Pesticide exposure in free-living native birds and the effects of acute dosing of fenitrothion and fipronil on physiological performance in selected species

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"This thesis is presented as part of the requirements for the Award of the Degree of Doctor of Philosophy of the University of Wollongong"

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CERTIFICATION

I, Karen Josephine Fildes, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

ABSTRACT

Chapter 1

This chapter introduces the main classes of pesticides with a brief overview of their mode of toxic action. Cholinesterase inhibiting chemicals and their known effects on birds are reviewed in detail. The benefits and limitations of the use of lethal compared to sublethal toxicological endpoints to assess pesticide impacts on avian species in toxicological research are discussed. It is argued that there is a critical need for investigations of sublethal effects to consider biochemical and physiological components of ecologically relevant traits. The use of pesticides for locust control, particularly fenitrothion and fipronil, by the Australian Plague Locust Commission, is discussed. The chemical properties and mode of toxic action of these two pesticides are reviewed and the potential impact of their application on Australian native bird species is assessed.

Chapter 2

Cholinesterase (ChE) inhibiting pesticides are applied throughout Australia to control agricultural pests. Blood plasma ChE activity is a sensitive indicator of exposure to organophosphorus insecticides in vertebrates. To aid biomonitoring and provide reference data for wildlife pesticide-risk assessment, plasma acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were characterised in nine native bird species: brown songlarks (*Cinclorhamphus cruralis*), budgerigars (*Melopsittacus undulatus*), clamorous reed warblers (*Acrocephalus stentoreus*), double-barred finches (*Taeniopygia bichenovii*), king quails (*Coturnix chinensis*), Richard's pipits (*Anthus novaeseelandiae*), white-plumed honeyeaters (*Lichenostomas penicillatus*), willie wagtails (*Rhipidura leucophrys*) and yellow

throated miners (*Manorina flavigula*). The plasma of all species contained AChE and BChE except in king quail where no AChE was present. The lowest detectable plasma AChE activity was 0.10 μmols/min/ml in budgerigars and the highest was 0.86 μmols/min/ml in clamorous reed-warblers. BChE activity in the plasma ranged from 0.37 in double-barred finches to 0.90 μmols/min/ml in white-plumed honeyeaters and clamorous reed-warblers. The lowest proportion of AChE was found in budgerigars (12.8%) and highest in willie-wagtails (67.8%). Apart from king quail AChE activities in all species were within the range reported for other avian species. The absence of AChE in king quail has not previously been reported for any bird species.

The effect of sampling time on plasma ChE was assessed in budgerigars and zebra finches (*Taeniopygia guttata*) and seasonal effects were examined in zebra finches. No diurnal variation in ChE activity was found at any time of day in either species although there was a significant difference in all ChE activity between seasons in zebra finches.

Chapter 3

Huge aggregations of flightless locust nymphs pose a serious threat to agriculture when they reach plague proportions, but provide a very visible and nutritious resource for native birds. Locust outbreaks occur in spring and summer months in semiarid regions of Australia. Fenitrothion, an organophosphate pesticide, is aerially sprayed to control locust plagues. To evaluate fenitrothion exposure in birds attending locust outbreaks, we measured total plasma cholinesterase (ChE), butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activities in four

avian species captured pre- and post-fenitrothion application and ChE reactivation in birds caught post spray only. Eleven of 21 plasma samples from four species had ChE activity below the diagnostic threshold (two standard deviations below the mean ChE activity of pre-spray samples). Granivorous zebra finches (*Taeniopygia guttata*) and insectivorous white-winged trillers (*Lalage sueurii*) had significantly lower mean plasma total ChE, BChE, and AChE activity post-spray, while two other insectivores, white-browed (*Artamus superciliosus*) and masked woodswallows (*Artamus personatus*), did not. Cholinesterase was reactivated in 19 of the 73 plasma samples collected, and in one of three brain samples. We conclude that native bird species are exposed to fenitrothion during locust control operations. This exposure could have detrimental impacts as locust outbreaks and avian reproductive events are both stimulated by heavy summer rainfall, leading to co-occurrence of locust-control and avian breeding activities.

Chapter 4

The effect of fenitrothion exposure on birds was examined by measuring aerobic metabolism, blood haemoglobin (Hb) content, plasma cholinesterase activities and body mass for up to 21 d post-dose. Peak metabolic rate (PMR) was measured in a flight metabolic chamber in three dose groups of house sparrows (*Passer domesticus*) (100 mg/kg = high, 60 mg/kg = medium, 30 mg/kg = low), and one dose group each of zebra finches (*Taeniopygia guttata*) (3 mg/kg) and king quails (*Coturnix chinensis*) (26 mg/kg). Aerobic metabolism was measured during a 1 h exposure to sub-freezing thermal conditions in low dose house sparrows and king quails (26 mg/kg). Fenitrothion had no effect on metabolic rate during cold exposure or on Hb at any time. By contrast, aerobic performance during exercise in sparrows was

reduced by 58% (high), by 18% (medium), and by 20% (low) 2 d post dose. House sparrows (high) had the longest recovery period for PMR (21 d) and plasma cholinesterase (ChE) activity (14 d). House sparrows (high) and treated king quails had significantly lower body weight at 48 h post-dose whereas body mass was invariant in zebra finches and house sparrows (medium and low). Cholinesterase was maximally inhibited at 6 h post dose, and had recovered within 24 h, in house sparrows (low), king quails and zebra finches. Exercise PMR in zebra finches and king quails was reduced by 23% at 2 and 3 d post-dose, respectively, despite these birds being asymptomatic in both behaviour and plasma ChE activities.

Chapter 5

We examined the sublethal effects of the fipronil based pesticide Adonis 3UL ® insecticide on birds by measuring exercise induced peak metabolic rate (PMR) in zebra finch (*Taeniopygia guttata*) and king quail (*Coturnix chinensis*), and during a 1-h exposure to sub-freezing conditions in king quail. Exercise induced peak metabolic rate was measured in zebra finch pre-dose and at one, two, ten and twenty days after treated birds ingested 17.5 mg/kg Adonis 3UL ® mixed with canola oil and control birds received canola oil alone. Peak metabolic rate measurements were taken during exercise pre-dose and two, six and fourteen days after king quail received 30 mg/kg Adonis 3UL ® or canola oil alone. Peak metabolic rate after was not affected by fipronil in Adonis 3UL ® or by sham treatment in birds of both species. We conclude that the administered sublethal dose of fipronil did not affect exercise performance in zebra finch or in king quail nor was there evidence of fipronil induced thermoregulatory effects in king quails.

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1 INTRODUCTION

1. 1 Pesticides

The economic, safety and health benefits that result from the use of pesticides are numerous and pervasive (Cooper and Dobson 2007), however so too are the associated risks. Through bioaccumulation and the potential to contaminate air and water the unintentioned adverse effects of pesticides can be far reaching (Everts 1997). Further, species throughout the animal kingdom share many basic processes in their neurological functions. Hence, the neurotoxic actions of many pesticides can adversely affect a wide range of organisms via similar mechanisms, and thus, are potentially biocidal (Shankland 1976). The importance, therefore of well-informed management decisions regarding pesticide use cannot be underestimated. To this end, there is an urgent need to gain a comprehensive understanding of the true ecological consequences of pesticide exposure in non-target organisms. The primary aim of this dissertation is to contribute to this understanding through toxicological research that integrates biochemical and physiological characterisation of animals before and after pesticide exposure in the laboratory and in the field.

The main classes of insecticides that are currently applied throughout the world include the pyrethroids, organochlorines, organophosphates and carbamates, all of which are neurotoxicants (Newton 1998). Pyrethroids historically were extracted as pyrethrins from chrysanthemum flowers. Over the past two decades many synthetic pyrethrin-like materials have become available. Because pyrethroids are potent insecticides with low mammalian toxicity and are stable in sunlight, they have become widely used (Heinzow 1996). They exert their neurotoxic action by affecting

sodium channels in nerve membranes resulting in prolonged sodium influx during neural stimulation, which in turn, causes repetitive neuronal discharge and hyperexcitation (Wolansky 2007).

Organochlorine (OC) insecticides, also known as chlorinated hydrocarbon compounds, were extensively used in the mid 1940s to 1960s (Gard and Hooper 1995). Dichlorodiphenyltrichloroethane (DDT) is one of a group of OCs that interact with neuronal membranes by altering their permeability for potassium, sodium and calcium-mediated processes. Other OCs, specifically cyclodienes and cyclohexane compounds, antagonantagonise the neurotransmitter γ–aminobutyric acid (GABA). When OCs inhibit these functions, repolarisation of nerves is disturbed resulting in uncoordinated nervous excitation (Heinzow 1996).

The physicochemical properties of organochlorine compounds render them environmentally persistent, further accentuating their potential to bioaccumulate in body fat and persist in food chains due to their lipophilicity and slow metabolic degradation (Heinzow 1996, Klemens et al. 2003). These compounds also have strong estrogenic and enzyme-inducing properties that interfere with vertebrate reproductive functions (Jaspers et al. 2005). Furthermore, the volatility of OCs at relatively moderate temperatures makes their effects more widespread than initially believed. For example, DDT is volatile at temperatures found in warm regions, but condenses in high-latitude locations due to their lower temperatures. Consequently OCs applied at lower latitudes can transfer to, and accumulate in, wildlife species of colder, high-latitude areas due to this global distillation process (Heinzow 1996). Because of their environmental persistence and long-term negative impacts on

reproduction, OCs have been banned in most developed nations but are still used extensively in some developing countries (Klemens et al. 2003, Gard and Hooper 1995, Riseborough 1986).

