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Sensitivity of the dasyurids, *Sminthopsis crassicaudata* (Gould 1844) and *S. macroura* (Gould 1845) to the organophosphorus insecticide, fenitrothion, and its impact on locomotory and thermogenic performance in *S. macroura*

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Sensitivity of the dasyurids, *Sminthopsis crassicaudata* (Gould 1844) and *S. macroura* (Gould 1845) to the organophosphorus insecticide, fenitrothion, and its impact on locomotory and thermogenic performance in *S. macroura*



Paul Story (B. App. Sc.)

This dissertation is submitted in fulfilment of the requirements for the degree of Master of Science (Research) within the School of Biological Sciences, University of Wollongong

March 30, 2015

Declaration

I, Paul Geoffrey Story, declare that this thesis is submitted in accordance with the rules and regulations of the University of Wollongong in fulfilment of the degree of Master of Science (Research) within the School of Biological Sciences. I certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university and that, to the best of my knowledge and belief, it does not contain any material previously published or written by another person where due reference is not made in the text.

All experiments were undertaken, animals caught, transported and housed, in accordance with the regulations laid down by the University of Wollongong's Animal Care and Ethics Committee, the Queensland Environment Protection Agency and the New South Wales National Parks and Wildlife Service. The relevant animal ethics permissions and wildlife permit numbers are given at the end of each chapter. All research was conducted in accordance with the National Health and Medical Research Council's "Australian Code of Practice for the care and use of animals for scientific purposes".

Signature: P. Story

Date: 30.3.2015

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between control and fenitrothion-treated animals at that specific
time point.

Abstract

The scarcity of information on the effects of pesticides on Australian native vertebrates constrains the development of biologically relevant risk assessments in Australia for the registration of insecticides. This thesis investigates the sensitivity of two dasyurid marsupials, *Sminthopsis crassicaudata* (Gould 1844) and *S. macroura* (Gould 1845) to an organophosphorous (OP) insecticide, fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenol)-phosphorothioate), a common locust control agent in Australia. The effects of fenitrothion on a suite of physiological measures that affect the ability of animals to survive in free-living conditions (namely, locomotory and thermogenic functions, metabolic performance, body mass, haematocrit and haemoglobin levels as well as plasma and brain cholinesterase activity) are investigated in relation to time since exposure in *S. macroura*.

The concern that endemically old and unique Australian vertebrate fauna might display high sensitivity to pesticides used for locust control provoked examination of the acute oral toxicity of fenitrothion for the fat-tailed dunnart *S. crassicaudata* and the stripe-faced dunnart, *S. macroura* in *Chapter 2* of this thesis. Using the Up-and-Down method for determining acute oral toxicity, *S. crassicaudata* and *S. macroura* were found to have estimated median lethal doses (LD_{50}) of 129 mg kg^{-1} (95% confidence interval (CI) = $74.2 - 159.0$) and 97 mg kg^{-1} (95% CI = $88.3 - 120.0$) respectively. These values are 10 - 14 times lower than the reported LD_{50} values for a similar-sized eutherian mammal, *Mus musculus* (L. 1758) ($LD_{50} = 1100 - 1400 \text{ mg kg}^{-1}$) and lower than all other reported mammalian LD_{50} values. Such wide inter-specific variation in sensitivity to fenitrothion may be a consequence of underlying differences in the metabolic pathway for fenitrothion detoxification in mammals and a possible explanation for the increased toxicity of fenitrothion to dunnarts, as compared with

other mammals, is proposed. The unexpectedly high sensitivity of these Australian marsupials to fenitrothion emphasises the importance of adequately evaluating the risks of pesticides to endemic Australian fauna.

Agricultural pesticides applied for locust control have the potential to exert structural and functional effects on Australian arid zone ecosystems by impacting endemic and evolutionarily unique species occupying the same habitat as the target insect. In *Chapter 3* I examined the impact of fenitrothion on a suite of physiological measures that affect the ability of animals to survive in free-living conditions; namely, locomotory and thermogenic functions, metabolic performance, body mass, haematocrit and haemoglobin levels. Plasma and brain cholinesterase activity in relation to time since exposure to pesticide were also determined. An orally applied dose of 90 mg kg^{-1} fenitrothion reduced running endurance in *S. macroura* by 80% the day after exposure concomitantly with a reduction of approximately 50% in plasma and 45% in brain acetylcholinesterase activity. These adverse effects disappeared by 10 days post-exposure. Maximal metabolic rates reached during running were unaffected by pesticide as were body mass, haemoglobin and haematocrit levels. Maximal cold-induced metabolic rate (measured as the peak 2 min metabolic rate attained during cold exposure), the time taken to reach peak metabolic rate upon cold exposure, the cumulative total oxygen consumed during the shivering thermogenesis bout and body temperature before and after cold exposure were unaffected by fenitrothion. Dunnart rectal temperature showed a reduction of up to 5°C after exposure to fenitrothion, but returned to pre-exposure levels by 10 d post dose. Such physiological compromises in otherwise asymptomatic animals demonstrate the importance of considering performance-based measures in pesticide risk assessments.

Acknowledgements

I was once asked in a job interview if I had always known that I wanted to work in the field of biology. My answer was an unequivocal, “Yes”. My family history has its roots in the cattle industry of the western Queensland and the Northern Territory border country and the stories of hardship within this industry have trickled down through my family for decades. At the same time, the environmental resilience, biodiversity (some of which remains largely unstudied even today) and sheer beauty of western Queensland grassland ecosystems has mesmerized me since my first visit in the early 1980s. Time spent around the dinner table listening to the stories of cattle mustering in western Queensland and the Northern Territory left me wondering about the biological resilience of these ecosystems, their productivity and sustainability in the face of stressors from agricultural production systems. To be honest, as an undergraduate, I would never have imagined that I would be fortunate enough to have completed a postgraduate thesis in the very subject that I spent many hours pondering as a child. But none of this would be possible without the support of many very important people.

Thanks must go to my supervisors, Professors Bill Buttemer and Kris French, whose advice and guidance were pivotal to this work being completed. Thanks must also go to Professor Lee Astheimer and Dr. Mike Hooper, my other supervisors in the early years. I also thank Dr. Marijka Batterham from the University of Wollongong’s Statistical Consulting Service for advice on data analysis and Dr Karen Fildes for undertaking acetylcholinesterase assays.

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Chapter 1. Review of the effects of organophosphorus insecticides on mammals and implications for Australian free-living dasyurids

1.1 Introduction

Organophosphorus (OP) insecticides have been in use world-wide for over seven decades and came to prominence within the agricultural sector largely due to the withdrawal of organochlorine (OC) pesticides in the 1950s and 60s [1]. They were first introduced into Australia in 1971 [2]. The case for replacing OCs with OPs was strengthened by the fact that OPs are highly susceptible to chemical hydrolysis and can degrade rapidly in biological systems [3], thereby reducing their potential to adversely affect the environment. Consequently, unlike OCs, OPs do not accumulate readily in food chains [2]. Central to the global acceptance of OP pesticides was experimental evidence that demonstrated their high insecticidal potency and low mammalian toxicity due to their rapid biotransformation within mammalian bodies [4]. The agricultural use of fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenol)-phosphorothioate), a commonly used OP in locust control in Australia, gained approval based on risk assessments from overseas environmental research [5]. However, the absence of information on the effects of this pesticide on Australian native vertebrates casts doubt on the adequacy of such risk assessments to accurately reflect the Australian ecosystems they are intended to protect [6].

The toxicological effects of OP insecticides result mainly from the accumulation of the neurotransmitter acetylcholine (see below, *Mode of action of organophosphorus insecticides*) within the synaptic cleft, specifically at nicotinic and muscarinic post-synaptic receptors [7]. Acetylcholine is a major neurotransmitter within mammalian central and peripheral nervous systems and consequently affects almost every

physiological and behavioural aspect of mammals [8]. Because of the ubiquitous distribution of cholinergic innervation in vertebrates, it is not surprising that chemicals that disrupt acetylcholine-based signalling, such as OPs, have broad-ranging effects. These include impairment to thermoregulation, compromised endocrine function and its subsequent effects on metabolism and reproduction, modified chronobiology, impaired sensory perception and longer-lasting physiological and histological changes including neuronal damage and pesticide-induced muscle neurosis [9]. Similarly, OPs affect behavioural characteristics of mammals including activity levels, food and water intake, performance of learned tasks, learning and memory and aggression (see [2, 9-11] for a full review of these topics). Organophosphorus insecticides can also have pronounced ecological consequences, particularly in reducing reproductive output of individuals and survivability at the population level [2, 12-14].

Given the wide-ranging effects of OPs on mammalian physiology and behaviour, it is somewhat daunting to select those most pertinent for examining sublethal consequences of OP exposure on Australian native mammals. It seems logical that the measures chosen should be sensitive to any compromises in functional capacity and thus reveal reductions in overall vitality of the species studied. In this light, examination of OP effects on aerobically intense activities should reveal the extent and duration of physiological impairment following sublethal exposure to pesticides. Suitable physical activities in this regard are thermogenic performance when exposed to extreme rates of heat loss and running endurance in mammals. Because these activities reveal an animal's aerobic capacity, which, in turn, require the integrated function of a suite of enzymes, cells, tissues, organs and organ systems [15], such variables provide a meaningful measure of an animals overall vitality. With this objective in mind, the aim of this thesis is to establish both the sensitivity of selected dasyurids to fenitrothion as

well as the consequences of sublethal exposure to this OP on aerobic performance. I will discuss the mode of action of organophosphorus insecticides, the effects of OPs on locomotory and thermogenic performance of vertebrates in laboratory and field-based scenarios and the potential ecological implications of compromises in these functions for Australian endemic vertebrates.

The two studies that follow outline the acute oral toxicity of fenitrothion to the dasyurids, *Sminthopsis crassicaudata* (Gould 1844) and *S. macroura* (Gould 1845) and then quantify the sub-lethal effects of an ecologically realistic dose of this pesticide on locomotory and thermogenic performance in one of these species, *S. macroura*. These two chapters have been written as papers and, as such, some repetition in the introduction and methods sections may be expected. The first chapter has been published as;

Story PG, Hooper MJ, Astheimer LB and Buttemer WA (2011). The acute oral toxicity of the organophosphorus pesticide, fenitrothion, to fat-tailed and stripe-faced dunnarts and its relevance for pesticide risk assessments in Australia.

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The second chapter in this thesis has been accepted for publication in *Environmental Toxicology and Chemistry* as;

Story PG, French K, Astheimer LB and Buttemer WA Fenitrothion, an organophosphorus insecticide, impairs locomotory function and alters body temperatures in *Sminthopsis macroura* (Gould 1845) without reducing metabolic rates during running endurance and thermogenic performance tests.

Environmental Toxicology and Chemistry

During the course of this investigation, limitations of current pesticide risk assessments in adequately predicting effects on non-target organisms have become

apparent. For example, the lack of comprehensive insect residue data, from which to calculate experimental pesticide doses, makes it difficult to ensure that the physiological parameters studied here encompass a full range of realistic pesticide exposure scenarios. Consequently, I conclude this thesis with a set of potential topics for future research that may assist in refining further investigations into the environmental impacts of commonly used agricultural pesticides in Australia.

1.2 Mode of action of organophosphorus insecticides

The primary indication of exposure to OP compounds is the inhibition of cholinesterase enzyme activity and this biomarker has been widely used in the diagnosis of impacts caused by these compounds [2]. The main function of acetylcholinesterase (AChE) is to hydrolyse acetylcholine, a neurotransmitter essential to the normal function of most vertebrate nervous systems [16], thus terminating its effects on neural signalling. Acetylcholine (ACh) is stored in vesicles inside the axon terminal near the presynaptic membrane of a nerve cell in both the peripheral (PNS) and central nervous systems (CNS). Arrival of an action potential near the axon terminal causes the vesicle to fuse with the presynaptic membrane, discharging acetylcholine into the synaptic cleft. The discharged ACh binds fleetingly to receptors on the postsynaptic cell inducing a change in the membrane potential and initiating a nerve impulse dependent on the cell type [17].

Proper functioning of the cholinergic nervous system, however, requires that postsynaptic activities accurately reflect the pattern of signals sent from presynaptic cells, thereby requiring appropriate termination of the neurotransmitter to ensure that acetylcholine is switched off promptly after the presynaptic cell falls quiet [17]. Unlike other neurotransmitters that are typically removed from the synaptic cleft by

reabsorption, ACh is enzymatically removed from the cleft and postsynaptic receptors by hydrolysis through the activity of cholinesterases [18]. This occurs through the hydrolysis of ACh to acetate and choline by acetylcholinesterase (AChE), which then diffuse back into the presynaptic cell. Because every AChE molecule is capable of hydrolysing up to ten molecules of ACh per millisecond, most of the neurotransmitter is removed from the synaptic cleft within a few hundred milliseconds of its release from the nerve terminal [17, 19]. Thus, AChE is critical for normal functioning of the cholinergic nervous system.

Increased ACh levels consequent to the inhibition of AChE underlies many, but not all, of the neurological, behavioural and physiological manifestations that are associated with exposure to OP insecticides [19]. Many physiological actions involving ACh are initiated by its binding to two classes of receptors located in the plasma membrane; nicotinic (nAChRs) and muscarinic receptors (mAChRs) [20]. Nicotinic AChRs function as acetylcholine-gated channels and are located at the motor endplates of skeletal muscle fibres, autonomic ganglia and, to a lesser extent, in some CNS cell bodies and dendrites [21]. These receptors modulate the effects of ACh transmission on nervous, cardiovascular, immune, thermoregulation, respiration, gastrointestinal tract, and neuromuscular system functions as well as playing a role in proper cognitive function, vigilance and pain perception in mammals [22].

Muscarinic AChRs are G protein-coupled receptors [20] and have been the subject of more study than their nicotinic counterparts [19, 23-28]. These are mainly located on the effector cells of cardiac and smooth muscle, although there are some mAChRs located on CNS cells and dendrites [21]. Multiple subtypes of these receptors exist, each with individual pharmacological and functional profiles, although these roles have been difficult to discern because of the lack of specific receptor subtype selectivity

expressed in experiments to date and the fact that most tissues or cell types can express two or more mAChR subtypes [20]. The subtypes of mAChRs, their primary tissue distribution and responses have been classified according to Table 1 [29, 30].

Table 1. Primary tissue distribution, effectors and responses of muscarinic acetylcholine receptor subtypes (Source [30])

Muscarinic receptor subtype	Primary tissue distribution	Effectors and responses
M₁	Neuronal (ganglionic)	Phosphoinositide synthesis and cGMP formation
M₂	Cardiac (smooth muscle)	cAMP inhibition, opening of K ⁺ channels in heart muscle and CNS
M₃	Glandular (smooth muscle)	Phosphoinositide synthesis and cGMP formation
M₄	Brian striatum	cAMP inhibition
M₅	Glandular (smooth muscle)	Phosphoinositide synthesis and cGMP formation

The ultimate cause of death from AChE inhibition is usually asphyxiation, although the cause of asphyxiation will vary depending on the species and type of OP involved in the poisoning [31]. The inactivation of AChE in the synaptic cleft results in hyperstimulation of nAChRs and the desensitization of these receptors leading to paralysis of the intercostal and diaphragm muscles. This is often accompanied by

overstimulation of inhibitory of mAChRs by ACh in heart muscle and blood vessels and the inhibition of the breathing centre in the brain leading to death [26].

Acute toxicity from OP exposure is generally associated with a depression in brain AChE activity of greater than 50-70% whereas inhibitions in excess of 20%, or two standard deviations below the reported norm, have been used as criteria of exposure to anticholinesterase pesticides following their application [7]. Plasma ChE can be inhibited to levels close to zero without severe inhibition of brain AChE or overt toxic effects [32] and several studies have indicated that inhibition of greater than 50% in living vertebrates is not necessarily life threatening, at least in laboratory settings (e.g. [33-35]).

Although the primary effect of OPs on native Australian mammals would be expected to be similar to those of mammals from other continents, namely inhibition of cholinesterase (ChE) activity, the associated alterations in physiology, behaviour, reproduction and survival may differ (see below), particularly due to the unique lineage of mammals inhabiting Australia [2, 6, 36, 37].

1.3 Effects of OPs on locomotion

Australia's arid zone is characterised by low and infrequent rainfall events and, although not studied to the same extent as more mesic environments, mammalian population dynamics in Australia's arid regions are driven by the availability of resources rather than by predation, habitat structure or competition [38, 39]. Thus the spatial and temporal distribution of resources and the ability of animals to exploit them is a critical factor in determining the success of species inhabiting these regions. The intermittent distribution of resources in arid regions dictates that animals inhabiting these ecosystems develop life history strategies that can dynamically exploit the mosaic

of resources generated within habitats over a wide range of spatial and temporal scales [40].

The high mobility of Australian, arid zone, small mammal species may be an adaptive response to the low productivity and variable rainfall of Australian deserts [41], relative to other ecosystems [42]. In such an environment, a life history strategy geared towards locating food-rich “refuges” during times of drought that offer sufficient resources for survival and reproduction would be evolutionarily favoured over the establishment of permanent home ranges, burrows and foraging sites [42]. In arid habitats, such refugia can be geographically widespread and ephemeral in nature [43]. Consequently, the maintenance of locomotory function would be critical in enabling small mammals to rapidly exploit the transient resources with their available habitat and transitory impairments in locomotory performance could impede an individual’s ability to utilise resource mosaics, thereby increasing the likelihood of localized population extinctions.

A large number of terrestrial mammal species occupying arid habitats in Australia are remarkably mobile. Individual red kangaroos (*Macropus rufus*, Desmarest 1822) have been shown to cover distances of over 200 km over several weeks [44] and undertake shorter movements (~ 50 km) while foraging [45]. Similarly, dingoes (*Canis lupus* L. 1758) range across individual territories averaging 77 km² with the longest distance for an individual animal movement recorded at 250 km [46]. Smaller mammals are also known to be highly mobile, with rodents (sandy-inland mouse, *Pseudomys hermannsbergensis* (Waite 1896), ash-grey mouse, *P. albocinereus* (Gould 1845) and spinifex hopping mouse, *Notomys alexis*, Thomas 1922) and dasyurids (the lesser hairy-footed dunnart, *S. hirtipes*, Thomas 1898) documented as moving more the 10 km in “between trapping sessions” in Australia’s arid northern interior [42], although

the period between trapping sessions is unquantified in this study. Other studies using radio-tracking methods on arid zone dasyurids and rodents have documented overnight movements between 700 m and 2 km for *P. hermannsbergensis*, *N. alexis* and *S. youngsoni*, McKenzie and Archer 1982, with a young male *S. youngsoni* travelling approximately 5.7 km over several weeks [40]. In another study, *S. youngsoni* have been reported to have average overnight movements of over 400 m, with one male moving over 2.5 km in a single overnight session [47]. Another Australia dasyurid, the fat-tailed dunnart, *S. crassicaudata*, has been shown to move distances greater than 5 km, with average daily movements of 400 - 600 m [48]. In the aforementioned studies, there was little evidence that these movements were associated with dispersal after reproduction, but rather appeared to increase either during or after rainfall events [49]. Importantly, home ranges measured in these studies were described as unstable and continually shifting, thereby suggesting that mobility is an important life history attribute for species in these habitats.

