (n = 11) *Sminthopsis macroura* (Gould 1844) brain, liver, plasma and subcutaneous and tail fat ($\mu\text{g kg}^{-1}$). Error bars represent ± 1 standard error.

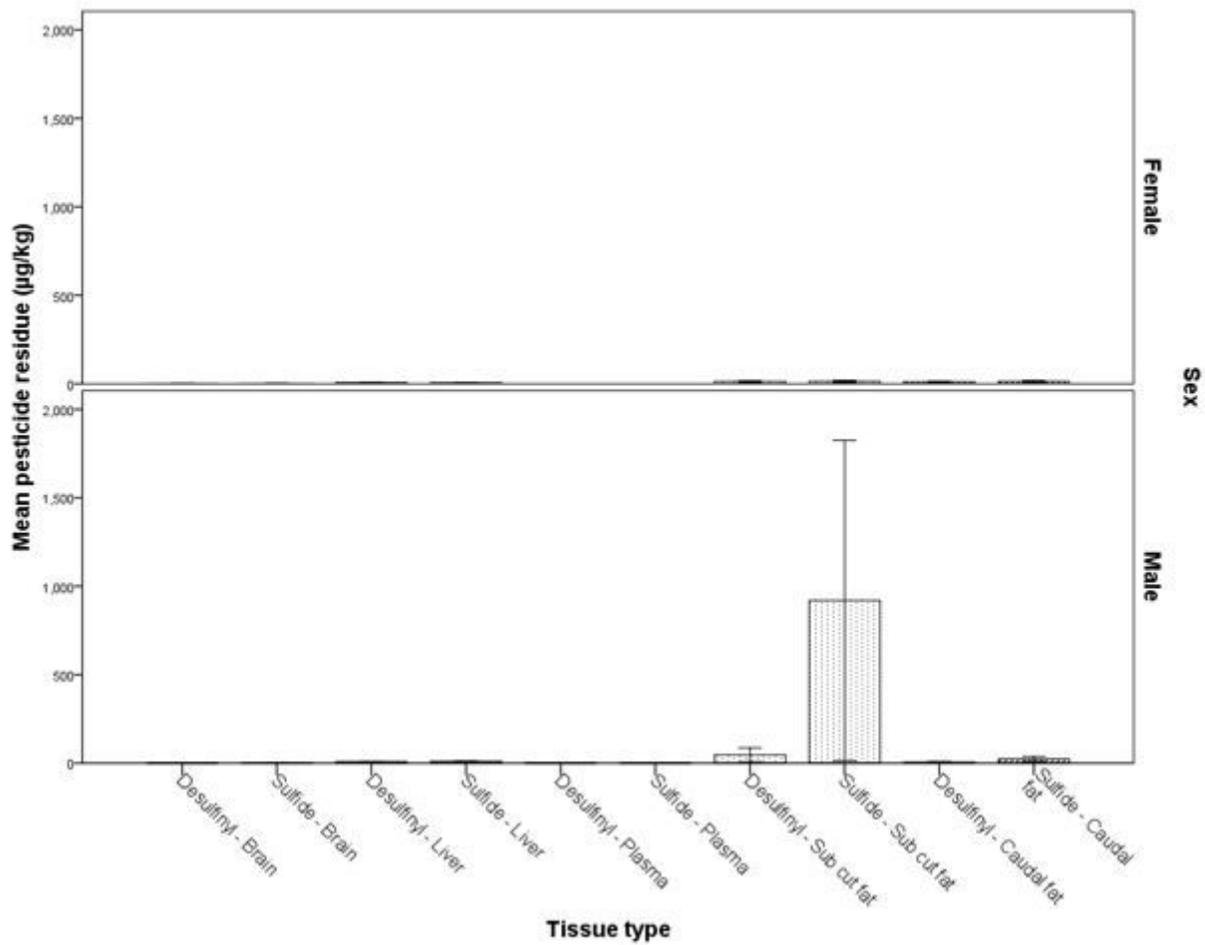


Figure 7

Mean desulfinyl and sulfide metabolite residues in male (n = 7) and female (n = 11) *Sminthopsis macroura* (Gould 1844) brain, liver, plasma and subcutaneous and tail fat ($\mu\text{g kg}^{-1}$). Error bars represent ± 1 standard error.