In Europe, North America and Australia, the organophosphate (OP) and carbamate pesticides have largely replaced the organochlorines and are currently the most widely used pesticides internationally (Elliott et al. 1996). Organophosphates, as a class, include all insecticides containing phosphorus, and are toxic to both invertebrates and to vertebrates (Grue et al. 1997). Their mode of action is the inhibition of acetylcholinesterase (AChE), the hydrolytic enzyme necessary for termination of cholinergic synaptic transmission (Murphy et al. 1986). Carbamate insecticides, derivatives of carbamic acid, also inhibit AChE.

Acetylcholine (ACh) is one of the most ubiquitous neurotransmitters, occurring in both invertebrates and vertebrates, where it activates processes centrally in the brain and peripherally, notably at the neuromuscular junction (Habig and Giulio 1991, Shankland 1976). ACh is released into the synaptic cleft and binds transiently to membrane receptors that initiate an excitatory response in the receiving cell. Acetylcholinesterase, produced by postsynaptic cells and available at ACh receptor sites (on neurons or muscle cells), catalyzes the hydrolytic breakdown of ACh to its component compounds acetate and choline, thus facilitating rapid removal of the neurotransmitter from the cholinergic synapse, making the ACh receptor available for further stimulation (Michelson and Zeimal 1973). Acetylcholinesterase has a high specificity for ACh and is the functionally dominant cholinesterase (ChE) for neural

and neuromuscular functions, whereas butyrylcholinesterase (BChE), is not involved in neural transmission but often co-occurs in blood (O'Brien 1976).

When AChE is inhibited by anticholinesterase compounds, there is a build up of ACh at the synapse leading to a disruption of normal nervous system function (Thompson et al. 1991). It is well established that OPs inhibit cholinesterase activity by reacting with a specific serine within the catalytic centre of the enzyme to produce O, O-dialkyl phosphoserine and in this phosphorylated form the enzyme is unable to hydrolyze choline esters (Kennedy 1991). Although phosphorylated ChE is inactive, it can spontaneously dephosphorylate to its active form. This permits restoration of cholinergic functions, particularly muscarinic ones, in OP-exposed animals but at a very slow rate. The rate of dephosphorylation can be increased pharmacologically by exposing phosphorylated ChE to pyridine-2-aldoxime methiodide (2-PAM) (Wilson et al. 1992). This method can be used to determine the extent of exposure to OPs by comparing plasma or brain ChE activity before and after incubation with 2-PAM.

Many tissues in the body are innervated cholinergically and in vertebrates cholinergic receptor sites are located on all autonomic ganglia, cardiac muscle, smooth muscle, glands and central nervous system cell bodies and dendrites (Michelson and Zeimal 1973). Thus, pesticides inhibiting AChE can affect a myriad of physiological and behavioural processes (Grue et al. 1997). Major organs affected by AChE inhibition, and the resultant disruption to cholinergic transmission, are divided into those with muscarinic ACh receptors and nicotinic ACh receptors (Grue et al. 1997). Muscarinic receptors are found on effector cell membranes associated with, among other tissues, endocrine glands, cardiac muscle and smooth muscles in

the lungs and gastrointestinal tract (Grue et al. 1997). With the inhibition of ACh, responses at the muscarinic receptors are enhanced and their persistence increased with hyperexcitation (which in the case of the lungs results in bronchial constriction) or inhibition (for example vasodilation) (Grue et al. 1997). Overall physiological symptoms caused by hyperexcitation of the muscarinic receptors include increased saliva and mucus production, increased heart rate while contraction force is decreased, bronchial and respiratory muscle over-stimulation, and subsequent paralysis. Acetylcholinesterase also plays an important role in terminating the action of ACh on nicotinic receptors and is therefore highly concentrated at the neuromuscular junction. Disruptions to this process will result in hyperexcitation of the muscle leading to continuous contraction, followed by inhibition and eventual neuromuscular transmission failure and resultant muscle paralysis (Panenic et al. 1999). Therefore acute toxicity resulting from inhibition of AChE and its inability to hydrolyze AChE can lead to death by respiratory failure and/or cardiovascular arrest. Alternatively nonlethal exposure to anti-ChE compounds can lead to a variety of deleterious sublethal effects that can alter behaviour and compromise physiological well-being due to combined effects at both muscarinic and nicotinic receptors (Panenic et al. 1999).

The measurement of an animal's blood or tissue ChE activity is extensively used to diagnose exposure of animals to organophosphorus or carbamate insecticides (Fairbrother et al. 1991). Diagnostic work in wildlife toxicology has primarily utilised a colorimetric method developed by Ellman et al. (1961) that exploits the reaction of acetylthiocholine iodide (AThCh) with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), yielding a coloured end-product detectable spectrophotometrically. Blood

and tissues, including brain and muscle, have been used effectively to diagnose OP and carbamate exposure in the field (Goldstein et al. 1999a, Hunt et al. 1995, Wilson et al. 1991, Hooper et al. 1989) and to investigate ChE inhibition effects in laboratory studies (Table 1.2).

1. 2 Pesticide effects on birds

Bird abundance has been widely used as an indicator of environmental degradation, particularly in Britain where records of avian population numbers have been kept for centuries by recreational bird watchers (Hardy et al. 1987, Newton 1998). Widespread monitoring of bird populations during the past 30 years has shown that most of the avian species typically found on British farmland have declined (Newton 2004). A similar pattern has been identified in Western Europe (Donald et al. 2002) and North America (McLaughlin and Mineau 1995). This monitoring together with appropriate research and experimentation has played a crucial role in highlighting some of the long-term consequences of intensive agricultural practices including increased pesticide use.

Agricultural chemicals have been confirmed as a major factor in reducing the entire spectrum of biodiversity in farmed landscapes through both direct and indirect effects (Newton 1998). Pesticides have obvious direct effects on insect pests as well as non-target insect populations. Non-target vertebrates can also be directly affected by agricultural chemicals through exposure via oral, dermal and respiratory pathways. Such exposure can result in a wide range of physiological and behavioural effects depending on the dose and the toxicity of the compound (Newton 1995). In addition, vertebrates that prey on insects, including pest species, are affected indirectly by reduced food availability following pesticide application (Hart et al.

2006, Peveling 2003a).

Direct effects of pesticide exposure can be divided broadly into lethal and sublethal categories. A sublethal effect refers to any alteration in behaviour or physiology that could ultimately compromise an individual's ability to survive and/or reproduce (Newton 1998). The assessment of potential toxic impacts of pesticide exposure on non-target wildlife involves measuring dose-related effects of a given pesticide on mortality (lethal effects) or on functions such as reproduction, endocrine, immune, thermoregulation, or behavioural traits (non-lethal effects).

1.2.1 Avian mortality as an endpoint in toxicological research

Mortality caused by organophosphate and carbamate insecticides has been reported for numerous bird species (Table 1.1; Henny et al. 1998). A well documented mass mortality event occurred in Argentina, where approximately 6000 Swainson's hawks (*Buteo swainsoni*) were discovered dead between 1995 and 1996 (Goldstein et al. 1999b). The deaths were attributed to poisoning by monocrotophos that had been used to control grasshopper outbreaks. In the period 1985-1995 there were 520 incidents of raptor mortality resulting from ChE-inhibiting pesticides in the U.S.A., U.K. and Canada (Mineau 1999). Secondary poisoning of raptors from granular insecticides was reported in 1990 in the Fraser Delta, Canada, many months after the chemicals had been applied (Elliott et al. 1996). Similarly, deaths of black-billed magpies (*Pica pica*) and red-tailed hawks (*Buteo jamaicensis*) were attributed to exposure to famphur, an organophosphate insecticide, up to three months after cattle had been treated for cattle warbles (Henny et al. 1985). Interestingly, magpie populations declined in the western states of U.S.A. between 1968 and 1979, which corresponds with the widespread use of famphur (Henny et al. 1985). Magpies and

other species of birds were also found dead after cattle were treated with famphur in the U.K (Felton et al. 1981). Further, Franson and colleagues (1996) attributed deaths of red-tailed hawks from 1975 through to 1992 to carbamate or organophosphate pesticides.

As a consequence of mortality incidents, the focus of early ecotoxicological research was aimed at establishing non-lethal application levels. Initial field investigations were primarily aimed at identifying mortality associated with pesticide use and laboratory studies were focused on the lethal potential of specific chemicals (Hooper and La Point 1994). It is important to note that pesticide-induced mortality assessment is relatively rare due to the intensive labour required to conduct widespread carcass counts post application. Furthermore, mortality estimates at sites of pesticide application may underestimate mortality due to the rapid mobility of birds.

The toxicity of chemicals is assessed on avian species generally through the use of the up and down method of pesticide dosing to estimate an LD50 (lethal dose for 50% of subjects) (Australian Pesticides and Veterinary Medicines Authority: formerly National Registration Authority (NRA) 1999; National U.S. Environmental Protection Agency 1993). The LD50 is a statistically derived single dose of a chemical expected to cause 50% mortality in a given population under specific experimental conditions (Duffus 1996). Obviously, potential pesticides cannot be tested on all species that may be exposed once they come into general use, therefore surrogate species such as Northern bobwhite quail (*Colinus virginianus*) and mallard ducks (*Anas platyhynchos*) or laboratory rats and mice are used and presumed to be

representative of free-living counterparts. The dominant form of laboratory studies assessing wildlife toxicity has involved establishing LD50s for a few representative, usually captive species, and investigating exposure through the use of biomarkers (Hooper and LaPoint 1994).