As outlined above (see 1.2 Mode of action of organophosphorus insecticides) mammalian locomotion relies, in part, on nicotinic AChRs functioning as acetylcholine-gated channels at the motor endplates of skeletal muscle fibres. Cholinergic innervation of this type is also necessary for the proper functioning of autonomic ganglia and, to a lesser extent, in some CNS cell bodies and dendrites [21]. Exposure to OP insecticides can therefore interfere with locomotory performance and this has been demonstrated in several studies across a range of taxa. Of particular relevance to Australian mammals, a dasyurid, *S. crassicaudata* showed more than 50% reduction in running endurance for five days after exposure to 30 mg kg⁻¹ fenitrothion [36]. These reductions in running endurance were not associated with any decline in metabolic rate, which demonstrates that fenitrothion-induced exercise fatigue was not due to limitations in the supply or

uptake of nutrient substrate or oxygen to muscles. It is therefore more likely that the premature fatigue resulted from impairment of cholinergic activation of nicotinic receptors as a result of suppressed AChE activity. It is noteworthy that the dunnarts in this study were otherwise asymptomatic following this sublethal dosing of fenitrothion [36]. Used in concert with standardised, laboratory-based risk assessment techniques, performance measures, such as running endurance, should be used to assess the extent and duration of physiological compromise within pesticide risk assessment and better quantify the risks of pesticides to free-living wildlife populations.

Performance-based measurements of pesticide effects have previously revealed physiological compromise in mammals. Rats exposed to the carbamate, physostigmine, showed reduced running times and increased core temperatures when exercised to exhaustion on a treadmill [50, 51]. These decrements in running endurance and increased core body temperatures were returned to pre-dose levels following the administration of the drugs atropine and diazepam [50]. Furthermore, the extent of these pesticide-related effects was attenuated when physostigmine was administered chronically, rather than acutely [52]. Physostigmine is a carbamate insecticide and, although not an OP pesticide *per se*, the mechanism by which carbamates exert their toxicity is very similar to OPs in that they inhibit acetylcholinesterases [53]. Another carbamate insecticide, carbaryl (1-naphthyl methylcarbamate), has been shown to reduce sprint speed in the meadow-jumping mouse, *Zapus hudsonius* (Zimmerman 1780), by up to 40% dose-dependently in both males and females when compared to unexposed control animals. The proportion of female *Z. hudsonius* cannibalising their young increased after exposure to carbaryl in this trial, revealing that behavioural traits can also be affected by exposure to organophosphorus and carbamate insecticides (see [2, 9] for full reviews).

Research measuring sprint performance in the western fence lizard (*Sceloporus occidentalis*, Baird and Girard 1852) after exposure to AChE-inhibiting pesticides showed highly variable results in relation to dosage. While lizards exposed to malathion, an OP, showed an increased sprint performance at a high dose (200 mg kg⁻¹), those given lower doses (0.2, 2.0 or 20 mg kg⁻¹) or the vehicle alone (corn oil) were statistically indistinguishable [54] from control animals. Exposure of the same lizard species (*S. occidentalis*) to the anti-cholinesterase pesticide, carbaryl, in a study comparing locomotory performance in terrestrial (flat linear running track) and arboreal (represented by a dowel rod 2.5 cm in diameter suspended ~ 2 m above ground) sprint simulations showed differing results. Exposure of lizards to lower doses used in this study (2.5 and 25 µg g⁻¹) stimulated their locomotory performance whereas the high dose (250 µg g⁻¹) inhibited sprint performance during terrestrial performance tests. Lizards exposed to carbaryl during arboreal tests showed no stimulatory response, but rather sprint performance was inhibited at the higher dose (250 µg g⁻¹) while the responses in sprint performance at lower doses were statistically indistinguishable from control animals [55].

House sparrows (*Passer domesticus*, L. 1758) exposed to ecologically realistic doses of fenitrothion showed dose-dependent reductions in both the plasma enzymatic biomarker, acetylcholinesterase, and flight peak metabolic rates, none of the doses administered affected thermogenic measurements during a 60 min cold exposure trial. In results similar to those reported for the fat-tailed dunnart, fenitrothion exposure had no effect on blood haematocrit or haemoglobin content [36, 56].

The potential for reduced running endurance to adversely affect free-living dunnart populations exposed to pesticides is substantial and highlights the importance of performance-based investigations. Locomotory impairment will reduce their ability to

forage, escape predators, maintain home ranges and reproduce, at least in the short term. However, the laboratory-based studies reviewed above serve as a cautionary tale against an over-reliance on field-based techniques to assess the risks of OP pesticides to wildlife populations. Although population-level risk assessments are expected to increase the level of ecological relevance when assessing the potential for environmental harm to occur [57], they can be problematic if the xenobiotic under investigation affects the locomotory function of the target species under study. The use of live trapping techniques to assess population parameters such as intrinsic rate of increase, immigration and emigration rates and home range size and maintenance for wildlife populations assume that sublethally exposure animals have an equal chance of being captured as those that have not been exposed. However, the potential for animals to be captured would be dependent on their activity [58] and therefore their locomotory ability, particularly in the case of commensal rodent species [59]. Alterations to mammalian activity levels during the assessment of potential pesticide impacts could lead to an underestimate of biomarker responses in the case of acetylcholinesterase as the more exposed animals with higher levels of enzymatic suppression may not be captured in the trappable population. This may lead to the risk associated with exposure being underestimated.

Conversely, in studies where population densities are being enumerated to calculate intrinsic rate of population increase, sublethally exposed animals with reduced activity may simply be assumed to be dead and omitted from the overall census data, in which case risk may be overestimated. This potential bias was tested in the only study in the literature to assess exposure to a sublethal dose of the OP, dimethoate, on the trappability of wood mice, *Apodemus sylvaticus* (L. 1758) and bank voles, *Clethrionomys glareolus* (Schreber 1780) in woodland habitat. The study found no

short or long term effects of dimethoate exposure on wood mice and bank voles despite significant suppression (up to 75%) in brain AChE levels in wood mice. No AChE biomarker data were collected from bank voles in the study [58].

1.4 Effects of OPs on thermoregulation

The body temperature (T_b) of mammals is regulated within narrow limits by means of thermoregulatory control nuclei within the hypothalamus. These nuclei within the preoptic area of the anterior hypothalamus receive temperature status from core and peripheral temperature sensors [60] via muscarinic cholinergic receptors [21] and integrate this information to effect appropriate physiological and behavioural responses to maintain a relatively constant body temperature. This thermoregulatory control centre functions like a thermostat in that it activates metabolic heat production and evaporative heat loss mechanisms, as appropriate, to maintain body temperature at a particular set point [61]. Because of its reliance on cholinergic-based processes, body temperature regulation is one of many homeostatic processes that can be affected by anti-ChE agents [62] as evidenced by studies mainly involving captive small mammals and birds [9]. Exposure to organophosphorus insecticides is known to either inhibit an animal's tolerance to cold or directly impair their ability to maintain internal body temperature [9] and anti-ChE compounds have been shown to directly affect the activity of cholinergic pathways responsible for temperature regulation [62].

One of the first studies examining the effects of ChE inhibition on mammalian thermoregulation found that cold-exposed laboratory rats given a single dose of the OP pesticide, dioxathion, had significantly lower plasma corticosterone levels than control rats [63], reducing their ability to respond to cold stress. Other early research showed that rats injected with sublethal doses of anti-ChE agents had falls in body temperature

of between 2 and 6 °C, with the lowest body temperature reached 3 - 6 hours following dosing and taking up to 24 h before body temperatures returned to predose levels [64, 65]. White-footed mice (*Peromyscus leucopus*, Rafinesque 1818) intubated with parathion showed reductions in rectal temperature of 9.7% and 9.2% for animals kept at 23 °C and 10 °C, respectively, after pesticide exposure [66]. Similarly, Long-Evans rats (*Rattus norvegicus*, Berkenhout 1769) maintained at ambient temperatures of 20 to 24 °C and exposed to diisopropyl fluorophosphate (DFP) doses $\geq 1.0 \text{ mg kg}^{-1}$ showed significant decreases in body temperature (T_b) of up to 4 °C and lasting up to 5 days [62]. More recent studies have shown that rats given a single intramuscular injection of diisopropyl fluorophosphate (DFP) showed a decrease in rectal temperature 6 h after injection, with gradual recovery about 24 h post-exposure [65]. In this study, ChE activity was measured from the anterior preoptic area of the brain, the part of the brain believed to be responsible for temperature regulation, and shown to be maximally depressed (77%) 4 h after injection.

Both hypothermic and hyperthermic responses to OPs have been documented in rats within a single study. Laboratory rodents injected with DFP doses ranging from 0.25 to 1.5 mg kg^{-1} showed a reduction in T_b , heart rate, motor activity and selected ambient temperature (T_a) for the first 24 h following exposure. Conversely, during the next 24 h period after exposure, T_b returned to, and then exceeded pre-dose levels [67]. Furthermore, the preference for these rats to select a reduced T_a concomitantly with hypothermia, in the first 24 h following pesticide exposure is suggestive of a reduction in the hypothalamic set point controlling T_b .

Studies measuring reductions in body temperature and ChE suppression following exposure to anti-cholinesterase agents have demonstrated that the effect of OP exposure is complex, involves lowering the hypothalamic set point in treated

animals and that toxicity in these individuals can be moderated by activity selecting different ambient temperatures [62, 67-71]. For example, rats treated with DFP (0 - 2.0 mg kg⁻¹) showed vasodilation of the tail (measured as an increase in tail surface temperature) indicating that the hypothalamic set point for the rats body temperature had been reached and that heat dissipation through the tail was necessary to reduce T_b. Although rats exposed to the pesticide had decreased body temperatures (up to 5 h post exposure) and increased tail temperatures (for up to 1 h post exposure) they did not select a warmer ambient temperature when placed in a temperature gradient. Data from this study suggest that the effects of OP exposure in rats may be mediated by alterations to the hypothalamic set temperature or via the breakdown of cholinergic innervation necessary for the feedback from temperature sensing throughout the body [69].

One of the responses available to some mammals to reduce thermoregulatory costs when coping with low ambient temperatures is torpor [72]. This is characterised by both a reduction in body temperature and metabolic rate and is widely used by small mammals as an energy conservation tool during periods of food shortage and low ambient temperatures [73]. Although body temperature is reduced in animals undergoing torpor, it is regulated at this lower set point by increasing metabolic rate when body temperature approaches a particular “species-specific threshold” [60]. This requires the proper functioning of cholinergic innervation to integrate and relay information on ambient and body temperatures to the hypothalamus [60]. Torpor is critical for the survival and reproduction of small insectivorous marsupials, such as *S. macroura*, in Australian arid zone grasslands [74]. Individual free-living *S. macroura* have been observed to utilise torpor at temperatures ranging from 0 °C to 35.5 °C, resulting in estimated savings in daily energy expenditure of up to 89% [74],

significantly higher than energy savings measured under laboratory conditions [75].

Because torpor still relies on an effective cholinergic network to maintain the new set point temperature, OP exposure has the potential to interfere with their ability to utilise torpor, and thus, has ramifications for the energy requirements of free-living dunnarts.

While there is ample evidence that anti-ChE substances can adversely affect mammalian and avian thermoregulation, it is also clear that thermoregulatory demands can affect the lethality of a given OP dose. For example, Japanese quail (*Coturnix japonica*, Temminck and Schlegel, 1849) dosed with parathion (4.0, 5.7, 8.0, 11.3, 16.0 and 22.6 mg kg⁻¹) showed up to a two-fold increase in acute oral toxicity in birds exposed to either heat (37 °C) or cold (4 °C), compared to birds maintained at a thermoneutral temperature (26 °C). Additionally, quail given a low dose of parathion and maintained at high temperature showed significantly greater inhibition of AChE activity (42%) as compared to birds dosed at the same rate but maintained at 26 °C (12%) [76]. In a separate study, bobwhite quail (*Colinus virginianus* L. 1758) exposed to the OP chlorpyrifos, displayed even greater temperature-dependent toxic effects to this pesticide. Although the dose of chlorpyrifos given to these quail was shown to be the sole determinant of mortality when quantifying the LD_{50, 4h} of the administered dose, ambient temperature and body weight were all factors in determining the ChE inhibition of pesticide-exposed birds [77]. Specifically, cholinesterase suppression intensified with cold stress, suggesting that sublethal effects of pesticide exposure in animals surviving a toxic insult could be amplified, despite having the same lethal endpoint.

Cold stress has also been shown to increase mortality in Wistar rats receiving a daily dietary exposure of 4000 ppm malathion, approximating 240 mg kg⁻¹. While remaining otherwise asymptomatic (appearance, growth, food intake) rats in the treatment group showed significantly lower time to death (between 10 and 13 h) when kept at 1.5 ± 1 °C,

without affecting metabolic rates in pesticide-exposed animals [78]. The interaction of ambient temperature and OPs has also been examined on a range of functions in American kestrels (*Falco sparverius* L. 1758). After birds were administered the OP, methyl parathion, brain and plasma ChE activities were measured along with plasma corticosterone concentration and cloacal temperature in birds maintained at thermoneutral (22 °C) or cold (-5 °C) environments over a 10 h period. Brain and plasma cholinesterase inhibition in excess of 50% was associated with transient but pronounced hypothermia 2 h after intubation with methyl parathion, which also elevated plasma corticosterone concentration in treated birds. Cold intensified the effects associated with methyl parathion toxicity [79].

Given the large seasonal and diurnal variation in ambient temperatures experienced by desert-dwelling small mammals when locust control is underway, it's important to recognize the ability of ambient temperature to alter the toxicity of the pesticides being sprayed.

1.5 Implications for pesticide risk assessments in Australia: Why be concerned about the Dasyuridae?

The mammalian fauna of the Australian arid zone is characterised by a high species richness of insectivores, most of which are dasyurids, and contains about four times the number of insectivorous mammals found in North American desserts [80]. The Dasyuridae, with 14 extant species, are amongst the most diverse of the ten families of marsupial in Australia and a large proportion of these species occur in Australia's arid and semi-arid regions. Within New South Wales, they have been particularly susceptible to range reductions. Three species have become extinct and three more have contracted to occupy 10 - 50% of their former ranges [81]. Two out of three

extinctions occur in the arid, open plains where locust control occurs, and only one species in this region, the fat-tailed dunnart, *S. crassicaudata* (Gould 1844), has been classed as common. Additionally, the application of agricultural pesticides has been identified as a “threatening process” in New South Wales and “active management” has been recommended to prevent further attenuation and loss of the remaining species of dunnarts [81]. The status, therefore, of Australia’s dasyurids amplifies the potential for chemical control agents to affect these species and it is therefore unfortunate that there is a paucity of knowledge concerning the ability of both individuals and populations to deal with the application of agricultural chemicals [82].

At present an assessment of the hazard of OP insecticides to wildlife is based on knowledge of their environmental fate, persistence, application rate and toxicity [1]. Toxicity data generated on laboratory animals are often used to predict the toxic hazard to individual animals and/or populations in the wild. Qualitative extrapolation from laboratory to wild species can successfully be used to identify the types of effects pesticides have on a wide range of animals living in natural conditions. However, quantitative extrapolation of such data are often either inadequate or largely invalidated [1] because either interspecies sensitivity to pesticides varies or the effects of toxicity and differences in exposure patterns between laboratory and wild animals are largely unquantified. For example, laboratory studies on chronic oral toxicity involve receiving a constant dose either in feed (e.g. [34]), at regular intervals by oral dosage (e.g. [4, 83]) or gavage (e.g. [84, 85]) while the exposure of free-living animals is likely to be intermittent and depend upon the bio-availability of the compound. Based upon the limited evidence available, errors in the direct extrapolation of dose-response data from laboratory to field may be as large as three orders of magnitude [7, 86].

Locust control is primarily undertaken in the arid and semi-arid regions of

Australia where rainfall is the primary limiting factor for populations of the Australian plague locust, *Chortoicetes terminifera* (Walker 1870) [87]. Similarly, significant rainfall periods in these regions provide a “convenient and apparent” resource focus for various species of vertebrates [80] and this convergence of opportunistic feeders and locust control [88-90] exposes a range of native Australian vertebrates to acridicides through surface contact and/or by ingestion of sprayed vegetation, locusts, or non-target insects [2]. Such an overlap may be a significant evolutionary response to the limited resources available to species in arid ecosystems and, as such, the high mobility of Australian arid zone small mammal species may be an adaptive response to the low productivity and variable rainfall of Australian deserts [41], when compared to other systems [42]. In such an environment, a life history strategy geared towards locating food-rich refuges during times of drought that offer sufficient resources for survival and reproduction may be a safer evolutionary investment than the establishment of permanent home ranges, burrows and food sources [42]. What little evidence exists on movement in arid zone Australian small mammals indicates that several species of dunnart can cover significant linear distances in relatively short periods of time [40, 42, 91]. Under these circumstances, a transitory impairment in locomotory performance could impede an individual’s ability to utilise resource mosaics thereby enhancing the opportunity for localized population extinctions.

Numerous dasyurid species are located within arid regions subjected to pesticide application for locust control and some of these (e.g. the Kowari, *Dasyuriodes byrnei* and the bilby, *Macrotis lagotis*) are listed as endangered or vulnerable [81, 92]. Highly variable rainfall patterns in the arid and semi-arid regions of Australia have been demonstrated to influence the abundance and mobility of dasyurids including the species used in the current study, *S. crassicaudata* and *S. macroura* [80, 93-96].

Maximal population densities of *S. crassicaudata* have been correlated with times of dense vegetation cover approximately 12-15 months after a high seasonal rainfall event [97]. Moreover in both of the previous studies, the dasyurid populations were shown to be extremely fluid with population peaks not sustained beyond a few months. This raises the issue of the relationship between the effects of pesticides and density-dependence within arid-zone dwelling vertebrate populations. Pesticide-induced mortality may not necessarily affect abundance if it replaces density-dependent mortality, and the impairment of reproduction may affect population numbers only if annual population turnover is high and the pesticide widespread. Largest impacts may occur when the pesticide acts after density-dependent mortality has reduced population numbers but before breeding has occurred and members of the population that would have survived and reproduced are lost [1]. Mortality would then potentially be increased overall and the reproductive capacity of the population would be decreased. Under such circumstances the ability of a population to recover would then depend on the severity of the pesticide induced mortality, persistence of the chemical in the environment, immigration and any other factors limiting the rate of recruitment as well as the remaining population density [98].

Dasyurid marsupials are highly successful inhabitants of Australia's arid zone and most species are insectivorous [45]. The diets of dasyurids have been well documented as exhibiting a lack of dietary specialisation possibly as a consequence of an unpredictable supply of rainfall [80, 94, 95]. For example, the diets of two sympatric species of *Planigale spp.* were quantified *in situ* over a two-year period using faecal analysis in concert with an analysis of prey abundance estimated from light and pitfall traps [99]. Both *P. gilesi* (Aitken 1872) and *P. tenuirostris* (Troughton 1928) are generalists with respect to prey type and size and the prey items found in the faecal

samples were found to reflect those species of available prey. This study also noted that both these species were unlikely to take prey larger than their own body size [99]. A strong positive relationship between dasyurids and their prey items suggests that dietary generalism is an important characteristic allowing dasyurids to survive in the Australian arid zone where rainfall, and subsequently the abundance and quality of food resources, are unpredictable [100]. This is reinforced by additional research showing that small dasyurids can take prey up to 75 mm [101]. *C. terminifera* nymphs are generally between 5 and 30 mm in length and meet the prey selection requirements of dasyurids, particularly when nymphal densities during periods of locust control can be up to several thousand per square metre [102]. Consequently, a combination of small size (some as low as seven grams), high metabolic requirements, a primarily insectivorous diet [45, 100], combined with the added ability to gorge feed on intoxicated locusts (PG Story, Australian Plague Locust Commission, Canberra, Australia, unpublished data) make them vulnerable to the effects of pesticides used for locust control. Additionally, the habitat requirements of some dasyurids (e.g. *S. crassicaudata*, *S. hirtipes*, *S. macroura*, *S. youngsoni*, *P. gilesi* and *P. tenuirostris*) overlap substantially with that of *C. terminifera* [2] and so the co-occurrence of dunnart populations and locust spray operations is highly likely throughout the locust control season (October-March).