The establishment of lethal endpoints for non-target species and identification of mechanisms of toxicity to a given pesticide are obviously important components of risk-assessment studies, but sublethal effects in ecological contexts are equally, if not more important. Studies using mortality as an endpoint are not designed to assess the ecological consequences of sublethal effects. This is primarily due to the uncertainty surrounding field exposure levels and apparent interspecific differences in susceptibility to the same pesticide. Exposure under field conditions is usually accompanied by a wide range of biotic and abiotic parameters that are not considered in typical toxicological assessments. For example, Mineau et al. (1994) analysed 134 avian reproduction studies conducted on northern bobwhite and mallard ducks which are the two model species used in reproductive assessment required by the USEPA (USEPA 1982). The studies involved 69 different chemicals, 19 of which were found to cause developmental effects at levels lower than those giving rise to detectable parental toxicity. These results emphasise the importance of determining doses giving rise to sublethal effects instead of basing pesticide application on consideration of LD50s alone.

Despite known limitations in their pertinence to ecological contexts, assessments of wildlife chemical toxicity have been dominated by studies determining percent mortality over a standardised interval of time. Laboratory investigations to assess

toxicity usually involve LD50 studies conducted with standard species that are usually domestic strains (Heinz 1989). Field studies are conducted in restricted areas over short periods of time as the development of large scale field research for the assessment of hazards to wildlife is an expensive and time consuming process (Greig-Smith 1991, Hooper et al. 1990). In order to gain a comprehensive understanding of interactive environmental effects on survivorship of pesticide-exposed animals, it is necessary to conduct studies that involve a refined integration of field and laboratory exposure assessments. However such investigations are again expensive and time consuming. Consequently, potential effects on non-target wildlife are often poorly understood when a chemical is approved by government agencies for agricultural use.

Table.1-1 Organophosphate and carbamate (anticholinesterase) pesticides used for agricultural purposes that have resulted in mass mortalities of bird species worldwide (*n* = number of individuals found dead)

Pesticide	Agricultural Use	*n*	Birds	Reference
Carbofuran		14	Great egrets (*Nycticorax nycticorax*), Great blue herons (*Ardea herodias*), Black-crowned night herons (*Casmerodius albus*)	(Hunt et al. 1995)
Diazinon, Dasanit, Dursban, Carbofuran and Parathion	Applied to turf and crops	>400	Various species	(Stone 1979)
Diazinon	Applied to turf	57	Canada geese (*Branta Canadensis*)	(Frank et al. 1991)
Famfur and Fenthion		8	Bald eagles (*Haliaeetus leucocephalus*) Great horned owl (*Bubo virginianus*) Red-tailed hawks (*Buteo jamaicensis*)	(Henny et al. 1987)
Famfur	Against cattle warbles, U.S.A.	38	Black-billed magpie (*Pica pica*)	(Henny et al. 1985a)
Fensulfothion	Against pasture pests, New Zealand	394	White-backed magpie (*Gymnorhina tibicen*), Black-backed gull (*Larus dominicanus*), Harrier Hawk (*Circus approximans*)	(Mills 1973)
Monocrotophos	Against voles in alfalfa, Israel	400	Raptors	(Mendelssohn and Paz 1977)

(Table 1-1 Continued)

Monocrotophos	against grasshoppers, Argentina	6000	Swainson's hawks (*Buteo swainsoni*)	(Goldstein et al. 1999b)
Monocrotophos	Illegally used on rice Louisiana, USA Texas, USA	>100	Various species of waterfowl	(White and Mitchell 1983)
Monocrotophos	Illegally used on rice, Louisiana	1100	Various species of songbirds and waterfowl	(Flickinger et al. 1984)
Parathion	Against worms on vegetables	>200	Laughing gulls (*Larus atricilla*)	(White et al. 1979)

1. 3 Considerations of sublethal effects in toxicological research

The effects of pesticides on wildlife are considered to be of concern when there is clear evidence that exposure results in mortality or has adverse effects on reproductive potential (Heinz 1989). In the case of ChE-inhibiting pesticides, however, the disruption to cholinergic synaptic transmission can cause biochemical effects in many body tissues, resulting in physiological perturbations and/or behavioural changes that can indirectly result in mortality or reduce reproductive potential even at sublethal levels of exposure. The complexity of biochemical interactions at the physiological level, together with the complexity of interactions between a given individual and its environment, make sublethal effects more difficult to quantify than direct lethal effects. Consequently such investigations are much less common than those concerning mortality.

Toxicological studies concerned with ecologically relevant pesticide effects must examine physiological and behavioural responses that could compromise survival and reproduction in free-living birds. For example, sub-lethal exposure to anti-ChE pesticides can adversely affect thermoregulation (Grue et al. 1997). The interaction

between low temperatures and pesticide toxicity is often associated with a decrease in ability to withstand the cold (Martin and Solomon 1991, Maguire and Williams 1987a). Maintenance of a stable body temperature requires a dynamic balance between rates of heat production and heat loss (Maguire and Williams 1987b), which are regulated by hypothalamic control centres that effect muscular thermogenesis during exposure to cold and cooling mechanisms that are activated when hot (Gordon 1994). Because anti-ChE activity perturbs cholinergic functions, it can potentially affect thermoregulatory responses to the cold directly via their effects on hypothalamic neural functions (Grue et al. 1991, Hissa and Rautenberg 1975), and indirectly through inhibitory effects on shivering by skeletal muscles (Bicudo et al. 2001). In addition, OP pesticides are associated with short-term anorexia (Grue et al. 1982), which would deplete energy reserves and further reduce thermoregulatory capacity.

Exposure to cold has been demonstrated to amplify the toxicity of the OP parathion in northern bobwhite quail and American kestrels (*Falco sparverius*) (Rattner et al. 1987, Rattner and Franson 1984). Similarly, the mortality rate of cold exposed mallard ducklings in a group fed 100 ppm (parts per million) OP pesticide was 85% compared to 32% mortality in untreated controls (Fleming et al. 1985). Chlorpyrifos mortality was also amplified by cold stress in bobwhite quail (Maguire and Williams 1987b) and body temperature was significantly lower in zebra finches (*Taeniopygia guttata*) dosed with 11.36 mg/kg fenitrothion (Holmes 1988). In all these studies thermoregulatory capacity was impaired by anti-ChE compounds and the compound's toxicity increased with decreasing ambient temperature.

Locomotor performance is another ecologically relevant physiological trait observed to be adversely affected by exposure to sublethal levels of ChE-inhibiting pesticides. Deficits in locomotory speed and endurance have been observed in two mammalian species and a decline in locomotory speed and agility has been found in five reptilian species as a result of sublethal OP exposure. Experiments conducted with fat-tailed dunnarts (*Sminthopsis crassicaudata*) found that running endurance declined by over 50% for up to five days after dosing with 30 mg/kg fenitrothion (estimated LD50 was 129 mg/kg; Buttemer et al. 2008) and dietary exposure to carbaryl in poison baits for seven consecutive days reduced running speed in the meadow jumping mouse by up to 50% (*Zapus hudsonius*) (Punzo 2003). Malathion at 200 mg/kg had no effect on sprint velocity of western fence lizards (*Sceloporus occidentalis*) (Holem et al. 2006) but 25 mg/kg carbaryl exposure slowed sprint speed and impaired arboreal climbing performance (DuRant et al. 2007). Carbaryl also slowed swimming velocity in swamp snakes (*Seminatrix pygaea*) and diamondback water snakes (*Nerodia rhombifer*) (Hopkins et al. 2005). Inhibition of ChE caused by sublethal exposure to anti-ChE compounds is thought to cause accumulation of ACh at the neuromuscular junction with transient over excitation and later neuromuscular transmission failure (Panenic et al. 1999). The effect of anti-ChE compounds on locomotory performance may be due to these changes at the neuromuscular junction and be a generalised effect of anti-ChE compounds.

A decline in physiological performance could be significantly detrimental to avian species under free-living conditions. Because flight requires a large aerobic capacity (Chappell et al. 1999) and has a high-energy requirement, a reduction in flight performance could restrict foraging ability and energy uptake and, through decreased

nutrition and resulting muscle catabolism, could create a feedback loop that further reduces locomotory ability. The resulting reduction in food consumption and consequent depletion in energy reserves can affect an individual's ability to escape predators, migrate, find shelter and/or thermoregulate effectively (Table 1.2.) (Martin and Solomon 1991, Patnode and White 1991). Thus, it is imperative that sublethal effects of pesticides be evaluated in the context of physiological and behavioural traits that are needed for free-living animals to survive and breed successfully.

Physiological compromise caused by sublethal exposure to anti-ChE compounds can also result in changes to behaviours that are critical for reproductive success and the survival of young and thus impact at the population level (Ortego et al. 2007). Although low doses of ChE-inhibiting compounds can cause short-term hyperactivity in both birds and mammals (Grue et al. 1997), the most frequently documented behavioural effect of intoxication is decreased overall activity levels. Laboratory investigations have shown depression in behaviours such as flying, singing, preening, foraging and food consumption following anti-ChE exposure (Table 1.2). Avian breeding systems depend upon a set of intricate and critically timed behavioural interactions, first between a male and female during courtship and mating and then between parent(s) and offspring. Thus, depending upon the time of exposure, the documented pesticide-induced changes in thermoregulatory ability, behaviour (Table 1.2), or decline in locomotory performance could adversely affect survival and/or breeding success in free-living birds.

Effects on reproductive success that have been reported following exposure to anti-ChE pesticides during reproduction include: changes to reproductive behaviour

(Busby et al 1990, White et al 1983, Grue et al. 1982) embryonic mortality and malformation (Meenely and Wyttenbach 1989, Swartz 1981, Paul and Vadlamudi 1976) reduced egg production, lower egg weight (Bennett et al. 1990, Gile and Meyers 1986) and decreased nestling weight and survival (Stromborg et al. 1988, Grue and Shipley 1984, Rattner et al. 1982). The consequences of these effects on reproductive output have been investigated in some free-living species. Reduced reproduction in tree swallows (*Tachycineta bicolor*) and eastern bluebirds (*Sialia sialis*) were consistently found in birds nesting in OP and carbamate pesticide-sprayed apple orchards in Canada over a six-year period (Bishop et al. 2000). Similarly, American robins (*Turdus migratorius*) and song sparrows (*Melospizsa melodia*) nesting in Christmas tree plantations sprayed with OP pesticides experienced nestling mortality twice that of birds breeding in unsprayed plots, despite the absence of differences in adult mortality between these sites (Rondeau and Desgranges 1995).

Busby et al. (1990) investigated the behavioural responses of breeding birds to fenitrothion (sprayed at 420 g/ha) and found that negative influences included territory abandonment, inability to defend territory, disruption of normal incubation, and clutch desertion. In this study the majority of breeding attempts were disrupted and reproductive success in the sprayed area was only one-third that in the control area. Similarly OP-induced behavioural changes were found to adversely affect reproduction in European starlings (*Sturnus vulgaris*) (Grue et al. 1982). Further, laughing gulls dosed sublethally with parathion (6 mg/kg) spent significantly less time incubating than control birds two and three days after pesticide intake (White et

al. 1983), thus making the clutch more susceptible to predation or developmental failure.

Table 1-2 Studies on captive birds demonstrating the effects of organophosphate pesticides on behaviour

Pesticide	Species	Behavioural Effect	Author(s)
Acephate	White-throated sparrow (*Zonotrichia albicollis*)	Produced aberrant migratory behaviour in adults	(Vyas et al. 1995)
Chlorfenvinphos	House sparrow (*Passer domesticus*)	Increase in seed dropping rate	(Fryday et al. 1994)
Chlorfenvinphos	European starlings (*Sturnus vulgaris*)	Significant reduction in activity, flying performance was not significantly different between control and dosed birds	(Fryday et al. 1995)
Chlorfenvinphos	European starlings	Use of cover increased, feeding and flying decreased	(Fryday et al. 1996)
Dicrotophos	European starlings	Perching increased, flying, foraging, singing and displaying decreased	(Grue and Shipley 1981)
Fenitrothion	White-throated sparrows	Singing frequency reduced & song structure changed	(Varty 1980)
Fenitrothion	Zebra finch (*Taeniopygia guttata*)	Perch hopping decreased	(Holmes and Boag 1990)
Fenitrothion	White-throated sparrows	Hopping and singing declined, increase in foraging in low dosage group	(Forsyth and Martin 1993)
Fenthion, Methyl Parathion, Dicrotophos, Fenitrothion,	Common grackle (*Quiscalus quiscula*)	Feeding decreased	(Grue 1982)
Fenthion, Dicrotophos	European starlings	Increased resting, decreased flying, singing, feeding	(Hart 1993)
Methyl parathion	Bobwhite quail (*Colinus virginianus*)	Increased susceptibility to predation by domestic cat, decreased activity	(Galindo 1985)

Investigations into the consequences of sublethal exposure to anti-ChE compounds highlight how assessment of mortality alone underestimates the potential for negative

impacts on free-living individuals. Compromised physiological performance such as reduced locomotor capacity or impaired thermoregulatory abilities can result in changes to behaviour that could compromise survival and reproduction in free-living birds at much lower exposure levels than LD50s would indicate. Therefore, physiological investigations of impacts at the individual level have the potential to provide a more comprehensive understanding of the real consequences of pesticide exposure in free-living birds and the subsequent potential for negative effects at the population level.

1. 4 Locust control, pesticide use and implications for native birds in Australia

The Australian Plague Locust Commission (APLC) was established by the Australian Commonwealth Government in 1974 to research, monitor and control populations of locusts that pose an interstate threat to agricultural systems in eastern Australia (Story and Cox 2001). The APLC has responsibility for containing locust outbreaks in the eastern half of Australia (approximately 2 million km^2) (Hunter and Deveson 2002). Of the locust species residing in Australia, three pose a threat to agriculture when their populations increase and reach plague proportions: the Australian plague locust (*Chortoicetes terminifera*, Walker), the Spur-throated locust (*Austracris guttulosa*, Walker), and the migratory locust (*Locusta migratoria* , L.) (Scanlan et al. 2001). Spring and summer rains and resultant grass growth provide optimal conditions for egg laying and locust development. Under such conditions, several generations of locusts can mature to adulthood in the course of a season (Hunter et al. 2002, Hunter et al. 2001).

Fenitrothion (0,0-dimethyl 0-4-nitro-m-tolyl phosphorothioate), an organophosphate pesticide, has been used for locust control by the APLC since the early 1970's.

Although the total application of pesticides by the APLC varies from season to season depending on rainfall and other weather conditions, the average annual amount of fenitrothion applied from 1980 to 1998 was estimated to be 60 – 80 tonnes per year (NRA 1999). Aerial spraying of locust bands and swarms has been the dominant form of application. The APLC currently spray fenitrothion at 267 g/ha, which is well below the spray rate of 560 g /ha, above which marked increases in avian mortality have been reported (Story and Cox 2001). Fenitrothion does not typically bioaccumulate in vertebrates and studies have reported that residues in tissue or fat are not detectable within 14 days following exposure (Gilmour et al. 1999, Sancho et al. 1998). Fenitrothion can degrade into several metabolites including fenitrooxon, which is created during metabolism and after exposure to ultraviolet light, and can be more toxic than the parent compound. (Greenhalagh et al. 1980). Demethylfenitrothion is formed in acidic conditions while 3-methyl-4-nitrophenol, which is more water soluable and therefore has greater mobility than the parent compound, is formed in alkaline environments. When fenitrothion is broken down by microbes, such as bacteria and fungi, the metabolite aminofenitrothion can be formed (Greenhalagh et al. 1980). Fenitrothion degrades rapidly in aquatic environments with a half-life of 1.5-2 days (Weinberger et al. 1982) and does not accumulate in soils, with a similar half-life of 2-3 days (Gilmour et al. 1999).

Fenitrothion, as with many pesticides, is widely variable in its toxicity to birds. Avian LD50s from captive studies range from 25 mg /kg in red-winged blackbirds (*Agelaius phoeniceus*); 34.5 mg /kg in ringneck pheasants (*Phasianus colchicus*); 140 mg /kg in Japanese quail (*Coturnix japonica*) and 1190 mg/kg in mallard ducks (NRA 1999). Fenitrothion sensitivity in free-living birds can therefore be expected to

be equally as variable. Incidents of avian mortality in free-living species are known to have occurred in Senegal where fenitrothion was aerially applied at 485 g/ha and in Canada when applied above 210 g/ha (NRA 1999). Mortality and deleterious sublethal effects are a function of exposure, which is often related to differences in habitat preference, physiological condition and foraging behaviour among individuals and species, making realistic generalisations concerning risks to avian wildlife difficult to predict (Grue et al. 1997). However, LD50 data together with the above mentioned mortality incidents suggest that the risk to native Australian birds from fenitrothion exposure during locust control could be significant.

Information on avian responses to fenitrothion in the field in Australia is limited, as extensive and systematic searching for avian casualties has not occurred. However 15 dead black kites (*Milvus migrans*) were found in 1992, 24 hours after APLC control operations had taken place. Examination of stomach contents revealed that they had been gorging on locusts and the stomach contents contained between 26.3 and 91.5 mg /kg fenitrothion (NRA 1999).

In addition to fenitrothion, the APLC also uses fipronil [(±)-5-amino-1 (2,6-dichloro-alpha, alpha, alpha-trifluoro-p-tolyl)-4-trifluoromethyl-sulfinylpyrazole-3-carbonitrile] for locust control. It is one of a relatively new group of pesticides known as the phenylpyrazoles (or fiproles). Fipronil, the first pesticide in this class, is effective at very low application rates against insects resistant or tolerant to pyrethroid, organophosphate and carbamate insecticides (Gunasekara et al. 2007). Phenylpyrazoles as a class were introduced in 1990 and registered in the US in 1996. They exert their neurotoxic action on similar processes as the cyclodienes and

cyclohexane OC compounds, by blocking the γ-aminobutyric acid (GABA)-gated chloride channels in the central nervous system. GABA is a major inhibitory neurotransmitter in the nervous system (Ikeda et al. 2001, Hainzlet al. 1998). Exposure to fipronil produces hyperexcitation at low doses and convulsions and death at high doses (Gunasekara et al. 2007). Insects are highly sensitive to fipronil including many lepidopteran species as well as termites, thrips, ants, cockroaches, fleas, ticks and locusts and the pesticide is very effective at low doses (Pevelling et al. 2003, Tingle et al. 2003). Consequently, the APLC can apply fipronil at only 3 g/ha, which is nearly a 90-fold lower spray rate than for fenitrothion.