With some notable exceptions [6, 34, 36] there is a general dearth of literature concerning the effects of OP insecticides on Australian mammalian fauna. Because a large proportion of Australia's small mammals are highly endemic and evolutionarily old, it is unlikely that their susceptibility to pesticides can be inferred from studies of distantly related mammals from very different evolutionary histories [6]. There are many effects associated with exposure to OP insecticides. These can be broadly categorised as being either lethal, resulting in the death of the animal or reproductive

impairment (defined as the subsequent death of eggs embryos or young, see [103]) or sublethal. Sublethal effects are characterised by a variety of responses including reduced tolerance to cold temperatures, impairment of endocrine function, reduced learning and memory, changes in levels of activity and aggression and/or a reduction in water and food intake [1, 9, 83]. All these effects could impact on survival and reproductive output, but have been seldom investigated in vertebrate fauna.

The research projects that follow are designed to establish the sensitivity of selected dasyurids to fenitrothion and investigate the consequences of sublethal exposure to this OP on aerobic performance, a laboratory-based performance parameter with particular relevance to free-living populations routinely subjected to pesticide exposure as a result of locust population management in Australia's arid rangelands. I will also discuss the potential ecological implications of compromises in these functions for Australian endemic vertebrates.

Chapter 2: The acute oral toxicity of the organophosphorus pesticide, fenitrothion, to fat-tailed (*Sminthopsis crassicaudata* (Gould 1844)) and stripe-faced (*S. macroura* (Gould 1845)) dunnarts and its relevance for pesticide risk assessments in Australia

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2.1 Introduction

Fenitrothion is a broad-spectrum, organophosphorus (OP) insecticide used extensively throughout the world for the control of agricultural and forest pests. It was first introduced into Australia in 1971 and is considered the most cost-effective chemical currently available to the Australian Plague Locust Commission (APLC) for aerial locust spray programs [2]. Organophosphorus insecticides are generally esters of phosphorus-containing acids and, because of the susceptibility of esters to chemical hydrolysis, can degrade rapidly in biological systems. Therefore, insecticides such as the OP fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenyl)-phosphorothioate) tend not to accumulate readily in food chains, but can have acute environmental impacts on animals through interference with acetylcholinesterase, an enzyme required for the normal function of vertebrate and invertebrate nervous systems (see review by Story and Cox [2]).

The information often used to predict the environmental risk of pesticides to individual animals and/or free-living populations includes knowledge of its

environmental fate, persistence, application rate and toxicity, with the toxicity data usually attained from laboratory animals under controlled experimental conditions. This toxicological endpoint data, such as LD₅₀ values, can be modelled using species sensitivity distributions (SSD) (see Aldenberg *et al.* [104] for a review of SSDs and their use in ecotoxicology) and to statistically estimate percentiles as either levels of protection or the fraction of species affected by a given dose of toxicant. One commonly used percentile is the HD₀₅ value representing the hazardous dose for the 5th percentile of the modelled data, thereby affecting 5% of the species. This value is therefore considered to be protective of the remaining 95% of species [105]. The majority of risk assessments evaluating the hazard posed by pesticides on native Australian fauna are based on estimated toxicities of a standard range of non-endemic test species such as laboratory rodents, cats, rabbits, dogs, etc. (Table 2). A large proportion of Australia's marsupial fauna, however, is highly endemic and evolutionarily old and it is not known if their sensitivity to pesticides differs significantly from the non-Australian, eutherian mammals studied to date.

Central to the acceptance of OP pesticides is laboratory evidence demonstrating a high insecticidal potency and low mammalian toxicity due to their rapid biotransformation within mammalian bodies [5]. In Australia, the use of fenitrothion for locust control gained approval based on overseas environmental research [4]. Although the anti-cholinesterase effect of OP pesticides in Australian metatherian and eutherian mammals would be expected to be similar mechanistically to that demonstrated in other mammals [34], it is unclear how exposure to these pesticides might affect the physiology, behaviour, reproduction and survival of Australian endemic species at doses currently being applied for locust control in Australia.

Table 2. Acute oral fenitrothion median lethal dose (LD₅₀) values for a variety of vertebrates.

Test animal	Sex	Acute oral LD ₅₀ *(Lower 95% confidence interval - Upper 95% confidence interval, <i>n</i>) (mg kg ⁻¹ of body mass)	Reference
Rat	Male	681 (252 - 1840, 9)	[2]
Rat	Female	795 (178 - 3352, 8)	[2]
Mouse	Male	1105 (641 - 1906, 4)	[2]
Mouse	Female	1139 (761 - 1707, 4)	[2]
Guinea pig	Male	974 (263 - 3608, 3)	[2]
Dog (beagle)	Male/Female	>681	[106]
Cat	Male	142	[106]
Cattle	Male	>500	[107]
Mule deer	Unknown	727	[108]
Sheep	Male	>770	[107]
Pig	Unknown	>310	[107]

*Where multiple LD₅₀ values were available for a species, the geometric mean of the LD₅₀ values was used and the standard error (SE) and sample size given.

Previous research examining the effects of pesticides on Australian native mammals reported that stripe-faced dunnarts (*Sminthopsis macroura* (Gould 1845)) survived a dietary intake of monocrotophos at rates of two mg/kg over 18 d, despite a cholinesterase (ChE) inhibition of up to 92%. However, they also reported that the animals died when given a higher single oral dose of 8 mg/kg ingested in just four

minutes, resulting in the inhibition of brain ChE by 66 - 69%. Aerobic metabolism during cold exposure and exercise performance (measured as run duration and oxygen consumption while running at 1 m s⁻¹) has been quantified previously in the fat-tailed dunnart, *S. crassicaudata* (Gould 1844), before and after the ingestion of a 30 mg kg⁻¹ dose of fenitrothion [36]. That study demonstrated that sublethal doses of this pesticide impaired locomotory performance by over 50% for the first 3 d post-dose while leaving metabolic rates unaffected.

Of the numerous mammal families in Australia, the Dasyuridae are most likely to be affected by pesticides used for locust control. The combination of small size (some as low as seven grams), high food requirements, a primarily insectivorous diet [45, 100], and the tendency to gorge feed on intoxicated locusts (PG Story, Australian Plague Locust Commission, Canberra, Australia, unpublished data), make them potentially vulnerable to pesticide effects. Some dasyurid species have distributions that overlap with areas subjected to locust control and are listed as endangered or vulnerable by Australian environmental legislation. Some of these species, such as the Kowari (*Dasyuroides byrnei* Spencer 1896) are included in the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species as vulnerable and suffering a "declining population trend" [109].

The Up-and-Down Protocol (UDP) devised and recommended by the Organisation for Economic Cooperation and Development (OECD) for evaluating acute oral toxicity (Guideline 425) is a useful alternative to conventional LD₅₀ testing [110]. The UDP has been shown to produce a median lethal dose (LD₅₀) estimate similar to that achieved from conventional toxicity testing, but requiring significantly fewer animals to resolve it [111]. The LD₅₀ values derived from the UDP method are 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 [111]. The present study reports the acute oral toxicity of fenitrothion for the Australian native marsupials *S. crassicaudata* and *S. macroura* and compare these values to the established sensitivity of other eutherian mammals more commonly used in risk assessments based on species sensitivity distributions (SSD).

2.2 Material and Methods

2.2.1 Animal housing

Dunnarts used in this trial were sourced from a combination of a laboratory colony kept at the University of Wollongong and a cohort of animals collected from field populations in southwest Queensland. Those dunnarts sourced from our laboratory colony were first or second generation descendants from field-collected animals and so their response to pesticide exposure was not expected to differ significantly from wild caught dunnarts. All dunnarts were maintained in individual cages at a day: night cycle of 12:12 and a constant temperature of 23 °C. Dunnarts were fed with a blended combination of wet (canned) dog food (40%), dry cat kibble (40%) and water (20%) and provided with water *ad libitum*. Their diets were supplemented with live mealworms and crickets when available. Dunnarts were fasted overnight before the administration of fenitrothion doses and then observed periodically for the following 14 d. Food and water were returned to the dunnart's cages at the cessation of toxicological signs outlined in Figure 1.

2.2.2 Determination of acute oral toxicity

In total, 20 dunnarts were used in this experiment: 9 female *S. crassicaudata* and 7 female along with 4 male *S. macroura*. Typically, animals from one gender are used

for LD₅₀ estimates, but we lacked sufficient numbers of female *S. macroura* to determine this with one gender. Doses were administered as per the UDP schedule and were made by mixing fenitrothion with canola oil to produce a standard 0.2 ml volume for each individual dose, which was then delivered oesophageally via a gavage needle attached to a one ml syringe.

We followed OECD Guideline 425 to estimate the acute oral toxicity LD₅₀ value along with its confidence interval [110]. We used the main test of this guideline which consists of a single-ordered dose progression in which animals are dosed individually (one at a time) and observed, generally for a minimum of 48 h before a subsequent dose is administered to another animal. The interval between dosing can be altered, although subsequent doses should be delayed until one is confident about the fate of the previously dosed animal. In the present study, the dosing interval was set at 48 h, even though the time course for the toxicological signs associated with fenitrothion administration were mostly under 2 h.

The UDP protocol stipulates "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" [110]. Given that very little information was available on the toxicity of fenitrothion to Australian native marsupials, we commenced the dosing regimes at 175 mg kg⁻¹, as per the UDP protocol. The dose progression for each subsequent animal was determined by the short-term (48 h) fate of the previously dosed one. If a dunnart survived the dose given to it within the 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 2 and 3) as prescribed by the Acute Oral Toxicity (AOT) software program [110] used for the analysis of dosing data. The long-term fate of dunnarts, defined in

this study as the fate of animals at 14 d post-exposure surviving a given dose of fenitrothion, was also recorded.

Dosing continued until one of the three standard stopping criteria was met. Either three consecutive animals survived at the upper bound of dosing, five *reversals* occurred in any six consecutive animals tested (where a reversal is created by a pair of responses in a situation where a non-response is observed at a particular dose and a response observed at the next dose tested, or vice versa) or at least four animals have followed the first reversal and the specific likelihood ratios (see OECD [110]) exceed the critical value.

It was intended at the commencement of the present study that the stopping criteria using the identification of five reversals would be used to bring a halt to the dosing schedule and determine the LD₅₀ and associated confidence limits. However, after dosing several animals of both species, it became clear that obtaining the five reversals necessary was going to require more dunnarts than were available. Additionally, at this point in the experiment, the AOT software indicated that the final stopping criteria outlined above had been satisfied and so dosing was halted and LD₅₀ values and confidence limits were calculated.

All animals were observed during the entire duration of their toxicological signs and a time line was developed (Figure 1). After the stopping criteria were reached, an estimate of the LD₅₀ value and the associated confidence limits were calculated using the AOT software (Guideline 425) Statistical Program Version 1.0 [110]. All dunnarts were weighed daily and body mass data were analysed using a repeated measures analysis of variance [112].

2.2.3 Cholinesterase measurements and analysis

To provide an estimate of acetylcholinesterase (AChE) and total cholinesterase (TChE) activities in dunnarts exposed to fenitrothion, whole brain samples were taken either immediately after death in animals not surviving a given dose, or from animals euthanised at the end of the 14 d (long term) observation period for dunnarts surviving a given dose. Brain cholinesterase activities in unexposed animals were obtained from a control group within another study investigating the effects of fenitrothion on the locomotory and thermogenic performance of dunnarts.

Dunnarts were killed by CO₂ asphyxiation and all brains were quickly removed (within 15 min.) and placed in a -80 °C freezer for storage. Acetylcholinesterase and total ChE activities were determined from dunnart brain material using the method of Ellman *et al.* [113] as modified [114] for use in a 96-well spectrophotometric plate reader. Enzyme characterisation had been undertaken previously for these two dunnart species and full details of the assay procedure can be found elsewhere [36].

We tested cholinesterase data for normality using the Wilk-Shapiro index (all data were normally distributed) and then analysed for differences between groups using analysis of variance (ANOVA) with Tukey honestly significant difference (HSD) pair-wise mean comparisons applied post-hoc [112].

2.2.4 Species sensitivity distributions

Species sensitivity distributions were prepared by sorting LD₅₀ values in ascending dose order and ranked. The equation $(100 * rank) / (n+1) = centile$ was used to develop values for a probabilistic distribution. Each data set was plotted with the LD₅₀ values on the logarithmic *X*-axis and the resulting centiles plotted on the *Y*-axis. A logarithmic trend line was created for each distribution and extended to the *X*-axis. A

5% line was created for reference by solving each regression equation for $Y = 5$, with the corresponding X -value being the HD_{05} value for the line (following Aldenberg and Luttik [105]). The resulting HD_{05} values were then compared with and without the inclusion of the dunnart LD_{50} data.

2.3 Results

The progression of toxicological signs after dosing was similar for all dunnarts with slight differences noted for the onset of particular symptoms, although no discernible pattern was noticed for these variations. For example, a higher dose did not necessarily result in either an earlier onset, or an increase in the intensity of, toxicological signs. Toxicological signs observed in the fatalities differed only in the dunnart not recovering from the hyperventilation period (Figure 1), characterized as “rapid and shallow breathing.” Those dunnarts that did survive this phase exhibited a period of lethargy for a variable period of up to 100 min.

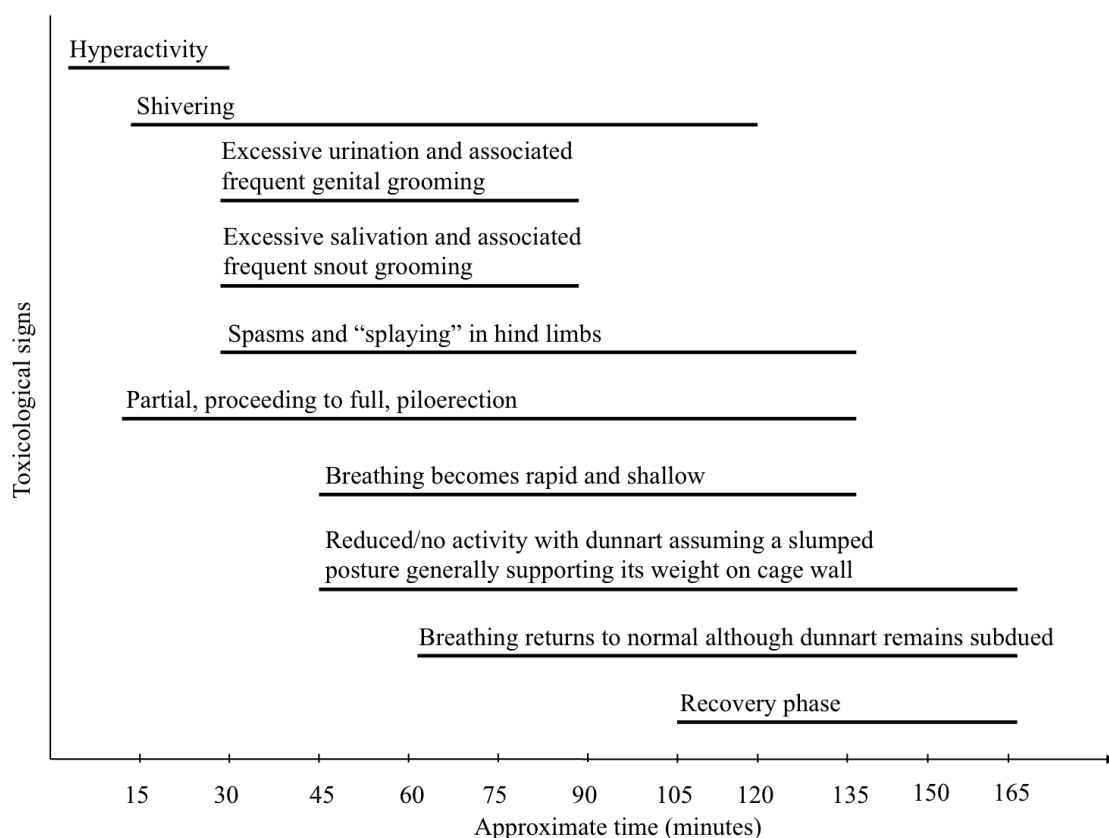


Figure 1. Summary of the temporal sequence of toxicological signs for all dunnarts following oral gavage with fenitrothion.

The dose progressions oscillated between 73 and 310 mg kg⁻¹ for both species. The short- (48 h) and long-term (14 d) fates of *S. crassicaudata* and *S. macroura* individuals used in the experiment are given in Tables 2 and 3 respectively.

Table 3. Dose progression given with short- (48 h) and long-term (14 d) fates of individual *Sminthopsis crassicaudata* dosed orally with fenitrothion.

Test animal	Gender	Dose (mg kg ⁻¹)	Short term (48 h)	Long term (14 d)
1	Female	175	X	X
2	Female	129	O	O
3	Female	175	O	X
4	Female	230	O	X
5	Female	310	X	X
6	Female	230	X	X
7	Female	175	X	X
8	Female	129	X	X
9	Female	97	O	O

(X=Death, O=survival)

Estimates of the LD₅₀ values calculated for *S. crassicaudata* and *S. macroura* were 129 mg kg⁻¹ (95% CI 74.2 - 159 mg kg⁻¹) and 97 mg kg⁻¹ (95% CI 88.3 - 120 mg kg⁻¹) respectively. These values are approximately 10 times lower than other previously published LD₅₀ data for *Mus musculus* (L. 1758), a murid species of similar body mass (Table 2).

Table 4. Dose progression given with short- (48 h) and long-term (14 d) fates of individual *Sminthopsis macroura* dosed orally with fenitrothion.

Test animal	Gender	Dose (mg kg ⁻¹)	Short term (48 h)	Long term (14 d)
1	Female	175	X	X
2	Female	129	X	X
3	Female	97	O	O
4	Female	129	X	X
5	Female	97	X	X
6	Female	73	O	O
7	Female	97	X	X
8	Male	73	O	O
9	Male	97	O	O
10	Male	129	X	X
11	Male	97	O	O

(X=Death, O=survival)

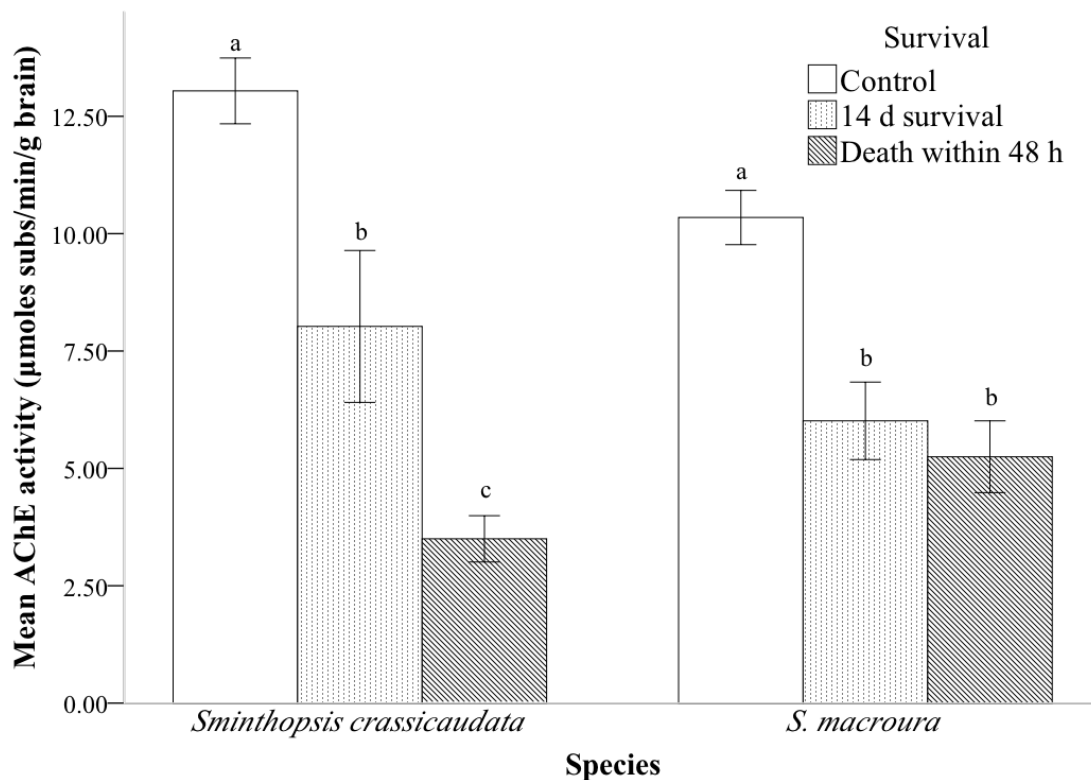


Figure 2. Mean brain acetylcholinesterase (AChE) activity for *Sminthopsis crassicaudata* (Gould 1844) and *S. macroura* (Gould 1845) in control dunnarts, dunnarts dying within the first 48 h post-dose and in those dunnarts who survived the long-term (14 d) observation period. Letters denote homogenous groups. Vertical bars show standard errors. AChE = acetylcholinesterase.