There is limited information on the effects of fipronil on non-target vertebrate species. Although insect GABA receptors are structurally similar to vertebrate GABAA and GABAC receptors, fipronil has a higher affinity for insect GABA receptors relative to vertebrate GABAA and relatively no affinity for GABAC receptors (Stehr et al. 2006). It is thought that these differences in receptor sensitivity result in fipronil being much less toxic to vertebrates, specifically mammals, than to invertebrates (Hainzl et al. 1998). However, the pesticide has recently been shown to impair the development of spinal locomotor pathways in fish (Stehr et al. 2006). The authors explored the toxicological effects of fipronil on embryos and larvae of zebrafish (*Danio rerio*), which displayed notochord degeneration and subsequent uncoordinated muscle contractions in response to touch. Transient adverse neuronal and behavioural effects have also been found in rats dosed with 250 mg /kg when synaptic excitability in sleep-wake phases were analysed (Szegedi et al. 2005). Further, Ohi et al. (2004) found that a dose of 70 mg /kg in rats impaired normal functioning of the endocrine system and caused adverse reproductive effects.

Fipronil has also been found to be detrimental to several species of lizards (Peveling and Demba 2003). Pevelling (2003) found significant adverse effects on body mass, locomotor activity and prey consumption in the lizard *Acanthodactylus dumerili*, with an LD50 estimated to be as low as 30 mg/kg fipronil. Studies on free-living avian species are lacking, although avian toxicity to technical grade fipronil is known to be highly variable. The reported LD50 in northern bobwhite quail is as low as 11.3 mg/kg while the chemical is virtually non-toxic to mallard ducks (Tingle et al. 2003). The environmental persistence of fipronil is low to moderate and it can accumulate in soils containing high organic matter. The dissipation of fipronil from such soils is via gradual microbial breakdown and its half-life can extend up to one year. Unlike OCs, its low vapour pressure and Henry's law constant make it unlikely to dissipate to the atmosphere (Gunasekara et al. 2007). Fipronil degrades to form four environmental metabolites; fipronil-sulfone, fipronil-sulfide, fipronil-amide and fironil-desulfinyl. These degradation products have been shown to have similar toxic potential (Hainzl et al. 1998, Schlenk 2001) and be more environmentally stable than the parent compound (Hainzl and Cassida1996).

While there has been limited quantification of the ecological consequences of pesticides in non-target vertebrates worldwide, even less is known about pesticide effects on native Australian species. Consequently, the Australian Pesticides and Veterinary Medicines Authority (formerly known as the National Registration Authority (NRA)) in its review on fenitrothion (NRA 1999) relied on field studies, incident reports and regulatory action from other countries (notably the USA and Canada). It is questionable, however, whether pesticide sensitivities of species from other parts of the world can be extrapolated to Australian species, due to the unique

ecology and phylogeny (Geffen and Yom-Tov 2000) of the Australian avifauna and the vastly different avian life history strategies that have evolved in response to the variable and often unpredictable climate (Ford 1989).

Much of inland Australia has low and unpredictable rainfall. Many of the endemic species residing there have evolved an array of life history strategies in response to unpredictable and low-amplitude variations in resource availability (Geffen and Yom-Tov 2000, Keast and Marshall 1954). These birds in comparison to their north-temperate counterparts, have smaller clutch sizes, high incidences of cooperative breeding, nomadism, and longer breeding and moulting periods which often overlap (Ford 1989, Yom-Tov 1987, Payne 1972, Keast 1968). Australian native bird species have a tendency to breed more in response to favourable local conditions than to particular times of the year. Opportunistic breeding is common among species from the semi-arid and arid zone, with breeding triggered in response to rain and the resultant increases in primary productivity, rather than in response to changes in seasons and day length (Astheimer and Buttemer 2002). Consequently, conditions favouring locust outbreaks will also trigger avian reproductive events. Thus locust outbreaks will often be associated with locally dense assemblages of breeding birds. It is therefore very important to understand the extent and duration of sublethal effects of pesticide exposure on avifauna for the future biomonitoring of ecological effects.

In order to realistically assess the potential impact that pesticide application during locust control may have on Australian native birds, exposure needs to be established and effects of such exposure must be investigated. When birds are present at the time

of pesticide application for locust control, exposure occurs dermally through contact with spray or sprayed vegetation, and by preening pesticide-exposed feathers. It can also occur through food consumption. Birds feeding on grasses, locusts, other insects or birds of prey feeding on smaller birds are all at risk.

Investigative research that measures a variety of biochemical and physiological endpoints in native fauna is vital to understanding the real consequences of pesticide exposure in free-living birds. Through an integration of field and captive studies, this dissertation i) evaluates plasma ChE characteristics in Australian native bird species to establish appropriate assay conditions and gain reference values for monitoring of anti-ChE exposure following locust control as well as other agricultural practices ii) assesses the effect of acute fenitrothion exposure in birds by measuring its effect on a variety of ecologically relevant physiological traits iii) examines the sublethal effects of Adonis, a formulation of fipronil used by the APLC for locust control, in birds by utilizing the same physiological metrics. It is hypothesised that there will be a wide variation in plasma ChE characteristics in native birds and that acute sublethal doses of fenitrothion and fipronil will have negative physiological effects on exposed animals.

It is hoped that through this research, those involved with pesticide registration and developing protocols for their use will be better equipped to make sound management choices between chemicals, timing of application and when circumstances require, consider appropriate alternatives.

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2 PLASMA CHOLINESTERASE CHARACTERISTICS IN NATIVE AUSTRALIAN BIRDS: IMPLICATIONS FOR MONITORING AVIAN SPECIES FOR PESTICIDE EXPOSURE IN AVIFAUNA

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I collected blood samples from captive birds and conducted all analysis of plasma samples for cholinesterase characterisation and activity. I completed all statistical analysis and drafted the manuscript. Assistance was received from J.K. Szabo in field work and sample collection, from M. J. Hooper in assay methods and verfication, from L. B. Astheimer and W. A. Buttemer in blood sample collection in captive birds, research design, logistics analysis and manuscript development.

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2. 1 Introduction

Approximately 8000 tonnes of organophosphate (OP) and carbamate insecticides are applied annually throughout Australia for the control of agricultural pests (Radcliffe 2002). These insecticides are anticholinesterase (anti-ChE) chemicals that essentially inhibit esterases, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Acetylcholinesterase is an enzyme that hydrolyses the neurotransmitter acetylcholine (ACh) and thereby terminates cholinergic synaptic transmission (Walker and Thompson 1991). Acetylcholinesterase has a high specificity for ACh and is inhibited at high substrate concentrations. Butyrylcholinesterase however, is a less specific esterase that has a higher affinity for the synthetic substrate, butyrylcholine than it has for ACh and is not inhibited at high ACh concentrations (Thompson and Walker 1994).

As ChEs occur among both vertebrates and invertebrates, there is potential for poisoning in a range of non-target species with organophosphate and carbamate pesticides (Fossi et al. 1996, Hill 1992, Wilson et al. 1992). The inhibition of AChE by such compounds causes a build-up of ACh in the synapse, leading to a disruption of normal nervous system function (Walker and Thompson 1991). Acute toxicity can result in death by respiratory and/or cardiovascular arrest and because cholinergic innervation of the body is nearly ubiquitous, sublethal exposures can lead to a range of biochemical, physiological and behavioural effects (Grue et al. 1997, Fryday et al. 1996).

Monitoring avian exposure to anticholinesterase compounds is particularly important when there is broad overlap of insect-control operations with high densities of birds.

Such a scenario occurs in eastern Australia, where we have previously established that a variety of Australian native birds are exposed to fenitrothion during pesticide application for locust control (Fildes et al. 2006). Apart from the present study there is little published information on cholinesterase activity in Australian native bird species, or Australian terrestrial vertebrates generally (Buttemer et al. 2008, Fildes et al. 2006, Bain et al. 2004, Story and Cox 2001). The lack of such data should be remedied, especially since biomonitoring of anti-cholinesterase chemical exposure can improve the effectiveness of risk assessments.

The measurement of blood or tissue ChE activity is extensively used as a tool in diagnosing organophosphorous or carbamate insecticide exposure in animals (Fairbrother et al. 1991). It is an accepted diagnostic convention that whole-brain ChE inhibition greater than 50% is indicative of anti-ChE exposure as the cause of death in avian mortality events (Hill and Fleming 1982, Ludke et al. 1975). Further, various adverse physiological and behavioural changes have been associated with inhibition of brain ChE activity within 40 to 60% of unexposed levels (Tamura et al. 2001, Fryday et al. 1996, Vyas et al. 1995, Hart 1993). Though little is known about the function of ChEs in the plasma, their inhibition can be used to assess anti-ChE exposure and aid in diagnosing behavioural or physiological effects (Thompson et al. 1991). Another value of plasma or serum ChEs as biomarkers lies in their use in determining exposure to anti-ChE compounds in a non-lethal manner. Such methods have been used successfully to monitor exposure to agricultural chemicals in a variety of free-living species (Strum et al. 2008, Parsons et al. 2000, Hooper et al. 1989). Since plasma ChEs are inhibited faster and more extensively than brain ChEs by anti-ChE compounds, exposure is more easily detectable via plasma ChE

inhibition. Further, due to the comparatively rapid recovery rate of plasma ChE activity, significant inhibition in the plasma indicates recent exposure (Gard and Hooper 1993). Accordingly the absence of plasma ChE inhibition following known exposure to OP insecticides is strong evidence that the exposure level is minimal and unlikely to cause adverse effects (Goldstein et al. 1999).

Because plasma ChE activity is highly variable among birds, reliable detection of ChE inhibition following exposure to anti-ChE chemicals requires establishment of reference levels of ChE activity for the species of concern (Fossi et al. 1994). When reference values are unknown, evidence of anti-ChE exposure can be deduced if plasma ChE activity can be chemically reactivated (McInnes et al. 1996, Wilson et al. 1992). However this technique requires an understanding of the appropriate assay conditions for each species before ChE activity reliably measured.

The primary aim of this investigation is to characterise the plasma ChEs, and establish the appropriate assay conditions, in nine avian species that typically co-occur with locust outbreaks in eastern Australia. Fildes et al. (2006) reported that insectivorous birds were exposed to fenitrothion during locust control, as well as granivorous and honeyeater species, indicating that a variety of exposure routes exist for birds at locust spray events. Reference values for these native birds will furnish baseline values for performing anti-ChE exposure assessments in avian species following OP or carbamate application for locust control or any impact study following agricultural pesticide application. The nine study species consist of birds from three orders including the granivorous king quails (*Coturnix chinensis*) of the order Galliformes, the granivorous budgerigars (*Melopsittacus undulatus*) of the order Psittaciformes and seven species from the order Passeriformes. The latter

group includes two species within the Parvorder Corvida (white-plumed honeyeaters (*Lichenstomus penicillatus*) and yellow-throated miners (*Manorina flavigula*), both of which feed on nectar and small insects and five species within the Parvorder Passerida (the insectivorous Australian pipits, (*Anthus novaeseelandiae*), brown songlarks (*Cinclorhamphus cruralis*), clamorous reed warblers (*Acrocephalus stentoreus*) and willie wagtails (*Rhipidura leucophrys*), as well as the granivorous double-barred finches (*Taeniopygia bichenovii*)) (Simpson and Day 2004). These species were chosen based on their common co-occurrence with plague locust control and their ease of capture at these sites.

Intraspecific variation in plasma esterases has been attributed to diurnal and seasonal effects in several species from the northern hemisphere (Rattner and Fairbrother 1991). Hence temporal and seasonal variation in Australian species has the potential to influence the diagnosis of anti-ChE exposure. Our secondary aim therefore, is to examine the effect of time of day on plasma ChE activity using captive-bred zebra finches (*Taeniopygia guttata*) and captive-bred budgerigars. The effect of time of year on plasma ChE activity in captive-bred zebra finches was also examined. This examination of temporal variation will benefit researchers during toxicological investigations utilising captive species. Zebra finches and budgerigars are appropriate study species as both are native to Australia and co-occur with locust outbreaks in the semi arid and arid zone regions but are also available from commercial suppliers and are relatively easy to maintain in captivity.

2. 2 Materials and Methods

All birds used to establish reference ChE activities (Table 2.1) were free-living apart from king quails, which were captive-bred and purchased from a commercial

supplier. Blood samples were taken from adult free-living birds at study sites in the Riverina region of central New South Wales (NSW), Australia during September 2001 and from sites in the Northern Rivers region of NSW, Australia during November 2001. The study sites were primarily utilised for cattle grazing and are habitats typically sprayed with pesticide during locust outbreaks. Other than aerially spraying fenitrothion to control locusts, anti-ChE pesticide application rarely occurs in these areas. Because no locust control was taking place in the study areas at the time of capture, birds were unlikely to have been exposed to ChE-inhibiting chemicals. Birds were captured in mist-nets between 6.00 am and 11.00 am and individually banded with numbered leg bands. A 200-µl blood sample (<2% of body weight) was taken from a brachial vein by venipuncture using a 25-gauge needle and collected into heparinised microhematocrit tubes. Following centrifugation, plasma was extracted and frozen in liquid nitrogen. On return from the field to the laboratory, the samples were stored in a –80^{o}C freezer until analysis. Samples from five of the free-living species were transported and analysed at the Institute of Toxicology, Texas Tech University. Plasma samples from all other species were analysed by the same individual at the University of Wollongong.

To examine the effect of time of day and season on plasma ChE activity, we obtained wild type, captive-bred zebra finches and budgerigars from a commercial supplier. Birds were individually banded with metal leg bands, housed in outdoor aviaries (3500 x 2100 x 2500 cm), and provided with food and water ad libitum at the University of Wollongong. Birds were allowed to adjust to caging conditions for at least two weeks before blood samples were taken. Zebra finches (sample size = 58) were divided into four groups and budgerigars (sample size = 10) were a single

group. Blood samples (200 μl) were taken from each group once a week at a different time of day over a four week period. Sampling times were at either: 6 am, 10 am, 2 pm or 6 pm. Blood was sampled a total of four times from each individual. To examine the effect of season on plasma ChE activity 58 plasma samples collected from zebra finches during the summer months of January and February were compared with 34 plasma samples collected during the winter months of June and July.

Diluted plasma samples were assayed using the Ellman et al. (1961) method as modified by Gard and Hooper (1993) for use in a 96-well spectrophotometric plate reader (BioRad, Crown Scientific, Minto, NSW, Australia) which was equipped with software for enzyme kinetic analysis (KC Junior, Bio-Tek Instruments, Winooski, VT, USA). Assay reagents were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). All plasma samples were assayed in triplicate for total ChE and AChE activities at 25^{o} C for 3 min (readings taken at 13 s intervals). Assay components were acetylthiocholine iodide (AThCh, the ChE substrate), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.05 M tris (pH 8.0) buffer and diluted enzyme with a total volume of 250 μl per microplate well. The assay was initiated by the addition of AThCh to all other components. Acetylcholinesterase was differentiated from BChE by preincubation (5 min, prior to AThCh addition) with the specific BChE inhibitor, tetraisopropyl pyrophosphoramide (iso-OMPA, 10^{-5} M, FC, prior to addition of AThCh). Butyrylcholinesterase activity was calculated as the difference between total ChE and AChE activities. Horse serum (Sigma-Aldrich), frozen in aliquots, provided a between-assay standard. Blank wells without added enzyme provided background colour formation. The increase in absorbance at 412 nm (ΔA/min),

corrected for blank, was converted to μmoles AThCh hydrolysed/min/ml plasma (or /g brain tissue) using the molar extinction coefficient 13,600/cm/M (Ellman et al. 1961).

Characterisation was conducted on pooled plasma samples from at least five individuals of each species since the volume needed exceeded the viable limit that any individual bird could furnish. The appropriate plasma dilution factor, substrate and iso-OMPA concentrations were therefore determined from the pooled plasma samples. The optimal plasma dilution was established so that total ChE activities achieved a ΔA/min value of 0.100 to 0.150. Iso-ompa inhibition of BChE was determined over a range of iso-OMPA preincubation concentrations from 10^{-2} M to 10^{-12} M. The optimal concentration used in subsequent ChE determinations was that at which all BChE was inhibited, but at which AChE activity remained constant. Substrate affinity was determined by measuring AChE and BChE activities over a range of AThCh concentrations from 10^{-2} M to 10^{-6} M, using peak AChE activity to choose the most appropriate assay concentration for both enzymes.

Once the plasma from each species was characterised using the pooled samples, mean ChE activity level for each species was determined using plasma samples taken from individual birds. Samples were diluted and assayed using the optimised concentration of assay components established through characterisation. Mean plasma ChE activities for all samples were calculated for each species.

The appropriate plasma dilutions for the assay were 5-fold in double-barred finches and budgerigars, 20-fold in king quails and 10-fold in all other species. In all species,

except king quails, a concentration-dependent decrease in ChE activity occurred as iso-OMPA concentration increased and a plateau of inhibition was reached at which BChE was inhibited and AChE activity remained. An iso-OMPA stock concentration of 10^{-4} M was chosen for use in all species with the exception of king quails, as this concentration led to activities being within the plateau of BChE inhibition. Variable quantities of residual AChE activity were demonstrated at the chosen plateau iso-OMPA concentration.

King quails were notable for their lack of measurable ChE activity at iso-OMPA concentrations above 10^{-5} M suggesting a lack of plasma AChE. The characterisation of king quail plasma was repeated four times with four different pooled plasma samples with consistent results. Consequently, the lack of a plateau in king quails precluded the use of iso-OMPA as a selective inhibitor of BChE in order to isolate AChE, therefore only total ChE could be measured in this species.

The optimized iso-OMPA concentration in all other species was used to assess substrate affinity. The AThCh concentration that led to maximum AChE activity in clamorous reed-warblers and double-barred finches was 316 mM. In all other species apart from king quails, optimum AThCh concentration was 100 mM. Plasma ChE in king quails did not reach maximal activity levels at concentrations of AThCh or BThCh up to 10^{-2} M.

Temporal effects on ChE activity were assessed for normality using the Shapiro-Wilk test. Since these data were normally distributed time of day effects in captive zebra finches and captive budgerigars were examined using an ANOVA for repeated

measures, while sampling time of year effects were examined using a Student's *t*-test. The significance level was $p < 0.05$; results are expressed as mean (± SE). Statistical procedures follow those outlined in Zar (1999) with analyses performed using JMP statistical software (Version 5.1, SAS Institute Inc., Cary, NC, USA).

2. 3 Results

The variation in mean plasma AChE activity across species ranged over an order of magnitude, from 0.089 μmols/min/ml in budgerigars to 0.860 μmols/min/ml in clamorous reed-warblers (Table 2.1). Plasma BchE activity in the plasma ranged from 0.414 μmols/min/ml in double-barred finches to 1.13 μmols/min/ml in white-plumed honeyeaters and clamorous reed-warblers. White-plumed honeyeaters, yellow-throated miners and budgerigars had a lower proportion of AChE to BChE in the plasma, with budgerigars displaying the lowest measurable AChE activity at 0.089 μmol/min/ml (9.13% of total ChE) (Table 2.1.); willie wagtails had the highest percentage of AChE at 60.2% of total ChE activity.