Brain AChE and total ChE activities were measured as biomarkers of fenitrothion exposure in dosed and control animals (Figures 2 and 3). These measures were separated into three groups representing control animals (C), those dying within the first 48 h of dosing (short-term fate, O) and those surviving the dose (long-term fate, X) and observed for a further 14 d.

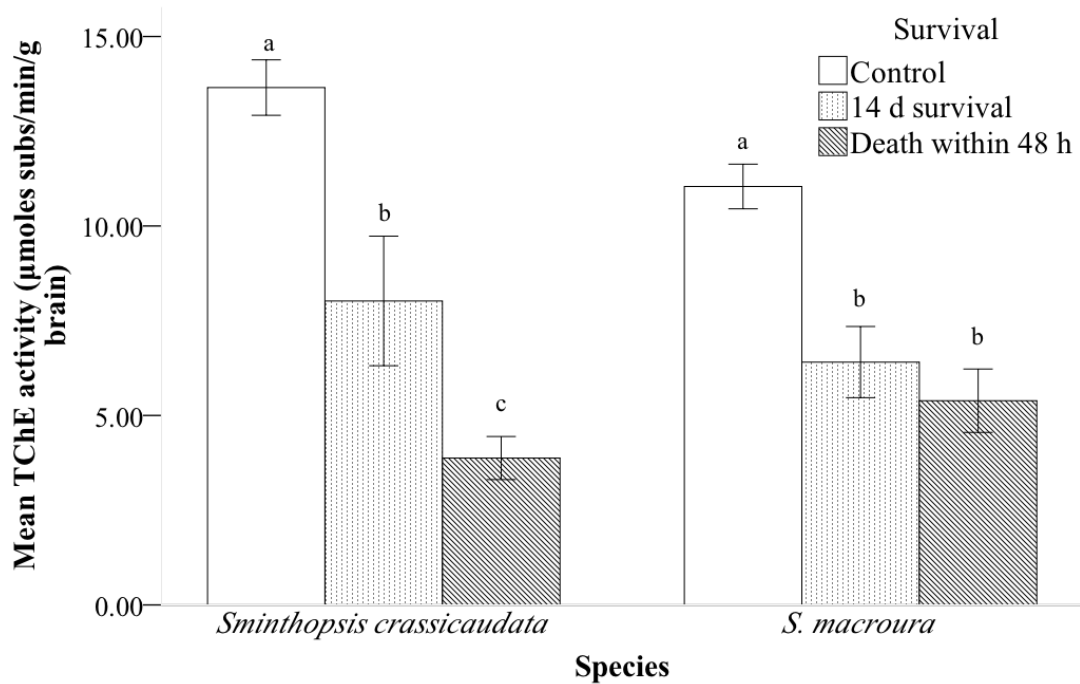


Figure 3. Mean brain total cholinesterase (TChE) activity for *Sminthopsis crassicaudata* (Gould 1844) and *S. macroura* (Gould 1845) in control dunnarts, dunnarts dying within the first 48 h post-dose and in those dunnarts who survived the long-term (14 d) observation period. Letters denote homogenous groups. Vertical bars show standard errors. TChE = total cholinesterase.

Brain AChE data obtained from *S. crassicaudata* and analysed using ANOVA, showed that AChE varied between all treatment groups ($F = 53.07$, $p < 0.01$, degrees of freedom $[df] = 2$) with a post-hoc Tukey's HSD test demonstrating that significant differences lay between all fate groups (Figure 2). Brain AChE activities in *S. macroura* also showed that significant differences existed between exposed and unexposed animals ($F = 14.31$, $p < 0.01$, $df = 2$). However, in this instance, a Tukey's HSD test applied post-hoc revealed that these differences existed only between exposed and unexposed dunnarts and no significant difference in suppressed brain AChE activity

was found between dunnarts surviving or dying after being dosed with fenitrothion (Figure 2).

A similar pattern of enzyme suppression was evident in the data for total brain ChE activity from both species (Figure 3). *S. crassicaudata* total ChE activity, analysed using ANOVA ($F = 49.14$, $p < 0.01$, $df = 2$) showed a distinct pattern of enzyme suppression and recovery with dunnarts sampled 14 d post dose showing an intermediate level of recovery of this enzyme biomarker. Total brain ChE activities obtained from *S. macroura* showed a similar trend to the AChE data given above. While analysis using ANOVA revealed differences among the mean total ChE activity of the fate groups ($F = 15.56$, $p = 0.00$, $df = 2$), no significant difference was evident, using Tukey's HSD test post-hoc, between dunnart brains in those animals having died within 48 h and those sampled at 14 d post-dose (Figure 3).

Weight variations experienced by dunnarts surviving pesticide ingestion were the same as those experienced in the breeding colony (T. Maddocks, University of Wollongong, Wollongong, Australia, unpublished data) and analysis of dunnart body mass data using 2-tailed, paired t tests [14] did not reveal any effect of fenitrothion on body mass at either 4 d post dose (*S. crassicaudata*; $t_{0.05(2)7} = 0.76$, $p = 0.941$; *S. macroura* $t_{0.05(2)5} = 1.116$, $p = 0.315$) or 6-9 d post dose (*S. crassicaudata*; $t_{0.05(2)7} = 1.441$, $p = 0.193$; *S. macroura* $t_{0.05(2)5} = -0.249$, $p = 0.315$).

Overall, the mean recorded time to death varied widely from a minimum of 0.37 h to a maximum of 32 h for both species (Figure 4) with no discernible dose-related pattern evident. With several fenitrothion doses restricted to a sample size of one dunnart (e.g., 129 and 310 mg kg⁻¹ for *S. crassicaudata* and 230 mg kg⁻¹ in the case of *S. macroura*) an appropriate statistical analysis of the time to death data was not possible.

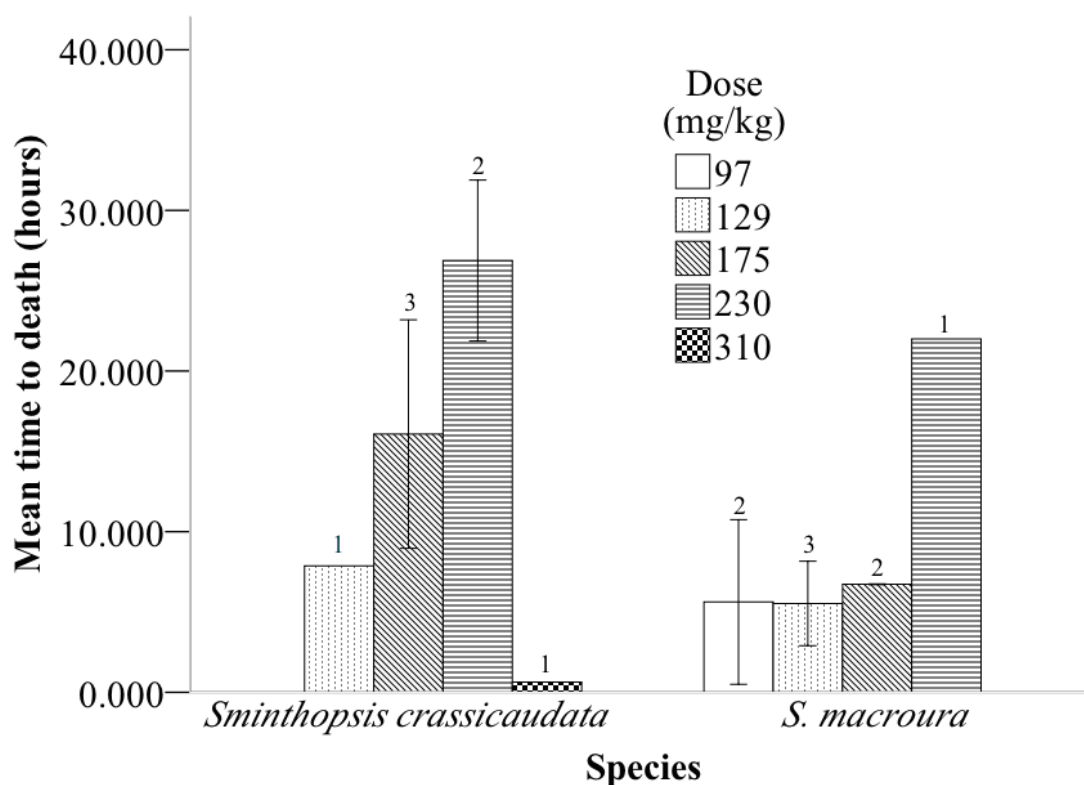


Figure 4. Mean time to death (given with standard error where $n > 1$) per dose for dunnarts of each species receiving a fatal dose of fenitrothion. Data for dunnarts surviving the administered dose is not included.

Comparative species sensitivity distributions were generated using mammalian LD_{50} data gathered from the scientific literature and the LD_{50} estimates generated from the present study (Figure 5). The HD_{05} value calculated from these distributions was reduced from 177 mg kg^{-1} to 93.5 mg kg^{-1} with the inclusion of LD_{50} estimates for the two dunnart species tested in the current study.

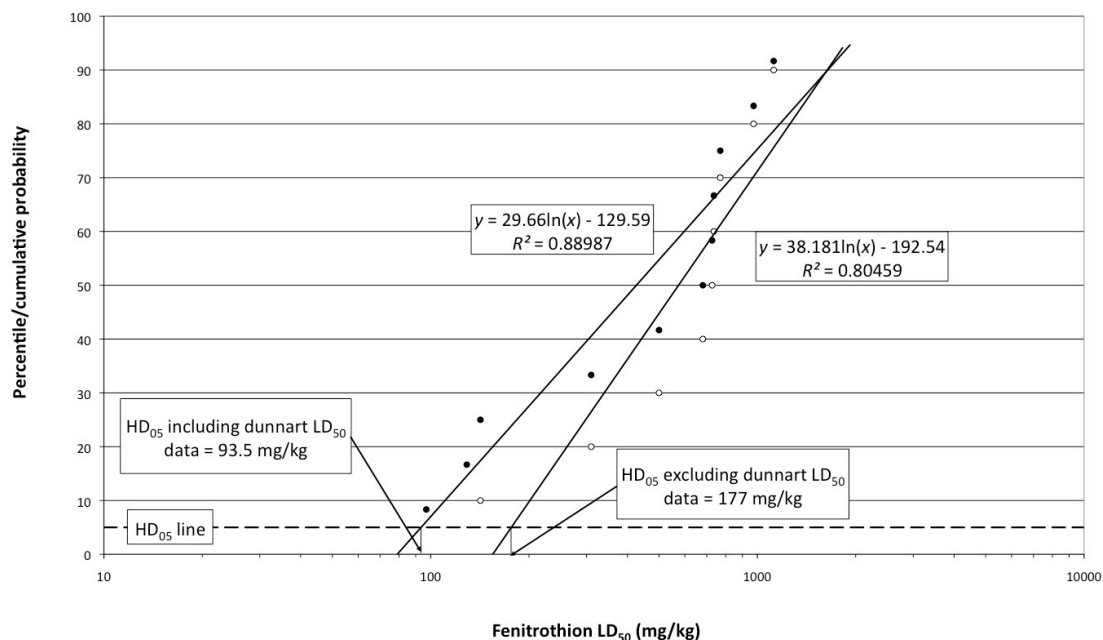


Figure 5. Species sensitivity distribution showing mammalian median lethal dose (LD₅₀) values and the calculated HD₀₅ (hazard dose) level, with and without dunnart values. HD = hazard dose.

2.4 Discussion

Both species of dunnart tested were substantially more sensitive to fenitrothion than all other mammals commonly used in vertebrate risk assessments for this pesticide. Such a high acute oral toxicity is of considerable concern as none of the commonly tested mammals used to develop environmental risk assessments, and subsequently to support the registration of this product, are native Australian species. Both dunnart species tested in the present study are commonly found throughout habitats in eastern Australia subjected to locust spray operations. Because they are highly insectivorous [45] they may be further at risk of fenitrothion exposure through gorge feeding on sprayed locust nymphs.

The signs of intoxication observed in dunnarts immediately after dosing suggest severe short-term locomotory impairments. These included a period of immobilization followed by an extended period of general lethargy for those dunnarts surviving the dose. For dunnarts not surviving the administered dose, no intuitive or logical pattern was observed in the time taken to reach death for either *S. macroura* or *S. crassicaudata*. The large range in time to death, combined with the extended period of locomotory impairment in survivors, would likely increase the susceptibility of sublethally exposed dunnarts to predation. Similar conclusions resulting from the reduction in locomotory functions experienced in *S. crassicaudata* given a 30 mg kg⁻¹ dose of fenitrothion by gavage have been previously described [36].

The extent of brain acetylcholinesterase and total cholinesterase suppressions observed in dunnarts dosed with fenitrothion (~50 - 70% suppression) within the present study are similar to those documented in previous research [36] investigating the effects of this pesticide on *S. crassicaudata*. Severe locomotory impairments were observed in dosed dunnarts concomitantly showing AChE suppressions of approximately 60% of pre-exposure levels [36]. While the lesser fenitrothion dose of 30 mg kg⁻¹ on *S. crassicaudata* showed full recovery of cholinesterase activities at 10 d post-dose [36], both brain AChE and total ChE remained significantly suppressed in *S. crassicaudata* and *S. macroura* at 14 d post-exposure during the present study, likely due to the increased doses of fenitrothion administered (73 - 310 mg kg⁻¹).

Fenitrothion residue levels previously quantified on nymphs of the Australian plague locust ranged from 3.3 to 83 mg kg⁻¹ [115]. As both *S. crassicaudata* and *S. macroura* have been shown to consume approximately 70% of their body mass (on average) over a 24 h period in feeding trials (PG Story, Australian Plague Locust Commission, Canberra, Australia, unpublished data) potential daily fenitrothion

consumption rates for both dunnart species range from 2 to 58 mg kg⁻¹ body weight, or up to 46 and 60% of the LD₅₀ values for *S. macroura* and *S. crassicaudata* respectively.

Reduced food and water intake, and subsequent weight loss, are common behavioural changes observed in mammals following exposure to organophosphorus pesticides [116-119]. For example, cottontail rabbits (*Sylvilagus floridanus* Allen 1890) significantly reduced their food intake for up to 2 d following intubation with 8 mg kg⁻¹ parathion [119], and similar reductions in food consumption have been recorded in several rodents exposed to OPs [116-118]. These studies also show that weight is eventually restored after sublethal dosing through compensatory increases in feeding, which offset the initial anorexic effect of OP intoxication [119]. Despite the lack of an obvious and consistent weight loss after dosing in the present study, it would be instructive to see if these species might develop a feeding aversion to food contaminated with pesticide. Such a response would be beneficial for a species showing a high sensitivity to OP pesticides, as secondary exposures to the pesticide would be reduced. This response, however, may compromise the energy reserves of such small animals, and prove to be as lethal as the pesticides themselves. For example, when Australian native hopping mice, *Notomys alexis* (Thomas 1922) and *N. mitchelli* (Ogilby 1838), were given a diet of hulled millet containing 668 mg kg⁻¹ monocrotophos, they quickly developed a food aversion to the contaminated seed and decreased their feeding rate and body weight [34]. Similar results were recorded for *S. macroura* ingesting a dietary dose of 2 mg kg⁻¹ body weight from the same study.

Fenitrothion is a neurotoxicant that acts by inhibiting cholinesterase enzymes [9]. Specifically, acetylcholinesterase enables rapid neurotransmission frequencies in both the peripheral and central nervous systems by hydrolysing the neurotransmitter, acetylcholine, after it has excited postsynaptic membranes [17]. Thus, exposure to anti-

cholinesterase compounds is likely to compromise cholinergic neural and neuromuscular functions if the normal rates of acetylcholine breakdown are disabled. There are two primary metabolic pathways catalysed by different enzymes that are of importance in determining the toxicity of fenitrothion. The first is an oxidative desulphuration, catalysed primarily by cytochrome P450 that converts the parent compound, fenitrothion, into the more potent anticholinesterase, fenitrooxon [53] (in some tissues this may lead to detoxification of some fenitrothion). The second chemical process involves the hydrolysis of fenitrooxon by an “oxonase” that detoxifies its anti-ChE character and increases its transport to the kidneys for subsequent excretion [120-122]. Differences in the toxicity of chlorpyrifos between young and adult Long-Evans rats (*Rattus norvegicus* Berkenhout 1769) have been attributed to young rats having less oxonase activity and therefore having a lower capacity to detoxify physiologically relevant concentrations of the chlorpyrifos-oxon after exposure [123]. The presence of sufficient oxonase activity to hydrolyse the oxon metabolite of OP insecticides is thus a critical step in the metabolism of OPs in mammals. Differences in the availability or functionality of dunnart oxonases may explain why native Australian marsupials studied here differ from *M. musculus* in their sensitivity to anti-cholinesterases. Examination of this possibility is currently being undertaken.

The fifth percentile (HD₅ or HD₅%) of a species sensitivity distribution can be selected as a concentration that is considered protective of most species in a community [124] and consequently is commonly used as an environmental quality criterion in risk assessments [125]. Although empirically derived, HD₅ values do not distinguish between the animals affected in terms of their ecological importance and thus, adverse effects on keystone or sentinel species are not given additional weighting when evaluating environmental risk to species as a whole. Instead, it is generally assumed

that protection of a majority of species with a conservative HD value would also be protective of an ecosystem exposed to these pesticides (see Aldenberg et al [126] for an elaboration of this argument). In the present study, the differences between the SSDs estimated with and without the acute oral toxicity values for dunnarts highlights the need to evaluate the in-situ effects of fenitrothion use on Australian native marsupials.

2.5 Conclusions

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 [127]. While there is often a 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. This 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.