Table 2-1 Plasma cholinesterase activity from selected Australian avian species (AChE = acetylcholinesterase, BChE = butyrylcholinesterase, SD = standard deviation, *n* = sample size, ND = not detected)

		Enzyme Activity (μmol/min/ml)		
Species	Measures	AChE	BChE	% AChE
Brown songlark	Mean	0.222	1.06	17.2
	SD	0.05	0.29	
	n	10	10	
Budgerigar	Mean	0.089	0.885	9.1
	SD	0.02	0.19	
	n	6	6	
Clamorous reed warbler	Mean	0.860	0.901	48.8
	SD	NA	NA	
	n	1	1	
Double barred finch	Mean	0.28	0.41	39.7
	SD	0.02	0.05	
	n	3	3	
King quail	Mean	ND	2.23	ND
	SD		0.05	
	n		9	
Australian pipit	Mean	0.174	1.075	13.9
	SD	0.01	0.10	
	n	3	3	
White-plumed honeyeater	Mean	0.327	1.130	23
	SD	0.06	0.30	
	n	7	7	
Willie wagtail	Mean	0.763	0.508	60.2
	SD	0.09	0.15	
	n	3	3	
Yellow throated miner	Mean	0.290	0.926	19.8
	SD	0.07	0.53	
	n	3	3	

Plasma ChE activity in zebra finches did not vary significantly with time of day. Total ChE and BChE activity in this species changed between morning and evening by as little as 0.5% and 2.3%, respectively (Fig. 2.1A; total ChE $p > 0.07$, BChE, $p > 0.1$). AChE activity displayed slightly greater variation throughout the day but this was also not significant ($p > 0.3$). Plasma ChE activity in budgerigars was more

variable than in zebra finches. Although the morning samples appeared to be generally higher in all ChE activities than the evening samples (Fig. 2.1B), this variation was not statistically significant for any ChE activity, nor at any time of day in this species ($p > 0.2$). Time of year however, did have an effect on all plasma ChEs in zebra finches (Fig. 2.2.), with plasma activity significantly higher during the summer months than during winter ($p < 0.001$).

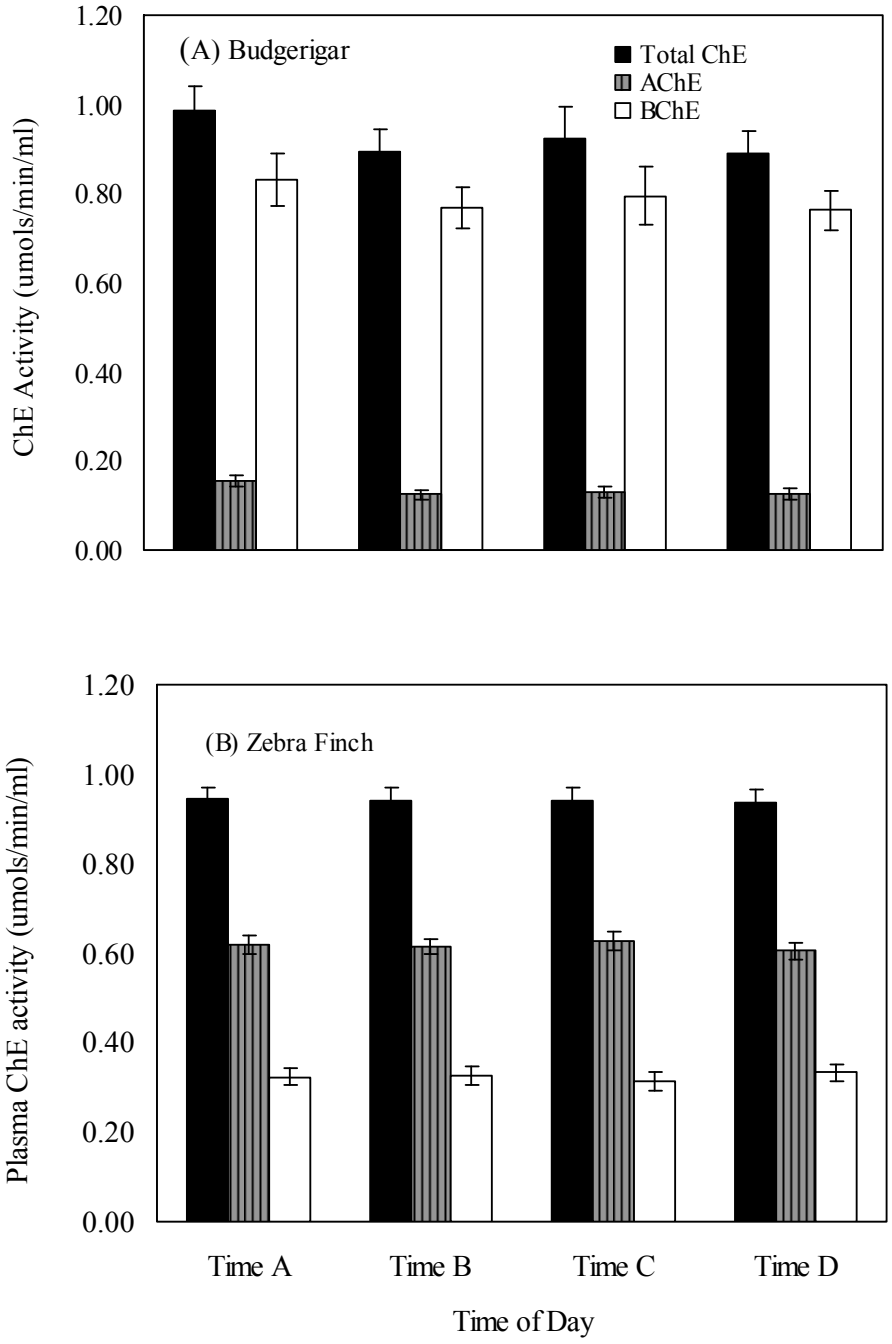

Figure 2-1 Mean plasma cholinesterase activity at four different times of day (± standard error) in (A) budgerigars (number of individuals = 10) and (B) zebra finches (number of individuals = 58; Time A = 6 am; Time B = 10 am; Time C = 2 pm; Time D = 6 pm).There were no significant differences in any enzyme activity at any time point

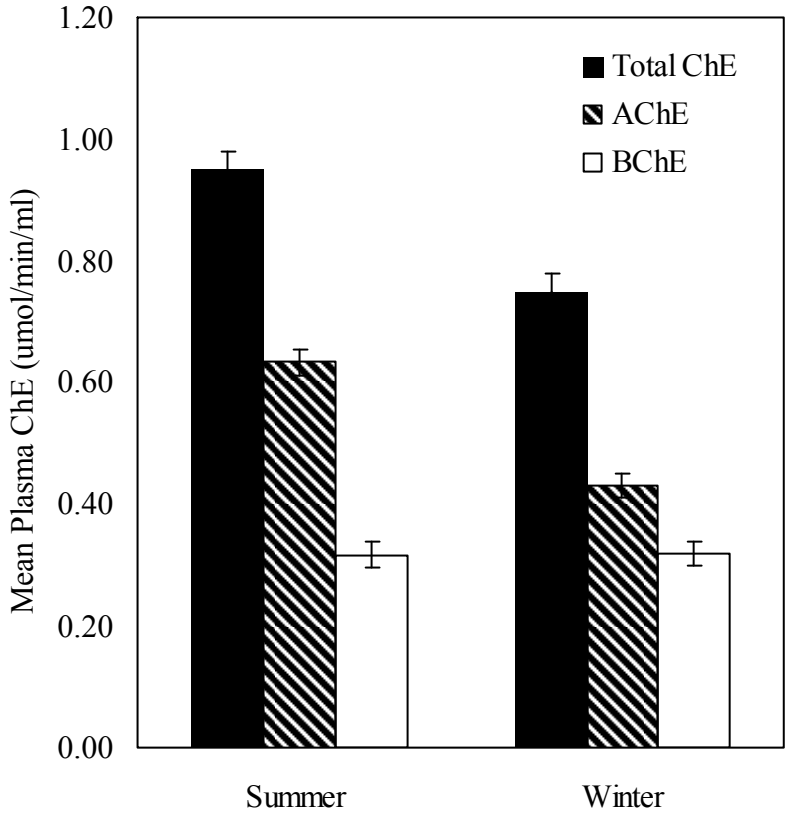

Figure 2-2 Seasonal changes in mean plasma cholinesterase activity in zebra finches (± standard error; number of individuals: summer = 58, winter = 34). All plasma ChE activities in winter were significantly lower than summer

2. 4 Discussion

The defining characteristics of AChE include the hydrolysis of AThCh, resistance to the selective inhibitor iso-OMPA and inhibition at high concentrations of AThCh (Radic et al. 1993). By this definition, all native bird plasma samples, excepting those from king quails, contained AChE, an iso-OMPA resistant fraction, and BChE, an iso-OMPA sensitive fraction. In all species except king quails, there was a concentration-dependent inhibition of a portion of the total ChE activity, with iso-OMPA as inhibitor, and there was little difference in the iso-OMPA concentrations at which the plateau of inhibition was reached. Unlike the other species, ChE activities

in the plasma of king quail was inhibited at low concentrations of iso-OMPA, with no evidence of any residual AChE activity that was resistant to the selective inhibitor. Further, hydrolysis was not inhibited at high substrate concentrations, therefore, there was no measurable AChE in the plasma of king quails, only BChE. Although very low AChE activity has been reported for several species, king quail are the first species to display a complete lack of AChE activity in plasma. Interestingly, the lowest reported AChE fraction in birds was found in another galliform species, the domestic chicken (*Gallus domesticus*), with AChE contributing only 3% to total ChE activity (Hooper 1988). However low AChE activity is not confined to galliform species; in tawny owls (*Strix aluco*) and red-winged blackbirds (*Agelaius phoeniceus*) AChE only accounted for 6% of total plasma ChE activity (Claudie et al. 2005, Wolfe and Kendall 1998)