Chapter 3: Fenitrothion, an organophosphorus insecticide, impairs locomotory function and alters body temperatures in *Sminthopsis macroura* (Gould 1845) without reducing metabolic rates during running endurance and thermogenic performance tests

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Fenitrothion, an organophosphorus insecticide, impairs locomotory function and alters body temperatures in *Sminthopsis macroura* (Gould 1845) without reducing metabolic rates during running endurance and thermogenic performance tests. *Environmental Toxicology and Chemistry*

3.1 Introduction

The Australian plague locust, *Chortoicetes terminifera* (Walker 1870), inhabits arid and semi-arid grassland agro-ecosystems across much of eastern Australia [87]. Because locust populations can increase rapidly and migrate large distances into productive cropping regions, management programs employ chemical pesticides to protect agricultural production [128]. In contrast to most agricultural pests, locust control is often undertaken in natural or semi-natural landscapes located within arid and semi-arid regions of Australia [14] inhabited by highly endemic and evolutionarily old fauna [129]. Chemical-based locust control therefore has the potential to expose structurally and functionally important species within these ecosystems to agrochemicals to which they have no previous exposure and subsequently have not developed any tolerance.

The organophosphorus (OP) insecticide, fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenyl)-phosphorothioate) has been used in eastern Australia for locust

control since the mid-1970s and is currently the most cost-effective chemical pesticide for this purpose [128]. Central to the global acceptance of OP insecticides is laboratory evidence of their high insecticidal potency and relatively low mammalian toxicity compared to pesticides such as organochlorines due to their rapid biotransformation within organisms [4]. Risk assessments used to predict the potential hazards of these pesticides to non-target mammalian species generally utilise lethality endpoint data generated from northern hemisphere eutherian mammals exposed to pesticides under controlled experimental conditions.

Fenitrothion is a neurotoxicant that acts by inhibiting cholinesterase (ChE) enzymes that are necessary for hydrolysing the neurotransmitter acetylcholine (ACh) in both vertebrates and invertebrates [128], as well as affecting enzymes that are important for cell signalling [130]. There are two major types of cholinergic receptors, muscarinic and nicotinic, and there are several subtypes of each. These receptor types are differentially distributed throughout the central and peripheral nervous systems, thus contributing to the complexity of effects that may occur from OP exposure [21]. Nicotinic receptors are found on postganglionic cells, specifically parasympathetic and sympathetic autonomic fibres associated with the cardiovascular system as well as being located on the somatic motor nerve fibres of skeletal muscles [53]. Muscarinic receptors are generally associated with effector cell membranes within the parasympathetic autonomic nervous system and are also associated with presynaptic terminals of the central nervous system.

Exposure to anti-ChE compounds prolongs ACh activity which, in turn, disrupts normal cholinergic neural and neuromuscular functions [6]. At muscarinic receptors, suppression of acetylcholinesterase (AChE) causes the enhancement or persistence of the characteristic response, either excitatory (e.g., bronchoconstriction) or inhibitory

(e.g., vasodilatation). At nicotinic receptors, the effect is first excitatory (e.g., muscular fasciculation) followed by inhibition (e.g., muscular paralysis) [131].

Due to the ubiquitous roles of AChE in most vertebrates [132], numerous physiological (thermoregulation, endocrine function, metabolism and reproduction, chronobiology, sensory perception) [133] and behavioural (activity, feeding, performance of learned tasks, learning and memory, aggression and therefore inter-individual interactions) systems of animals have the potential to be affected by exposure to OP pesticides [132]. Because the current risk assessment framework is biased towards lethal consequences, there is limited information regarding the sublethal effects of OPs, but studies examining this reveal substantial variation among species studied [134]. When considered in combination with the dearth of data on the susceptibility of Australian endemic species to this family of insecticides, adequately estimating risk to Australian endemic free living populations is problematic [6].

Research aimed at addressing these data deficiencies should utilise experimental variables relevant to free-living populations. Locomotory performance directly reflects an animal's ability to forage, evade predators, interact with other individuals of the same species and ultimately reproduce. Furthermore, because effective locomotion requires functional integrity of a range of physiological variables [135], exercise performance measurements give insight into the extent and duration of effects of OP pesticides on the suite of sensory, neural, metabolic and muscular functions involved with locomotion that are mediated by cholinergic innervation [36]. Experiments involving exposure of rodents to the OP dimethoate revealed it to significantly reduce locomotory function in the species studied (bank vole, *Clerthionomys glareolus* (Schreber 1780); European pine vole, *Pitymys subbterraneus* (de Selys-Longchamps 1836); root vole, *Microtus oeconomus* (Pallas 1776); pygmy wood mouse *Apodemus*

microps (Pallas 1811); harvest mouse, *Micromys minutus* (Pallas 1771) and house mouse *Mus musculus* L. 1758), but there were substantial interspecific differences in sensitivity and the time taken for recovery [134]. The only study quantifying the sublethal effects of fenitrothion on a native Australian marsupial showed running endurance of fat-tailed dunnarts (*Sminthopsis crassicaudata* Gould 1844) to be reduced by over 60% 24 h after ingestion by gavage, with no concomitant reduction of peak metabolic rate during the trial [36].

Fenitrothion has been shown to suppress avian flight metabolism in a dose-dependent manner. In a study evaluating the impact of fenitrothion ingestion in house sparrows, *Passer domesticus* (L. 1758), flight-related peak metabolic rate was reduced by 20, 18 and 58% in birds receiving 30, 60 and 100 mg kg⁻¹, respectively and the time taken for these values to return to pre-dose levels was also dose-dependent [56]. Concomitant reductions in total plasma cholinesterase activities reflected this pattern with *P. domesticus* individuals taking 2, 6 and 14 d to recover to predose levels in the 30, 60 and 100 mg kg⁻¹ dose groups, respectively.

In addition to its adverse effects on locomotory performance, OP exposure is also known to affect thermoregulation in a range of vertebrate species. White-footed mice (*Peromyscus leucopus* Rafinesque 1818) intubated with parathion showed reductions in rectal temperature of 9.7% and 9.2% for animals kept at 23 °C and 10 °C respectively after exposure to pesticide [66]. Similarly, Long-Evans rats maintained at an ambient temperature of 20 - 24°C and exposed to diisopropyl fluorophosphate (DFP) doses ≥ 1.0 mg kg⁻¹ showed significant decreases in body temperature of up to 4 °C and lasting up to 5 h [69]. This is likely a result of OP effects on cholinergic neuronal innervation, which is important for the integration of input from peripheral skin and core temperature receptors, as well as output to metabolic heat production and

evaporative heat loss processes [61]. In addition, muscarinic cholinergic receptors on the hypothalamus are involved with feedback pertinent for adjusting the hypothalamic set-point temperature via input from the peripheral nervous system [21].

The acute oral toxicity of fenitrothion to two species of Australian endemic insectivorous dasyurids, *S. crassicaudata* and *S. macroura*, has been quantified at 127 and 97 mg kg⁻¹ respectively, some 10-14 times lower than the LD₅₀ determined for *M. musculus*, a similar-sized eutherian mammal that is commonly presented in pesticide risk assessments [6]. While this increased sensitivity to pesticide is of concern *per se*, it also highlights the importance of research aimed at examining the sublethal effects of pesticide exposure and the need to quantify the ecotoxicological significance of such responses [2]. We have accordingly quantified the effects of fenitrothion on locomotory and thermogenic performance of *S. macroura* and discuss the implications of these effects on free-living populations.

3.2 Material and Methods

3.2.1 Experimental animals

The adult dunnarts used in this research project were sourced from a laboratory-bred colony at the University of Wollongong supplemented with animals collected from field populations in southwest Queensland, Australia. All animals were maintained at 23 °C on a photoperiod of 12L:12D and provided food (a blended combination of canned dog food, dry cat kibble and water) and water *ad libitum*. This feeding regime was supplemented with mealworms and crickets once per week.

3.2.2 *Experimental protocols*

Blood was sampled from all dunnarts 55 days before they ingested pesticide to determine their pre-dose plasma cholinesterase activities. All blood samples before and after dosing were collected between 12:00 and 16:00 to minimise potential circadian influences. Animals were trained to run within a stationary chamber placed on a treadmill (see description below). Those individuals unable to maintain a running speed of 102 cm sec⁻¹ for at least 2 min after two weeks of training were eliminated from the experiment. The 44 dunnarts that satisfied this selection criterion were trained for an additional four weeks and then ranked according to their running endurance, before being assigned to serve either as controls (22 animals) or to receive pesticide (22 animals), with running abilities being distributed equally among the two groups (see Table 5 below for sampling schedule). The metabolic response to cold temperature (see below) was quantified in all animals 5 days prior to pesticide or sham treatments.

One day after being gavaged with either pesticide (treatment group) or canola oil vehicle alone (control group; see details below), all dunnarts were run in the morning until fatigued. Approximately three hours later, a subset were randomly selected for removal from the control and treatment groups (see Table 5 for sampling schedule) and then placed in a refrigerated incubator to measure their metabolic rate during cold exposure (2 °C; see details below). These animals were then allowed to rewarm for an hour before taking blood and brain samples for cholinesterase determination. The remaining dunnarts were run again 1, 4 (except cohort 1, see Table 5 below), 7 and 10 days after dosing, with the exception of randomly chosen dunnarts being removed from their cohorts at scheduled dates to determine their thermoregulatory response to cold before taking blood and brain samples. All dunnarts were killed by CO₂ asphyxiation immediately after final blood sampling and their brain was then quickly removed,

placed in liquid nitrogen, and stored at -80 °C before subsequent cholinesterase determinations.

Table 5. Dunnart sampling schedule. Total sample size for running endurance measures at each time point is given without brackets. Dunnart numbers given in brackets represent animals extracted for thermogenic performance measures, AChE and blood characterization at each time point after pesticide exposure. Column titles represent days on which measurements were undertaken.

Cohort	Group	Pre-dose	D1	D4	D7	D10
1	Control	7	7 (3)	-	4 (1)*	3
	Treatment	9	9 (3)	-	6 (3)	3
2	Control	15	15 (2)	13 (5)	8 (4)	4
	Treatment	13	13 (3)	10 (5)	5 (3)	2
Total		44	44	23	23	12

** As a result of 3 dunnarts from the control group dying unexpectedly during experimentation with the first cohort of animals, a second cohort (cohort 2 above) of dunnarts was acquired to supplement low sample sizes.*

3.2.3 Pesticide dosing

Our selected dose of 90 mg kg⁻¹ fenitrothion is ecologically realistic based on our knowledge that *S. macroura* can consume up to 100% of their body mass daily while gorge feeding and that locusts may contain residue values of up to 80 mg kg⁻¹ fenitrothion after locust control operations [115]. The dose selected for this study is 3-fold higher than previous research in a closely related species, *S. crassicaudata*, that found oxygen consumption rate to be unaffected by fenitrothion during both peak

thermogenesis and exercise [36]. Dose-dependent avian responses to pesticide exposure in previous research [56] prompted us to increase the rate for the current study from 30 mg kg⁻¹ as previously investigated [36] to 90 mg kg⁻¹ fenitrothion in anticipation that the effects of fenitrothion exposure would be more persistent when animals were subjected to a higher dose. This dose is known to be sublethal to *M. musculus*, a similar sized eutherian mammal [5].

Fenitrothion was diluted in canola oil using a vortex mixer to obtain the appropriate mass-specific dose in a 0.2 ml volume, which was administered by gavage. Control animals were given 0.2 ml of canola oil alone via gavage. Dunnarts were fasted overnight before the administration of oil or fenitrothion doses and were monitored closely for two hours after dosing and then at 30 min intervals for the next six hours. Food and water were returned to their cages after the cessation of any toxicological signs associated with pesticide ingestion as described in [6].

3.2.4 *Running endurance measurements*

Dunnarts were trained to run within an inverted, stationary Perspex chamber placed on a modified treadmill set to a speed of 102 cm s⁻¹. While running, air was aspirated through the chamber at 2.0 L min⁻¹ and monitored using a mass flow meter (Sierra Systems Top-Trak). Oxygen content of inlet and outlet air was measured using a Sable Systems FC-1 oxygen analyser in conjunction with an A to D converter (Universal Interface, Sable Systems) and a Macintosh computer. Water and CO₂ were removed from the input air stream prior to gas analysis using Drierite and soda lime, respectively. Dunnarts were placed in the running chamber and the treadmill was then activated to rapidly reach a speed of 102 cm s⁻¹. Dunnarts were kept running until the first signs of fatigue, as evidenced by animals making contact with the rear of the

chamber with increasing frequency. Running duration times were recorded using a stopwatch along with continuous (1-sec intervals) recording of oxygen consumption rate ($\dot{V} O_2$) during each individual exercise session. Calculations of $\dot{V} O_2$ used appropriate corrections for the respirometer configuration we used [136] and we transformed these values to instantaneous $\dot{V} O_2$ following the procedures of [137]. This acknowledges the dynamic characteristics of gas mixing under the specific physical conditions of our measurements. The maximal metabolic rate achieved during running endurance (PMR_{run}) was calculated as the highest continuous 2 min instantaneous $\dot{V} O_2$ recorded while running. Dunnart body mass was measured prior to each treadmill session, and body temperature measured before and after each exercise bout using a rectal probe using a calibrated Type T thermocouple (Physitemp RET3 rectal probe for mice) connected to an electronic thermometer (Physitemp BAT-12). The ambient air temperature was maintained at 23 °C for the duration of the experiment.

3.2.5 *Shivering thermogenesis*

Thermogenic performance experiments were conducted in the afternoon following a running endurance measure made that morning for a given individual. Pre-dose measures were taken 5 days prior to pesticide exposure with post-dose measures taken at 1, 4, 7 or 10 days after dosing for a given individual.

Dunnarts were placed singularly in a 2 L airtight metal respirometry chamber fitted with ports for incurrent and excurrent airflow. These were placed within a temperature-controlled cabinet set to 25 °C and then provided a gas mix of 79% helium and 21% oxygen at a flow rate of 750 ml min⁻¹. The use of a helium-oxygen gas mixture allows maximal rates of heat loss to be elicited from small endotherms to occur at above-freezing temperatures [138]. This thus permits evaluation of maximal rates of

shivering thermogenesis while avoiding freeze damage to animal tissues during experimentation. All flow meters were calibrated to deliver the He-O₂ mix using a primary airflow calibrator (Gilian Gillibrator, Sensidyne, USA). Excurrent gas from the respirometry chambers was passed through water and carbon dioxide absorbents (Drierite and soda lime, respectively) and then to an oxygen analyser (Sable Systems, Oxzilla), with the output signals from the oxygen analyser and mass-flow controllers recorded on a Macintosh computer.

After placement in the temperature-controlled cabinet, dunnarts were allowed a 20 min adjustment period at 25 °C. The cabinet temperature was then lowered to 2 °C and the dunnart's metabolic rate recorded for the next 60 min. The maximal metabolic rate achieved during this time ($\text{PMR}_{\text{therm}}$) was calculated as the highest continuous 2-min rate of oxygen consumption ($\dot{V} \text{O}_{2 \text{ max}}$) recorded during the cooling period. The total amount of oxygen consumed throughout the entire 60 min period was termed the integrated metabolic rate (IMR) and the time taken to reach $\text{PMR}_{\text{therm}}$ is termed the time to maximum metabolic rate (TMM) following [36]. Rectal temperature of each dunnart was measured using a calibrated Type T thermocouple (Physitemp RET3 rectal probe for mice) connected to an electronic thermometer (Physitemp BAT-12) before (Tb_{in}) and after (Tb_{out}) each cold-exposure session. These measurements were made on all animals pre- and post-dosing with either fenitrothion or canola oil alone.

3.2.6 *Blood and plasma characterisation*

Approximately 150 μL of blood was collected infra-orbitally using heparinised, micro-haematocrit tubes. Blood haemoglobin (Hb) content was measured by transferring approximately 3 μL of whole blood from a heparinised capillary tube into a microcuvette for immediate analysis of Hb content (Hemocue HB 201, Abgleholm,

Sweden), with Hb measured to the nearest 0.1 g dL⁻¹. Microhaematocrit tubes were then centrifuged at 8000 rpm for 4 min to separate plasma from the red blood cell fraction. Haematocrits were measured as percentages on each tube using a purpose-built reader (Hawksley Micro-haematocrit Reader, England, UK), after which all plasma was removed and transferred to a cryovial using a Hamilton syringe. All plasma samples were quickly frozen and stored at -80 °C until used for cholinesterase analyses.

Brain AChE and total ChE values were assessed in brains from a subset of control and fenitrothion-treated animals, representing specific times post-treatment. Brains were removed from dunnarts immediately after euthanizing them by CO₂ asphyxiation and then immediately placed in a -80 °C freezer for storage. Total cholinesterase and AChE in whole brain homogenate and plasma were analysed using the method of [113] as modified by [114]. Details of the complete methodology for the characterization and quantification of dunnart plasma and brain AChE and total ChE are outlined in [36].

3.2.7 *Data analysis*

A BACI design was employed to measure the effects of fenitrothion ingestion on plasma AChE and total ChE in control and treatment dunnarts. Data collected using a repeated measures design (running endurance, maximal metabolic rate while running, rectal temperature before and after running endurance and shivering thermogenesis measurements) were analysed using mixed effects models (SPSS, IBM Australia St Leonards, New South Wales, Australia). After fitting the mixed models to combined datasets, split models were then run on individual datasets for control and fenitrothion-exposed dunnarts to examine within treatment effects. Post hoc differences were identified using individual t-tests.

Those data not collected using a repeated measures design but rather as pre- and post-dose samples (haematocrit, haemoglobin, plasma AChE and TChE, brain AChE and TChE, maximal metabolic rate attained during thermogenic challenge (PMR_{therm}) integrated metabolic rate attained during thermogenic challenge (IMR) and the time taken to reach maximal metabolic rate during the thermogenic challenge (TTM)) were analysed using ANOVA with Tukey HSD post-hoc analysis (SPSS, IBM Australia St. Leonards, New South Wales, Australia).

3.3 Results

3.3.1 Behavioural response to pesticide exposure

Dunnarts treated with pesticide underwent a sequence of toxicological signs identical to that previously described for *S. crassicaudata* [36] and *S. macroura* [6]. Initially, dunnarts began salivating and intensifying their grooming activity, which was progressively replaced by shivering, body tremors, and piloerection. Breathing was generally rapid throughout the progression of these toxicological signs. Although all individuals showed the same sequence of symptoms, the timing of the onset and duration of each of these responses varied between dunnarts. Approximately 30 - 60 min after ingesting the pesticide, dunnarts began to lose coordination in their rear legs, progressing in some animals to complete immobilization. These signs usually dissipated within 2 h of dosing and were followed by extended periods of lethargy and inactivity.

3.3.2 Effect of fenitrothion on body mass

Body mass increased over the duration of the trial in both control and fenitrothion-exposed dunnarts (Figure 6), despite a suppression in feeding activity

immediately following pesticide ingestion. There was no significant difference in the percentage change in dunnart body mass between control and pesticide-exposed dunnarts ($F_{0.05, 1, 21} = 0.042$; $p = 0.839$)

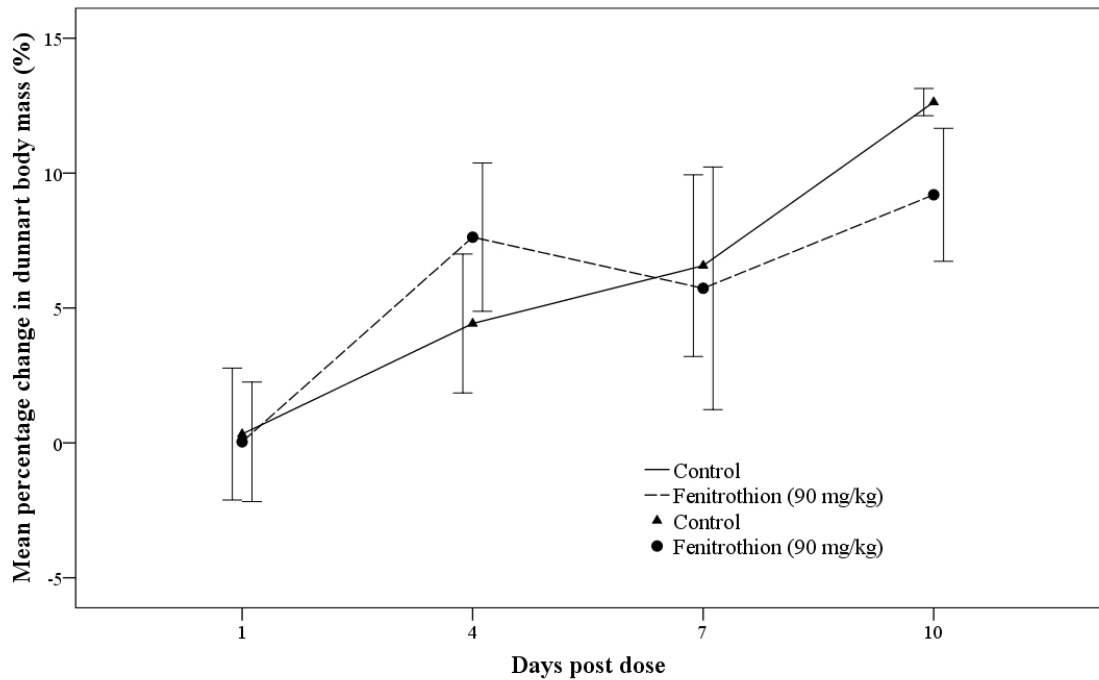


Figure 6. Percentage change in dunnart body mass at each post-dose time point from pre-dose values after exposure to 90 mg kg⁻¹ fenitrothion by gavage. Error bars represent ± 1 standard error and are offset for clarity

3.3.3 Running endurance, peak metabolic rate and body temperature change during exercise trial

Individuals showed very consistent running endurance times, despite large inter-individual variation in this measurement (range 1 - 21 min). After several weeks of treadmill training, running endurance for any given running event (x) by an individual correlated significantly with its immediate predecessor (x-1) ($F_{1, 42} = 331.64$, $p < 0.001$, $R^2 = 0.88$). After gavage, dunnarts in the control group appeared to show a further

“training effect” with increasing running endurance times, but this improvement was not statistically significant ($F_{0.05(53)3,12} = 0.406$; $p = 0.751$; Figure 7).

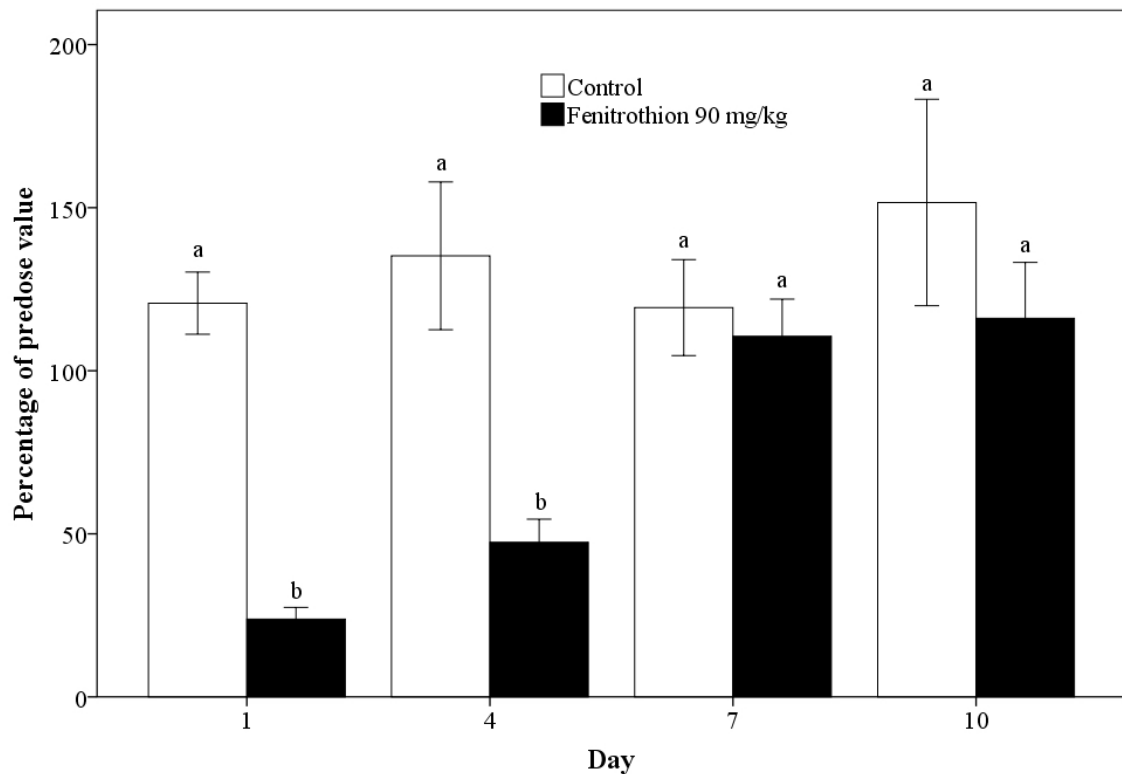


Figure 7. Dunnart running-endurance after exposure to 90 mg kg⁻¹ fenitrothion by gavage, expressed as percentage change from pre-dose levels. Data shown are means \pm 1 SE. Letters that differ above a pair of bars indicate statistically significant differences ($P < 0.05$) between control and fenitrothion-treated animals at that specific time point.

Dunnarts in the control and pesticide-treatment groups showed no statistically significant difference in running endurance before dosing (see below). After dosing, however, the fenitrothion-treated group showed reductions in running endurance of approximately 76 and 53% of pre-dose durations at 1 and 4 d post-exposure respectively. There was a significant interaction between treatment type and days sampled after

treatment ($F_{0.05 (102) 3, 22} = 6.02$; $p < 0.005$; Figure 7). When analysed separately, the fenitrothion-treated dunnarts had a significant reduction in running endurance after dosing ($F_{0.05 (48) 3, 8} = 26.237$; $p < 0.005$), whereas control animals were unaffected by the canola oil vehicle ($F_{0.05 (53) 3, 12} = 0.406$; $p = 0.751$).

Further, t tests (two-tailed) comparing running endurance times of control and pesticide-dosed animals at individual time points post dose showed significantly different times between control and treatment animals at post-treatment sampling days 1 ($t_{0.025 (44) 42} = 2.02$; $p < 0.005$) and 4 ($t_{0.025 (23) 21} = 2.08$; $p < 0.005$). Running endurance in fenitrothion-treated dunnarts returned to, or exceeded pre-dose levels and were statistically indistinguishable to control dunnarts at 7 ($t_{0.025 (23) 21} = 2.08$; $p = 0.645$) and 10 days ($t_{0.025 (12) 10} = 2.23$; $p = 0.402$) post-dose (Figure 7).

Metabolic rates attained by dunnarts during running endurance measurements averaged 4.01 ± 0.17 (SE) ml O₂ min⁻¹ for control and 4.40 ± 0.44 (SE) ml O₂ min⁻¹ for dosed animals before exposure to pesticide. Metabolic rates were unaffected by either fenitrothion or by the oil gavage that served as the control ($F_{0.05 (146) 4, 92} = 0.256$; $p = 0.905$), with metabolic rates at 1 d post-exposure averaging 4.78 ± 0.31 (SE) ml O₂ min⁻¹ and 5.10 ± 0.79 (SE) ml O₂ min⁻¹, respectively.

Mean dunnart rectal temperature (T_b) was consistently higher in dunnarts after running endurance measurements were completed throughout the experiment (Figure 8). Prior to gavage, the pre- and post-running (T_b) was indistinguishable between control and treatment animals ($t_{0.05, 21} = 0.53$; $p = 0.3$), with temperatures ranging from 34.2 to 37.2 °C in the control and 34.8 to 37.2 °C in the treatment group. Linear mixed model analysis of dunnart rectal temperature before (T_{b_{in}}) and after (T_{b_{out}}) running endurance measurements, using the starting temperature (T_{b_{in}}) as a covariate, revealed a

significant post-dose interaction between treatment and time ($F_{0.05(196) 6, 97} = 5.248$; $p < 0.005$).

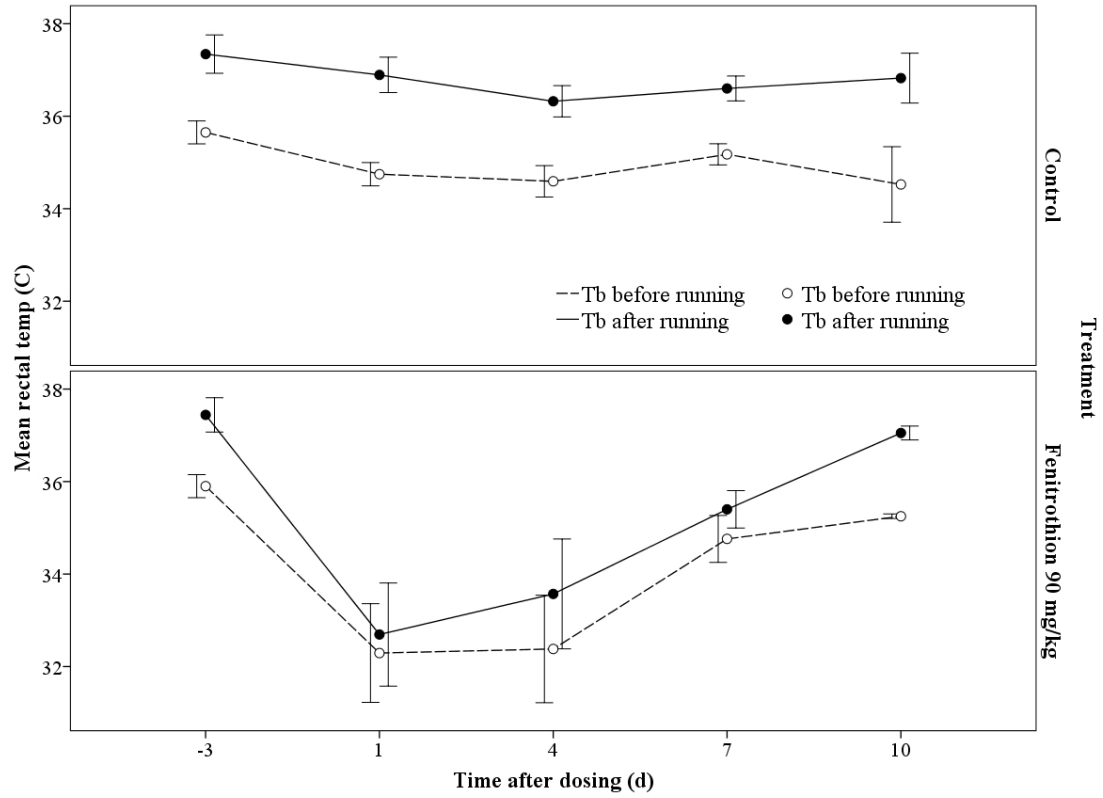


Figure 8. Dunnart rectal temperature before (T_b before running; open circles) and after (T_b after running; filled circles) running in control (upper panel) and fenitrothion-treated animals (fenitrothion 90 mg kg⁻¹; lower panel). Data shown are means \pm 1 SE . Error bars are offset for clarity.

Splitting the data file and applying the linear mixed effects model to the variable $T_{b_{out}}$, using $T_{b_{in}}$ as a covariate, showed pesticide-exposed animals to be significantly affected by treatment ($F_{0.05(149) 6, 44} = 3.689$; $p = 0.005$), but no effect of treatment on control animals ($F_{0.05(149) 6, 53} = 0.927$; $p = 0.483$). $T_{b_{out}}$ was significantly higher than $T_{b_{in}}$ in both the control ($F_{0.05(149) 1, 68} = 10.141$; $p < 0.005$) and pesticide-exposed dunnarts ($F_{0.05(149) 1, 58} = 451.951$; $p < 0.001$).

3.3.4 Shivering thermogenesis and body temperature

During shivering thermogenesis, oxygen consumption in all dunnarts increased as the chamber temperature was gradually decreased to 2 °C. Behavioural responses of dunnarts to cold exposure observed at the conclusion of thermogenic sessions were similar to that described previously [36], with dunnarts demonstrating significant piloerection and shivering as they were removed from the metabolism chamber.

Mean maximal metabolic rates (2 min) attained by dunnarts during these measurements were lower than those attained during locomotory performance tests ranging from 2.66 ± 0.23 to 3.88 ± 0.25 ml O₂ min⁻¹ for control and 2.60 ± 0.39 to 3.94 ± 0.62 ml O₂ min⁻¹ for pesticide-exposed dunnarts. Exposure to fenitrothion had no effect on dunnart peak metabolic rate attained during shivering thermogenesis ($F_{0.05, 4, 58} = 2.316$; $p = 0.068$). Total oxygen consumed during the course of the thermogenic challenge (IMR) ranged from 64.82 ± 9.03 to 101.76 ± 8.78 ml O₂ min⁻¹ in control and 61.78 ± 14.04 to 119.54 ± 29.95 ml O₂ min⁻¹ in pesticide-exposed dunnarts.

After treatment with fenitrothion or canola alone, dunnart IMR decreased in both control and treatment animals, but the IMR of both treatment groups was statistically indistinguishable ($F_{0.05, 4, 58} = 1.348$; $p = 0.263$). Similarly, the time taken for dunnarts to reach their peak metabolic rates while inside the respirometer was unaffected by fenitrothion ($F_{0.05, 4, 58} = 1.733$; $p = 0.155$) (Table 6).

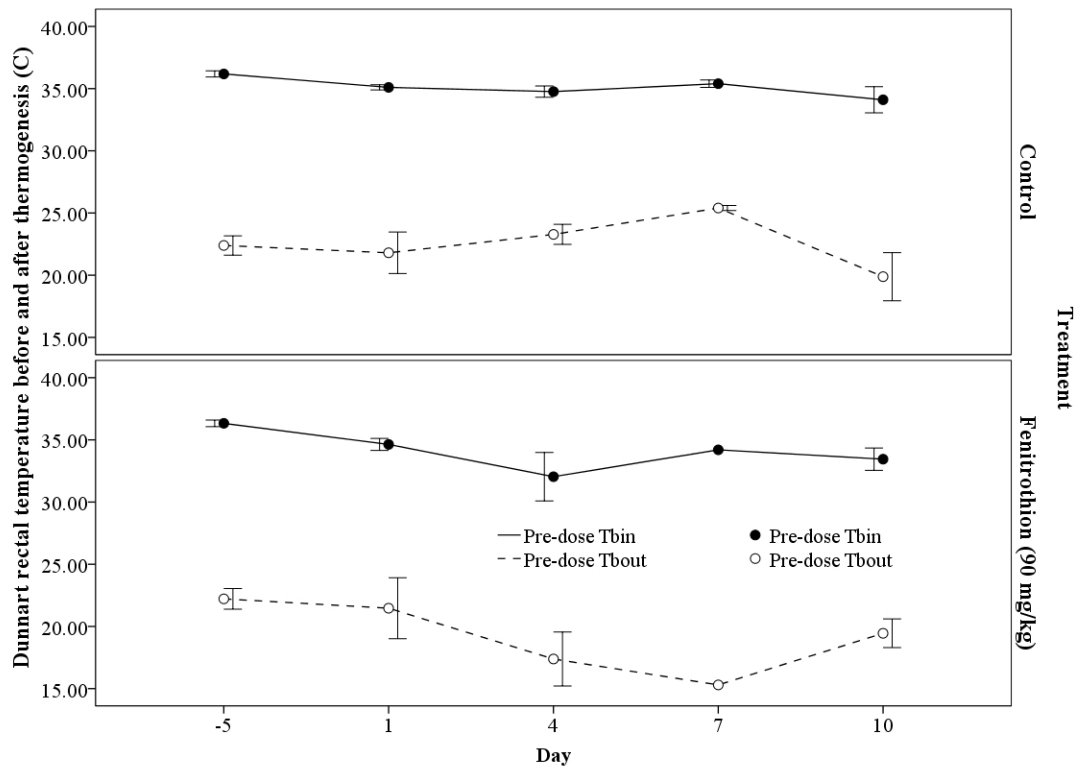


Figure 9. Dunnart rectal temperatures measured at the start (T_{bin} ; filled circles) and after (T_{bout} ; open circles) a 1-h shivering thermogenesis bout in animals treated with either 90 mg kg^{-1} fenitrothion (lower panel) or a sham dose of canola oil carrier only (Control; upper panel). Error bars represent one standard error and are offset for clarity.

Dunnart rectal temperatures fell significantly during thermogenic challenge in both control and pesticide-exposed groups ($F_{0.05, 1, 17} = 13.4$; $p = 0.02$), but there was no statistically significant effect of treatment on the extent of this hypothermia ($F_{0.05, 4, 12} = 2.96$; $p = 0.64$) (Figure 9).

Table 6. Pre and post treatment variables measured during thermogenic challenge to dunnarts exposed to fenitrothion 90 mg kg⁻¹ (treatment group) or to canola oil alone (controls) in relation to time before or after dosing (in days).

Variable (units)	Pre-dose value ± 1 se (n)		Post-dose value ± 1 se (n)			Statistical significance
	D-5	D1	D4	D7	D10	
<u>PMR (ml O₂ min⁻¹)</u>						
Control	3.59 ± 0.14 (17)	3.35 ± 0.22 (3)	3.88 ± 0.25 (5)	3.65 ± 0.32 (4)	2.66 ± 0.23 (5)	$F_{0.05, 4, 58} = 2.316;$
Treatment	3.52 ± 0.24 (17)	3.94 ± 0.62 (4)	2.60 ± 0.39 (4)	2.66 ± 0.66 (4)	3.27 ± 0.52 (5)	$p = 0.068$
<u>IMR (ml O₂)</u>						
Control	99.43± 6.1 (17)	84.45 ± 11.13 (3)	101.76 ± 8.78 (4)	91.43 ± 12.44 (4)	64.82 ± 9.03 (5)	$F_{0.05, 4, 58} = 1.348;$
Treatment	103.97 ± 11.91 (17)	119.54 ± 29.95 (4)	61.78 ± 14.04 (5)	68.45 ± 22.26 (4)	90.21 ± 17.59 (5)	$p = 0.263$
<u>Time taken to reach peak metabolic rate (PMR) (min)</u>						
Control	19.00 ± 0.87 (17)	19.50 ± 0.72 (3)	20.15 ± 1.25 (5)	21.35 ± 0.89 (4)	17.32 ± 2.3 (5)	$F_{0.05, 4, 58} = 1.733;$
Treatment	21.59 ± 1.35 (17)	18.38 ± 1.29 (4)	15.58 ± 3.92 (4)	19.18 ± 2.26 (4)	18.56 ± 1.78 (4)	$p = 0.155$

3.3.5 Plasma and brain acetylcholinesterase and total cholinesterase activities, haemoglobin and haematocrit levels

Although a decline in plasma AChE activity was observed one day after fenitrothion exposure, the maximal AChE suppression did not appear until 4 d post-dose (Figure 10).

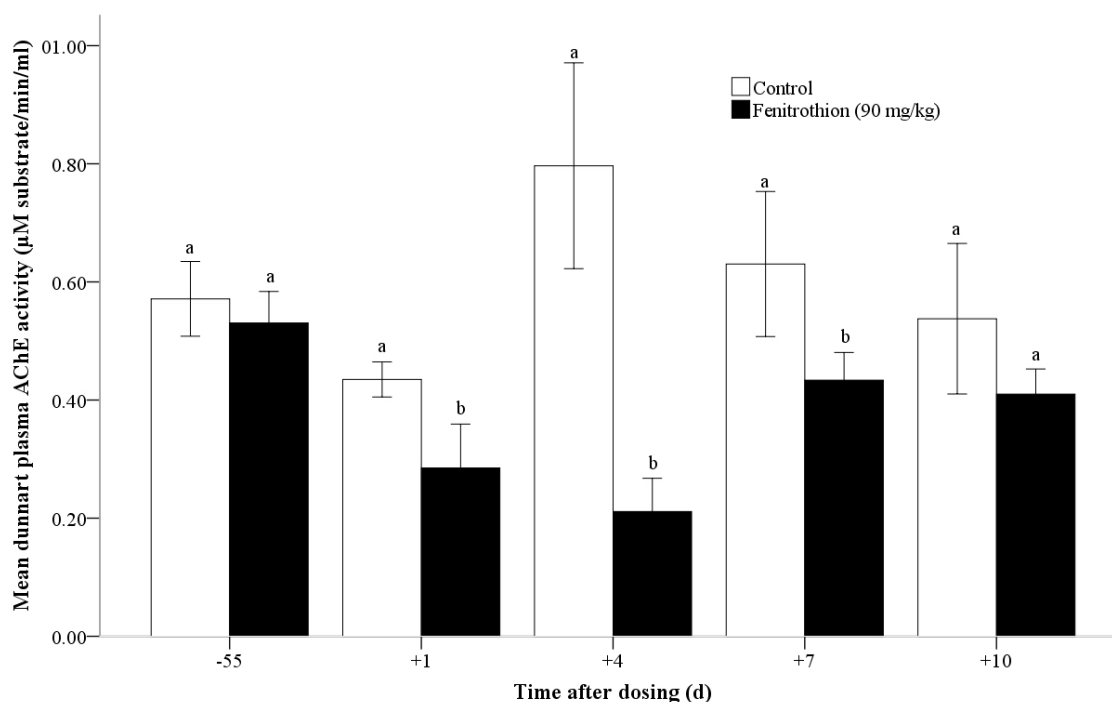


Figure 10. Dunnart plasma acetylcholinesterase (AChE) activity levels before and after pesticide exposure (90 mg kg⁻¹ fenitrothion) by gavage. Data shown are means ± 1 SE. Letters that differ above a pair of bars indicate statistically significant differences (P<0.05) between control and fenitrothion-treated animals at that specific time point.

Analysis of plasma AChE activity in control and pesticide-exposed dunnarts revealed a significant interaction between time and treatment ($F_{0.05, 4, 84} = 30925$; $p = 0.006$). Repeating this analysis on the split data file revealed no effect of time on

control dunnarts ($F_{0.05, 4, 42} = 1.04$; $p = 0.401$), but a significant effect of time on fenitrothion-exposed animals ($F_{0.05, 4, 42} = 6.003$; $p = 0.01$). Post hoc comparisons of control and pesticide-exposed dunnarts sampled at the same time points revealed no significant difference between them pre-dose ($t_{0.05, 40} = -0.05$ $p = 0.96$), significant differences at 1 d ($t_{0.05, 9} = 2.64$; $p = 0.02$) and 4 d post dose ($t_{0.05, 7} = 9.47$; $p < 0.001$), and did not differ statistically at 7 d ($t_{0.05, 6} = 1.32$; $p = 0.24$) and 10 d ($t_{0.05, 12} = 0.57$; $p = 0.58$) after dosing. The linear mixed model analysis of dunnart brain AChE activity in control and pesticide-exposed dunnarts (Figure 11) showed no interaction effect ($F_{0.05, 3, 20} = 0.519$), whereas analysis on a split data file (as outlined above) showed a significant effect of treatment on brain AChE activity ($F_{0.05, 1, 20} > 0.001$), but no effect of time since gavage ($F_{0.05, 1, 20} = 0.924$). Small sample sizes prevented comparison of treatment effects for each of the days in which dunnart brain ChE was sampled.

Mean haemoglobin levels decreased in all dunnarts during the course of the experiment (Table 2). Prior to dosing control and treatment animals had mean haemoglobin levels of 19.0 ± 0.5 and 18.6 ± 0.4 g dL⁻¹ respectively and these declined to 15.9 ± 0.5 and 15.3 ± 0.8 g dL⁻¹ respectively by 10 d post exposure. A two-way, between groups analysis of variance was conducted to explore the impact of treatment (fenitrothion) on dunnart haemoglobin levels and no effect of treatment was found ($F_{0.05, 4, 94} = 1.131$; $p = 0.348$).

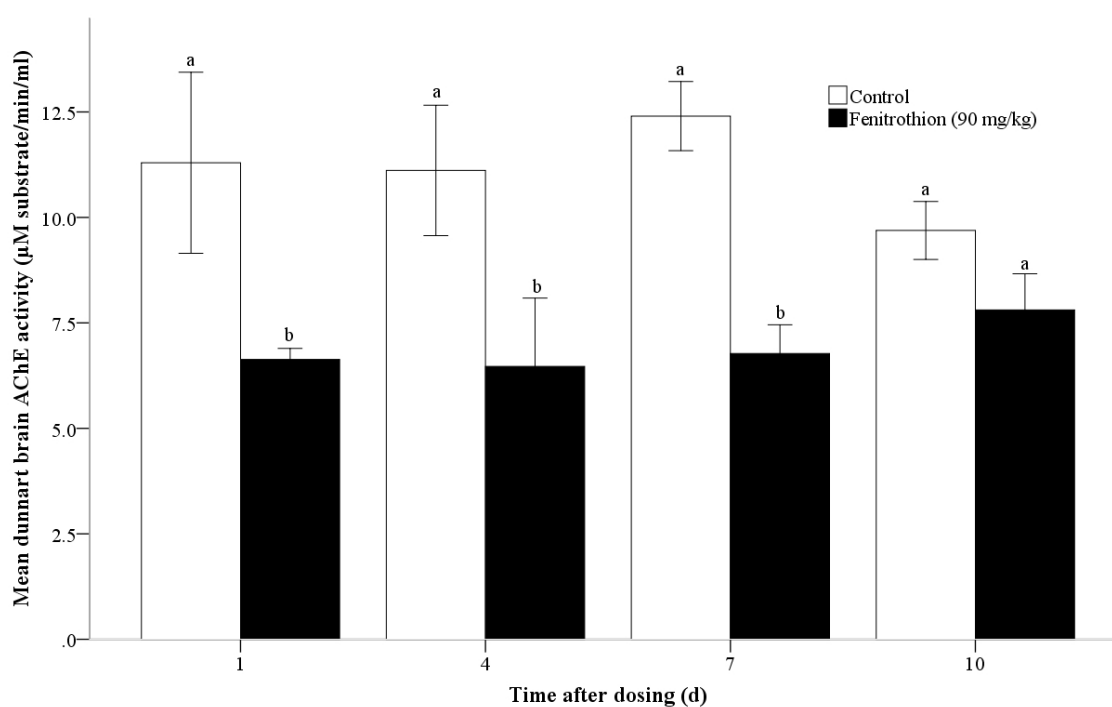


Figure 11. Dunnart brain acetylcholinesterase (AChE) activity levels before and after pesticide exposure (90 mg kg^{-1} fenitrothion) by gavage. Data shown are means \pm 1 SE. Letters that differ above a pair of bars indicate statistically significant differences ($P < 0.05$) between control and fenitrothion-treated animals at that specific time point.

A slight decline in mean haematocrit levels throughout the duration of the experiment was observed with levels measured prior to dosing of 52.9 ± 0.5 and 51.3 ± 0.7 % falling to 48.3 ± 1.2 and 47.7 ± 1.5 in control and treated dunnarts respectively (Table 5). No effect of pesticide was detected ($F_{0.05, 4, 93} = 1.636$; $p = 0.173$).

Table 7. Pre and post exposure haematocrit and haemoglobin levels in dunnarts exposed to fenitrothion 90 mg kg⁻¹ by gavage. Data are given as mean \pm SE.

Variable	Treatment	Time before or after treatment (days)				
		-5	1	4	7	10
Haematocrit (%)	Control	52.9 \pm 0.5	50.7 \pm 1.2	44.5 \pm 1.7	49.5 \pm 1.8	48.3 \pm 1.2
	Fenitrothion	51.3 \pm 0.7	46.5 \pm 0.2	58.5 \pm 4.8	46.7 \pm 2.5	47.7 \pm 1.5
Haemoglobin (g/dl)	Control	19.0 \pm 0.5	17.5 \pm 0.8	16.1 \pm 0.6	15.7 \pm 0.6	15.9 \pm 0.5
	Fenitrothion	18.6 \pm 0.4	17.8 \pm 0.7	13.9 \pm 0.8	16.0 \pm 0.3	15.3 \pm 0.8

3.4 Discussion

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. A significant overlap in habitat preferences between *C. terminifera* and *S. macroura*, the combination of dunnart's small body mass (some as low as 7 g) 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 [2]. Moreover, *S. macroura* has been recently found to be 10-14 times more sensitive to fenitrothion than a similar sized eutherian mammal, *M. musculus*, which is commonly referenced in pesticide risk assessments [6]. Such differences in pesticide sensitivity show the importance of understanding the responses of these dasyurids to sublethal exposure to pesticides, particularly given the ongoing need for chemical-based locust control.

Acute exposure of mammals to OP or carbamate insecticides often results in reductions in activity, commonly referred to as “behavioural slumps” (see [9] for a full review). The suppression of AChE by OP insecticides at the neuromuscular cleft results in elevated ACh levels causing the overstimulation and eventual desensitisation of post synaptic ACh receptors [9]. In this study, we used a dose three times higher (90 mg kg⁻¹) than used for a closely related species, *S. crassicaudata*, [36], to see if the higher dose would result in more extensive or longer lasting suppression in AChE and/or locomotory activities. The acute reduction of locomotory function in pesticide-treated *S. macroura* immediately following exposure was substantial, with running endurance reduced by 76% 24 h after exposure. This result represents a reduction in locomotory function approximately 10% greater than that found in previous research [36]. Further investigation, however, is required to determine whether these results fall within a dose-

dependency spectrum as has been previously identified across a range of other vertebrate taxa. Running endurance returned to pre-dose levels at 7 d after pesticide dosing. It's unclear if the training period used in the current study (~ 6 weeks) had any bearing on the ability of dunnarts to withstand the suppression of AChE within the synaptic cleft through increasing the density of nAChR, thereby enabling a more robust response to pesticide exposure. It would be informative to explore this possibility in future research.

Reductions in locomotory activity (measured as sprint speed) have been reported in the meadow jumping mouse (*Zapus hudsonius* Zimmerman 1780) in response to carbaryl (Sevin) exposure [139] and in adult male Sprague-Dawley rats (*R. norvegicus*) after exposure to physostigmine, a carbamate [51]. Dose-dependent reductions in swimming performance in black swamp snakes (*Seminatrix pygaea*, Cope 1895) and diamondback water snakes (*Nerodia rhombifer*, Smith and Brodie 1982) after exposure to 2.5 and 5.0 mg L⁻¹ carbaryl, have been reported, but no AChE activity measures were provided [140]. House sparrows (*P. domesticus*) gavaged with 30, 60 and 100 mg kg⁻¹ fenitrothion showed very significant dose dependent responses in their flight-induced peak metabolic rates (PMR), plasma AChE activities, and in the post-dose duration of pesticide-affected PMR [56].

The potential for reduced running endurance to adversely affect free-living dunnart populations exposed to pesticides is substantial and highlights the importance of performance-based investigations. Locomotory impairment will reduce their ability to forage, escape predators, maintain home ranges and reproduce, at least in the short term. Studies on movement patterns in arid zone small mammals indicate that several species of dunnart can cover significant linear distances in relatively short periods of time [40, 42, 91]. The high mobility of Australian, arid zone, small mammal species may be an

adaptive response to the low productivity and variable rainfall of Australian deserts [41], when compared to other systems [42]. In such an environment, a life history strategy geared towards locating food-rich refuges during times of drought that offer sufficient resources for survival and reproduction may be a safer evolutionary investment than the establishment of permanent home ranges, burrows and foraging sites [42]. In arid habitats, such refugia can be geographically widespread and transient in duration [43] and therefore the maintenance of locomotory function would be critical in enabling small mammals to rapidly exploit the resources within them. Even a transitory impairment in locomotory performance could impede an individual's ability to utilise resource mosaics, thereby increasing the likelihood of localized population extinctions.

The absence of any effect of fenitrothion on $\dot{V}O_{2\text{ max}}$, haematocrit or haemoglobin in the current study suggests that impairment of running endurance was not due to limitations in O_2 delivery or uptake by dunnart skeletal muscles. In addition to the reduction in synaptic ChE after OP exposure, there is evidence that OPs and their oxons bind reversibly to nicotinic ACh receptors (nAChRs) inducing nAChR desensitisation [26] and a subsequent reduction in ACh release into the synaptic cleft leading to continued cholinergic transmission failure during continuous or repetitive stimulation [141]. Additionally, previous research [36] suggested the lack of pesticide-induced effects on thermogenic variables mediated by muscarinic receptor-linked cholinergic innervation, as opposed to locomotory performance, mediated by nicotinic receptor-linked innervation, could largely be attributed to the differential responses of these receptor types to pesticide exposure. Specifically, spatial and temporal differences between muscarinic and nicotinic ACh receptors and their role in muscle recruitment reduces the likelihood that neuromuscular transmission failure would occur in shivering, as muscles involved in this activity are more widely distributed throughout

the body and require smaller levels of force to function than muscle fibres used for high-speed locomotion [36].

Dunnart rectal temperatures declined significantly in pesticide-exposed animals. These were as much as 5 °C lower at 1 d post-dose and then incrementally returned to pre-dose levels by 10 d post exposure. The body temperature of mammals is regulated within narrow limits by nuclei within the anterior hypothalamus. These cells receive and integrate input from core and peripheral temperature sensors [60] via muscarinic cholinergic receptors [21] and activate appropriate physiological and behavioural processes in an attempt to maintain a stable body temperature. Reductions in body temperature have been documented in a range of vertebrate species after exposure to organophosphorus insecticides. White-footed mice (*P. leucopus*) intubated with 10 mg kg⁻¹ parathion showed reductions in rectal temperature of 4 and 4.3 °C for animals kept at 23 and 10 °C, respectively [66]. Similarly, Long-Evans rats (*R. norvegicus*) maintained at an ambient temperature of 20 - 24 °C and exposed to diisopropyl fluorophosphonate (DFP) doses ≥ 1.0 mg kg⁻¹ showed significant decreases in body temperature of up to 4 °C, which lasted up to 5 h [69].

In the current study, reductions in rectal temperature experienced by pesticide-exposed dunnarts prior to running endurance measures contrasts with the absence of any fenitrothion-induced effects on parameters measured during shivering thermogenesis experiments. Fenitrothion had no effect on peak metabolic rate attained during cold exposure (PMR_{therm}), the time taken for each dunnart to reach PMR_{therm}, total metabolic energy expenditure (IMR) during the 1 h cold exposure, or rectal temperatures measured before and after thermogenic challenge (Tb_{in} and Tb_{out}). Within an animal's tolerable body temperature range, we would expect a decrease in body temperature to be accompanied by a concomitant decrease in oxygen consumption (Q10 effect [16]).

However, metabolic rates measured during locomotory and thermogenic challenges in the current study were unaffected by pesticide and these results mirror those from a previous study in which *S. crassicaudata* showed a similar lack of response to a lower dose (30 mg kg⁻¹) of the same pesticide [36].

There is evidence that AChE is important for mammalian thermoregulation through the proper functioning of cholinergic innervation to relay information on the ambient temperature from sensors in the peripheral nervous system to the hypothalamus [60]. Exposure to organophosphorus insecticides is known to either inhibit an animal's tolerance to cold or directly impair their ability to maintain internal body temperature [9]. Reductions in *S. macroura* body temperature were only evident in pesticide-exposed animals during the locomotory performance tests, which occurred between 8 and 11 am each day, whereas the thermogenic challenges took place in the afternoon. Because we did not monitor dunnart body temperatures and metabolic rates across a 24 h period, we cannot tell if the effects of fenitrothion on body temperature were indicative of a diel shift in hypothalamic temperature set point or a consequence of reduced thermoregulation.

It is also possible that the lower morning body temperatures in pesticide-treated dunnarts resulted from a change in the duration and/or frequency of overnight torpor. Dunnarts frequently utilise torpor, which is characterised by reductions in body temperature and metabolic rate. Torpor is widely used as an energy conservation mechanism during periods of food shortage and exposure to low ambient temperatures [73], although it is also known to occur when resources are not limited [142]. Free-living *S. macroura* have been observed to utilise torpor at temperatures ranging from 0 to 35.5 °C, resulting in savings in daily energy expenditure of up to 89% [74], which is significantly higher than energy savings noted from laboratory-based experiments [75].

It is unclear whether the reductions in dunnart body temperature that we measured resulted from impaired thermoregulatory capacity due to pesticide-induced interference with cholinergic innervation of the hypothalamus or represents an energy conservation response initiated by a reduction in feeding for approximately 24 hours post exposure. Clearly further research is needed to examine these possibilities.

In free-living populations, most mammals are usually mobile enough to select a more thermally benign microhabitat when faced with thermoregulatory challenges. Dunnarts experiencing reductions in core body temperature would be expected to respond behaviourally by selecting a higher ambient temperature to mediate the thermoregulatory response resulting from exposure to pesticides. However, the locomotory impairments demonstrated in the current study and reinforced by previous research [36], may compromise a dunnart's ability to select a warmer temperature gradient thereby interfering with proper thermoregulation and compromising its energetic balance.

Reductions in plasma and brain AChE activities of 50% and 45% of pre-exposure levels respectively (at 24 hours after dosing) in the current study were not as pronounced as those found in *S. crassicaudata*, where suppressions of 65% (plasma AChE) and 62% (brain AChE) were observed in the 24 hours following fenitrothion exposure. Suppressions in plasma and brain AChE measured fall within the 40 - 60% threshold range indicated by previous research as being indicative of sublethal exposure and resulting in effects on animal behaviour and physiology (see [9] for a review of the literature). Interestingly, the higher dose of 90 mg kg⁻¹ fenitrothion did not elicit an increased suppression of AChE (over results presented in [36] using a dose of 30 mg kg⁻¹) suggesting AChE response in *Sminthopsis spp.* may be one of threshold exceedence as opposed to dose-dependency.

The mammalian fauna of Australia's arid zone is characterised by a high species richness of insectivores, most of which are dasyurids. These marsupials represent nearly four times the number of species found in North American deserts [80]. Within New South Wales, dasyurids have been particularly susceptible to range reductions. Three species have become extinct and three more have contracted to occupy 10 - 50% of their former ranges [81]. Two out of three extinctions took place in the arid, open plains where locust control can occur and where only one species in this region, *S. crassicaudata*, is classified as common. The application of agricultural pesticides has been identified as a threatening process in New South Wales and active management has been recommended to prevent further attenuation of the remaining species of dunnarts [81]. The vulnerable status of many of Australia's dasyurids amplifies the potential for chemical control agents to adversely affect them. It is therefore critical that more understanding is gained concerning the ability of both individuals and populations to cope with exposure to agricultural chemicals [82].

3.5 Conclusions

Conventional toxicity testing used to generate data for pesticide risk assessment tends to fix exposure time (e.g. for the determination of median lethal dose values) to quantify mortality. However, exposure duration and intensity generally determine the lethal effects of toxicants and, while statistically convenient, occurs at the expense of gaining ecotoxicologically relevant information using performance-based examination of sublethal effects [143]. The duration of reductions in locomotory function and body temperature occurring concomitantly with significant reductions in acetylcholinesterase activities in fenitrothion-exposed dunnarts exceeded the 48 h period of median lethal dose studies used to support pesticide risk assessments. Moreover the parameters

measured above suggest relatively long-lasting ecophysiological impairments will be experienced by free-living dunnarts exposed to locust spray operations and should be considered when assessing the risk posed by pesticides to the environment.

Chapter 4: Conclusions and potential future research directions

Very little is known about the effects of carbamate and OP insecticides on Australian native vertebrate populations and, particularly, their ecotoxicological significance. Such effects are difficult to assess on wild species as pesticide intake rates are hard to quantify in free-ranging terrestrial animals and suitable reference levels in control animals are difficult to establish under free-living conditions [1]. Ultimately, the consequences of exposure to organophosphorus pesticides in free-living vertebrates need to be correlated with depression of acetylcholinesterase activity or with behavioural, physiological, or biochemical biomarkers to evaluate the broad-scale impacts of these insecticides on wildlife [9].

The ubiquitous presence of acetylcholine in vertebrates and the importance of acetylcholinesterase in the normal function of vertebrate central and peripheral nervous systems, makes it challenging to select the most functionally important biomarkers when studying the adverse effects of anticholinesterase pesticides. Numerous physiological systems can be affected by pesticide exposure and these effects can manifest themselves ecologically in many ways depending on the species, habitat and the quantum and duration of exposure. Consequently, locomotory and thermogenic performance were chosen as biomarkers of pesticide effects in the current study, as these traits require functional integrity of many vital systems and are also ecologically relevant.

Of the numerous mammal families in Australia, the Dasyuridae are the most likely to be affected by pesticide exposure resulting from locust spray operations. A significant overlap in habitat preferences of *Chortoicetes terminifera* (Walker 1870) and *Sminthopsis macroura* (Gould 1845), the combination of small body mass (some as low as 7 g) and high metabolic requirements, a primarily insectivorous diet and the ability to

gorge feed on intoxicated locusts make this species vulnerable to the effects of chemically based locust control [2]. Central to the acceptance of OP pesticides is laboratory evidence demonstrating a high insecticidal potency and low mammalian toxicity because of their rapid biotransformation within mammalian bodies [4]. In Australia, the registration of fenitrothion as a pesticide for locust control gained approval based on overseas environmental research [5]. Although the physiological pathways for anticholinesterase effect of OP pesticides in metatherian and eutherian mammals would be expected to be similar mechanistically, studies quantifying the sensitivity of marsupials to this group of pesticides have been lacking.

In *Chapter 2*, I quantified the acute oral toxicity of fenitrothion to *S. crassicaudata* (Gould 1844) and *S. macroura*, identifying median lethal dose values of 129 mg kg⁻¹ (95% confidence interval [CI] = 74.2 – 159.0) and 97 mg kg⁻¹ (95% CI = 88.3 – 120.0), respectively. These values are 10 - 14 times lower than values reported for a similar-sized eutherian mammal, *Mus musculus* (L. 1758), and lower than all previously reported mammalian LD₅₀ values. I suggested that such wide interspecific variation in sensitivity to fenitrothion may be a consequence of underlying differences in the metabolic pathways or their capacities for fenitrothion detoxification in mammals. Specifically, reductions in oxonase activity during the second stage of hepatic detoxification, as has been identified in weanling rats [123], may account for a lower capacity to detoxify physiologically relevant concentrations of the OP-oxon after exposure [123].

The unexpectedly high sensitivity of these two Australian marsupial species to fenitrothion emphasises the importance of adequately evaluating the risks of pesticides to endemic Australian fauna. The significance of lower acute oral toxicity values rests in their impact on standardised risk assessments by lowering the hazard values

calculated from species sensitivity distributions. As shown in *Chapter 2*, the fenitrothion hazard value calculated from a species sensitivity distribution that includes median lethal dose values for *S. crassicaudata* and *S. macroura* is reduced by nearly 50%, which, were it adopted by regulatory agencies, would reduce residue levels considered acceptable for fenitrothion use patterns.

The duration of reductions in locomotory function and changes in dunnart rectal temperatures identified in *Chapter 3*, occurring concomitantly with statistically significant acetylcholinesterase suppressions as a consequence of fenitrothion exposure, exceeded the 48 h period of medial lethal dose studies used to support pesticide risk assessments. Locomotory performance was reduced by up to 80% in the 24 h immediately following pesticide exposure. Although this reduction in running ability returned to predose levels by 7 d after dosing, the ability of free-living dunnarts to forage, escape predators, maintain home ranges and reproduce would have been reduced during this period. Dunnarts inhabiting Australia's arid zone have life history strategies that seek resource-rich refuges during times of drought and, thus, the high mobility of Australian arid zone small mammal species is an important trait for overcoming the low productivity and variable rainfall of Australian arid ecosystems [41].

The absence of pesticide-mediated effects on oxygen consumption rates in dunnarts suggests there were no limitations on the cardiopulmonary delivery of oxygen to muscles or their uptake. Thus, the consequent reductions in locomotory performance were more likely due to compromised neuromuscular functions as a result of acetylcholine accumulation within the synaptic cleft and the subsequent desensitising of nicotinic postsynaptic receptors.

Changes to dunnart rectal temperatures measured after exposure to fenitrothion are suggestive of a transient shift in daily body temperatures that have been shown to

occur in a range of other vertebrate species after exposure to organophosphorus insecticides in previous studies [66, 69]. Because dunnart body temperatures were not measured across a 24 h period, it is impossible to tell if the effects of fenitrothion on body temperature were indicative of a diel shift in body temperature or tolerance. Rather, body temperatures within a specific time period were lower in pesticide-treated dunnarts possibly reflecting an extension of the duration and/or frequency of overnight torpor. Given that torpor is widely used as an energy conservation mechanism during periods of food shortage and low ambient temperatures [73], and even when resources are not restricted [142], there may be implications for a dunnart's energy balance after exposure to fenitrothion. Considered together with locomotory deficits experienced by dunnarts after pesticide exposure that may suppress a dunnart's ability to secure sufficient food through foraging, there is considerable potential for additional adverse effects at the population level to result from broad scale pesticide application.

4.1 Future research directions

Conventional toxicity testing used for pesticide risk assessments tends to fix exposure time (e.g. for the determination of median lethal dose values) to quantify mortality limits [143]. However, exposure duration and intensity interact in determining the lethal effects of toxicants and focussing on short-term toxicological fate in acute oral toxicity studies, while statistically convenient, limits the ecotoxicological relevance of such results [143]. The duration of pesticide effects identified in the current study exceeded the “normal” duration of standard acute toxicological assessment. When combined with an increased sensitivity and an inability to develop a physiological tolerance to pesticides used for locust control, the potential exists for pesticide exposure to have a significantly negative impact on dasyurid populations. To

better understand the impact of pesticides on Australian mammal species, more robust environmental risk assessments need to be developed that expand the current test parameters and more realistically reflect exposure patterns and responses of Australian endemic species.

4.1.1 Improved locust pesticide residue data

Central to improving pesticide risk assessments is further information on the level and distribution of pesticide residue in prey items (e.g. locusts). Insect residue data for gorge feeding predatory species is sorely lacking throughout the world [144]. Current estimates for residue levels in insects in the European Union [145] are mainly based on pitfall-trapped insects in a variety of horticultural crops. Such data are unlikely to fulfil the requirements for risk assessments of locust and grasshopper control, especially given the specific nature of these spray programs (e.g., the use of ultra low-volume pesticide formulations in arid and semiarid ecosystems that have irregular vegetation structure and high pest densities). Recent research indicates that locusts accumulate pesticide residues through secondary uptake before they become debilitated and display symptoms of pesticide intoxication [144]. As a consequence, residue levels on vegetation and/or insect prey resulting from deposition alone may underestimate exposure in vertebrate predators consuming these prey items.

4.1.2 Evaluating pesticide dietary intake rates for gorge feeding predator species

The assessment of risk resulting from pesticide exposure is complicated by the fact that previous insect collections rarely consider biases in their method of sampling. Residue studies involving insects commonly capture invertebrates in pitfall traps, which may seriously underestimate residue levels by sampling active insects only; by sweep

netting, which may overestimate the residue level on locusts from contact with contaminated vegetation; or by collecting dead insects that desiccate rapidly, thereby increasing detected mass-specific residue levels [146]. Given the ability of arid zone species such as *S. macroura* and *S. crassicaudata* to gorge feed on super-abundant locust aggregations, the accurate estimation of pesticide exposure through dietary intake forms a critical component of any estimate of risk for insecticides used in arid environments. Similarly, the potential for predatory species to avoid pesticide-contaminated prey must also be quantified.

4.1.3 Quantifying the sensitivity of other native Australia vertebrate fauna to agricultural pesticides

The much greater sensitivity of dunnarts to fenitrothion as compared to murid rodents identified in *Chapter 2* underlines the importance of quantifying the acute response to pesticides on the particular species of interest. Although experimental restrictions can necessitate the need to extrapolate from a narrow range of organisms tested under standard laboratory conditions, the results of the current study show the limitations of this approach. Given that the standard set of test species for quantifying the acute responses of vertebrates to xenobiotic compounds does not include any Australian endemic species [6], there is a clear argument for evaluating the effects of pesticides on potential non-target species that are more phylogenetically inclusive.

4.1.4 Elucidation of hepatic detoxification pathways for OPs in marsupials

Acute oral toxicity values identified in *Chapter 2* for *S. macroura* and *S. crassicaudata* were 10 - 14 times lower than those quantified for a similar sized eutherian mammal, *M. musculus*, a species commonly represented in pesticide risk

assessments. Increased sensitivity to pesticide exposure has been identified previously in weanling rats and attributed to their much lower oxonase activity compared to adults during the second stage of hepatic detoxification of organophosphorus insecticides [147, 148]. This hydrolytic step detoxifies the anti-ChE character of the OP-oxon (generated in the first stage of hepatic detoxification process by oxidation via the cytochrome P450 group of isozymes) and increases its transport to the kidneys for subsequent excretion [123]. The presence of sufficient oxonase activity to hydrolyse the oxon metabolite of OP insecticides is thus a critical step in the metabolism of OPs in mammals.

Differences in the availability or functionality of dunnart oxonases may explain why native Australian marsupials studied differ from *M. musculus* in their sensitivity to anticholinesterases and warrants further study.

4.1.5 Effects of fenitrothion on diel temperature cycles and torpor in dunnarts and implications for dunnart energy balance

The body temperature of mammals is regulated within narrow limits by particular hypothalamic nuclei which integrate thermal status via muscarinic cholinergic receptors [21] and then effect appropriate physiological and behavioural responses that help maintain a constant body temperature. Exposure to organophosphorus insecticides interferes with cholinergic innervation in both the central and peripheral nervous systems and therefore has the potential to alter an animal's ability to moderate its body temperature. One of the mechanisms available to dunnarts to deal with low ambient temperatures is torpor [72], characterised by a reduction in body temperature and metabolic rate, and widely used as an energy conservation tool during periods of food shortage and low ambient temperatures [73]. Body temperature in animals undergoing torpor is regulated at or above a "species-specific threshold" by an increase in

metabolism [60] and requires the proper functioning of cholinergic innervation to relay information on peripheral and core body temperatures to the hypothalamus [60]. Torpor is critical for the survival and reproduction of small insectivorous marsupials, such as *S. macroura*, in Australian arid zone grasslands [74]. Individual *S. macroura* have been observed to utilise torpor at temperatures ranging from 0 to 35.5 °C, resulting in up to 89% savings in daily energy expenditure [74], significantly higher than energy savings predicted from laboratory-based experiments [75]. Suppressions in cholinergic activity that interfere with the communication pathways between the hypothalamus and peripheral sensors have ramifications for energy savings in dunnarts and warrant further investigation.

Several key questions concerning the impacts of OPs on the thermoregulatory function of mammals have not attracted much research. For example, little attention has been paid to examine whether temperature regulation differs in sensitivity to ChE inhibition compared to other AChE-dependent processes, such as motor activity, behaviour, memory and performance of learned tasks.

4.1.6 The role of “tolerance” of muscarinic AChE receptors and the potential for inter-generational effects of OP exposure in mammals

Previous studies (reviewed in [19]) have reported reductions in both the binding affinity [148] and density [149] of muscarinic AChE receptors (mAChRs) following exposure to organophosphorus insecticides. Such tolerance to anti-cholinergic agents can develop rapidly. Dose-dependent hypothermic effects in rats following administration of 10 and 20 mg kg⁻¹ pilocarpine (an AChE muscarinic receptor agonist) were reduced or eliminated with subsequent treatments of diisopropylflourphosphate (DFP) up to 6 h after the initial dose [150]. While it may be reasonable to expect

thermoregulatory effects to be transitory after OP exposure and to resume normal function once acetylcholinesterase activities are restored, these short-term effects have the potential to be expressed inter-generationally. For example, exposure to diisopropyl fluorophosphate (DFP) in pregnant rats has been shown to reduce the mAChR binding density in both maternal and foetal brains [151]. Although interpreted as the development of a tolerance to DFP-induced elevated acetylcholine (ACh) activity [151], alteration of mAChRs in developing progeny would likely have longer-term consequences given their roles in cholinergic innervation of cardiac and smooth muscle, proper functioning of the central nervous system and hypothalamic mediation of the endocrine system [21]. In Australia, locust spray operations occur predominantly from late spring through to early autumn and overlap strongly with the breeding cycle of numerous vertebrate species, including *S. macroura* (June-February [45]) in arid grassland ecosystems. Consequently, the potential exists for reproductively active *S. macroura* males and females to be exposed to pesticides. Unfortunately, effects of pesticide exposure on the reproductive capacity of mammals falls outside the scope of current risk assessments but is a profoundly important topic to investigate.

4.17 *Ecological implications of acute reductions in locomotory performance*

Sublethal exposure to fenitrothion in the current study suppressed locomotory function by up to 80% in the day immediately following dosing with this impairment returning to predose levels by 7 days post exposure. Reductions in locomotory performance after exposure to fenitrothion have been identified previously in *S. crassicaudata* [36] as well as in mice and rats exposed to another organophosphorus insecticide, dimethoate [134]. Suppression of locomotory function is likely to be ecologically important as it could adversely affect activities such as territory defence,

home range maintenance, foraging and animal-animal interactions necessary for maintaining normal population dynamics (e.g. aggressive home range defence, locating reproductively viable mates). Moreover, there are no extant data examining how these ecological parameters differ between species, or in how they are affected relative to level of exposure to particular pesticides. This warrants a field-based study examining the effects of pesticide exposure on life-history attributes of free-living animals using an experimental manipulation of pesticide dosage.

In conclusion, the application of agricultural pesticides has been identified as a threatening process to native mammals in New South Wales and active management has been recommended to prevent further population declines in the remaining species of dunnarts [81]. The status, therefore, of Australia's dasyurids amplifies the potential for pesticides to adversely affect these species, making it even more urgent to gain further knowledge concerning the ability of both individuals and populations to deal with the application of agricultural chemicals.

The assessment of agricultural and veterinary chemicals for registration in Australia is an evolving process, as both the amount of data submitted to support registrations increases and assessment methodologies and detection levels improve [29]. The current study has revealed that exposure of *S. macroura* to fenitrothion at levels an order of magnitude lower than known to be lethal in similar-sized murid rodents compromises their locomotory capacities for up to a week. This demonstrates that responses of Australian native species to pesticide exposure will likely fall outside scenarios encompassed by the current risk assessment framework used for pesticide applications. Consequently, there is an urgent need to evaluate the effects of pesticides on Australian endemic species, that differ phylogenetically from the standard range of

species represented in environmental risk assessments, and to ensure that such studies fully evaluate ecologically relevant endpoints in concert with laboratory-based investigations.

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Glossary

ACh	Acetylcholine. A neurotransmitter necessary for the proper functioning of cholinergic innervation in both the central and peripheral nervous systems.
AChE	Acetylcholinesterase. An enzyme used to hydrolyse acetylcholine within the synaptic cleft and modulate cholinergic impulses.
ANOVA	Analysis of variance.
AOT	Acute oral toxicity. Adverse effects of exposure to a xenobiotic substance, usually occurring within 14 days of exposure.
APLC	Australian Plague Locust Commission
AChRs	Acetylcholine receptors.
cAMP	Cyclic adenosine monophosphate. An intracellular second messenger derived from adenosine triphosphate (ATP).
cGMP	Cyclic guanosine monophosphate. A intracellular second messenger analogous to cAMP but affecting different physiological systems (e.g. signal transduction pathway involved in vision).
ChE	Cholinesterase.
CI	Confidence interval.
CNS	Central nervous system.
CO₂	Carbon dioxide.
DFP	Diisopropyl fluorophosphate is an organophosphorus insecticide, usually an oily, colorless liquid (chemical formula C ₆ H ₁₄ FO ₃ P).

Hb	Hemoglobin
HD₀₅	Hazard value (5%). Fifth percentile of a species sensitivity distribution that can be selected as a concentration considered protective of most species (i.e. the remaining 95%) in a community. Commonly used as an environmental quality criterion in risk assessments.
HSD	Honest(ly) significant difference. A multiple comparison statistical test used on raw data or in conjunction with an ANOVA (post-hoc analysis) to find means that are significantly different from each other.
IMR	Integrated metabolic rate calculated as the total amount of oxygen consumed throughout the 60 min period employed for shivering thermogenesis measures.
IUCN	International Union for the Conservation of Nature.
K⁺	Potassium.
LD₅₀	Median lethal dose of a toxin, radiation or pathogen required to kill 50% of a tested population after a specified test duration.
nAChRs	Nicotinic acetylcholine receptors.
mAChRs	Muscarinic acetylcholine receptors.
O₂	Oxygen.
OECD	Organisation for Economic Cooperation and Development.
OP	Organophosphorus.
OC	Organochlorine.
PMR	Peak metabolic rate.

PMR_{run}	The maximal metabolic rate achieved during running endurance measures, calculated as the highest continuous 2 min instantaneous \dot{V} O ₂ recorded while running.
PMR_{therm}	The maximal metabolic rate achieved during shivering thermogenesis measures, calculated as the highest continuous 2 min instantaneous \dot{V} O ₂ recorded during the cold challenge.
PNS	Peripheral nervous system.
SSD	Species sensitivity distribution used to graphically represent ranked percentile lethal dose data against the log of the corresponding dose for a xenobiotic compound. Used within probabilistic risk assessments to estimate protective hazard values.
T_a	Ambient temperature.
T_b	Body temperature measured as rectal temperature.
T_{b in}	Dunnart rectal temperature measured before commencement of running endurance or shivering thermogenesis experiments.
T_{b out}	Dunnart rectal temperature measured after commencement of running endurance or shivering thermogenesis experiments.
TChE	Total cholinesterase.
TMM	Time taken for each dunnart to reach it's maximal metabolic rate during shivering thermogenesis measures.
UDP	Up-and-Down Protocol (OECD Guideline 425). Alternative test to traditional LD ₅₀ assays used to determine the median lethal dose of a toxin, radiation or pathogen with a minimum numbers of animals

required to resolve the value.

VO₂ Rate of oxygen consumption during running endurance or shivering thermogenesis measures.

VO_{2 max} Maximal rate of oxygen consumption during running endurance or shivering thermogenesis measures.