Our study confirms BChE as the predominant esterase in avian plasma (Claudie et al. 2005) as five of the seven species characterised had BChE fractions higher than those for AChE. Apart from king quail, the lowest proportion of AChE, 9.13% of total ChE activity, was found in budgerigars which was similar to values reported in the eastern bluebird (*Sialia sialis*) and European starling (*Sturnus vulgaris*) by Gard and Hooper (1993). In the present study, BChE activity in white-plumed honeyeaters, Richard's pipits, brown songlarks and yellow-throated miners contributed to over 75% of total ChE activity in plasma. Similarly, high proportions of BChE have been reported in the plasma of barn owls (*Tyto alba*) and rock pigeons (*Columba livia*). Conversly, plasma AChE in willie wagtails averaged 60% of total ChE activity. These results confirm the wide variation in avian plasma ChE activity and the need

to establish reference values and appropriate assay conditions for each species being monitored for anti-ChE pesticide exposure.

Wide interspecies variation in ChE activity has been reported in 20 European raptor species with a suggestion of phylogenetic influence on some of the trends (Claudie et al. 2005). Similarly Westlake and co-workers (1983) demonstrated that brain and plasma AChE activies had discernible familial trends among the many species examined. Although we have characterised too few species as yet to evaluate phylogenetic trends in Australian native birds it is interesting to compare closely related species. White-plumed honeyeater and yellow-throated miner are both old-endemic species from the family Meliphagidae and both have similar ChE fractions in their plasma (Table 2.1) but very different ChE activity levels. Likewise, double-barred finches in the present study (Table 2.1) had similar plasma ChE fractions to free-living zebra finches of the same genus, with plasma in the latter species consisting of 53% AChE and 47% BChE (Fildes et al. 2006). That related species have similar fractions of ChE in the plasma is supported by other studies: white-browed woodswallows (*Artamus superciliosus*) and masked woodswallows (*A.personatus*) had similar fractions of 68 and 60% AChE respectively (Fildes et al. 2006) and snowy egrets (*Egretta thula*) and little blue herons (*E. caerulea*) both had 75% AChE in plasma (Parsons et al. 2000).

This study found no evidence that plasma ChE activity varies with time of day in zebra finches or budgerigars, but did distinguish a difference between summer and winter in all ChE activities in zebra finches. Cholinesterase activity in winter was 68% of activity displayed during the summer months. The latter result is not

unexpected as seasonal differences in serum and plasma ChE activity have been reported for avian species. There is little information available regarding circadian rhythms and seasonal variation in ChE activity however, and the existing information varies among species examined (Rattner and Fairbrother, 1991, Garcia-Rodriguez et al. 1987, Hill and Murray 1987). As much as a 22% difference in basal activity has been observed at different times of day in northern bobwhite hens (*Colinus virginianus*) (Rattner and Fairbrother 1991), while a marked diurnal variation in plasma carboxylesterase an enzyme closely related to ChE, was detected in European starlings (Thompson et al. 1988). ChE in blood samples taken over a 24-hour period from buzzards (*Buteo buteo*) demonstrated circadian rhythmic trends however samples from eagle owls (*Bubo bubo*) in the same study did not (Garcia-Rodriguez et al., 1987). The interspecies difference in temporal effects on plasma ChE activity precludes results from the present study being extrapolated to any other species. Nevertheless, information on the lack of daily variation in plasma ChE activity in captive zebra finches and budgerigars does allow for greater flexibility in sampling regimes in captive studies and demonstrates a further advantage of using these species as good native Australian representative species.

Interestingly, free-living zebra finches (Fildes et al. 2006) had plasma ChE activities 42% lower than the captive zebra finches in the present study measured at the same time of year (Fig. 2.2.). This difference in enzyme activity is likely to be due to a number of factors. In wild caught zebra finches, individuals of a certain age or sex may have been over-represented among those sampled, or they may have differed from captive-bred birds in regard to reproductive or nutritional status. All of these factors have been shown to affect avian ChE activities (Gard and Hooper 1993,

Rattner and Fairbrother 1991, Thompson 1991) and natural sources of variation would be greater in the wild than in the aviary. Such intraspecific variation further highlights the need to establish plasma ChE reference values from non-exposed animals collected at similar locations and at the same time of year to ensure interassay variation in measuring ChE activies is minimised (Fairbrother and Bennett 1988).

In conclusion, the variability in plasma ChE activity between species underscores the importance of characterising AChE activity before using the enzyme in monitoring schemes. Baseline measurements of ChE activity in Australian native bird species have to date been virtually absent in the literature. The present contribution begins to fill this void, aiding biological monitoring of non-target avian wildlife at risk of exposure to anti-ChE compounds.

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3 CHOLINESTERASE RESPONSE IN NATIVE BIRDS EXPOSED TO FENITROTHION DURING LOCUST CONTROL OPERATIONS IN EASTERN AUSTRALIA

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I conducted one third of the field work involved in this study and completed all analysis of plasma samples for cholinesterase characterisation and activity. I completed all statistical analysis and drafted the manuscript. I received assistance from M. J. Hooper in assay methods and verfication, from Paul Story in research design, from L. B. Astheimer and W. A. Buttemer in field work and blood sample collection, research design, logistics analysis and manuscript development.

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3. 1 Introduction

Modern intensive agriculture relies heavily on pest management with the use of pesticides that can significantly impact nontarget wildlife (McLaughlin and Mineau 1995). In Australia, three species of locusts can pose a serious threat to agriculture when their populations increase dramatically: the Australian plague locust, *Chortoicetes terminifera* (Walker), the Spur-throated locust, *Austracris guttulosa* (Walker), and the migratory locust, *Locusta migratoria* (L.) (Story and Cox 2001). Aerial spraying of locust bands and swarms with the organophosphate (OP) pesticide, fenitrothion, has been the Australian Plague Locust Commission's (APLC) dominant form of locust control in eastern Australia for the last twenty-five years (Story and Cox 2001) and is the only ChE-inhibiting pesticide in use by the commission.

In the present study the impact of locust control on nontarget bird species was investigated in semi-arid habitats of central and western Queensland, Australia. This region is characterised by low seasonality and unpredictable summer rainfall (Zann 1985). In contrast to highly seasonal regions, increasing resources are not always heralded by changes in day length or temperature and often, increases in primary productivity and insect abundance occur with the onset of erratic summer rains (Woinarski and Cullen 1984). Rainfall and warm temperatures stimulate the laying, as well as the synchronous hatching, of locust eggs (Hunter et al. 2001). Rainfall not only moistens the soil for the nymphs to emerge but also provides the green forage necessary for them to grow into adults (Scanlan et al. 2001). The nymphs form feeding bands that can eventually become kilometres wide and hundreds of metres long (Szabo 2005, Bain et al. 2004).

Australian birds of the arid and semi-arid zone have evolved to respond to unpredictable rainfall and the resulting increase in abundance of seed and insects (including locusts). Such environmental cues trigger physiological mechanisms that stimulate breeding, enabling these species to manage the natural variation in energy availability and reproduce while conditions are favourable (Hahn et al. 1992). Thus, locust outbreaks and avian reproductive events often co-occur, increasing the risk to avian populations of pesticide exposure from locust control operations. The huge aggregations of locust nymphs are a very obvious and nutritious food resource for birds, attracting a wide range of nomadic and semi-nomadic species (Szabo 2005). Many insectivorous species use locusts opportunistically, while some species, particularly raptors, gorge feed on locusts. Granivorous birds forage on grasses that also provide food for flightless locust nymphs (Szabo 2005).

The OP pesticide, fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenyl)-phosphorothioate) is a neurotoxicant that inhibits acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and other esterase enzymes (Grue et al. 1997). This inhibition is an effect widely used as a biomarker of acute exposure. Acute toxicity is associated with a range of signs including muscle tremors, ataxia and death through respiratory paralysis (Chambers 1992). Sublethal OP effects include: anorexia, loss of thermoregulation, endocrine impairment, and changes in sensory perception, memory and behaviour that may impact reproductive success, and affect survival of adults, offspring or both (Grue et al. 1991). Sensitivity to OPs varies widely between species and compounds, but birds as a class tend to be more sensitive than mammals (Mineau et al. 2001).

We used ChE inhibition and reactivation as biomarkers of exposure to determine the degree to which birds occurring at locust outbreaks are exposed to fenitrothion during locust control operations. Cholinesterase activity in blood plasma, although variable between species, is a useful non-lethal indicator of exposure to ChE-inhibiting insecticides while ChE reactivation is a valuable adjunct to the evaluation when baseline ChE activity is unknown (Wilson et al. 1992, McInness et al. 1996). Brain ChE activity and reactivation were analyzed in birds that died in the net or in the hand. Because there is little information on ChE activities for Australian birds, we characterised plasma ChEs of native bird species to confirm assay conditions for this and future pesticide studies.

3. 2 Materials and Methods

3.2.1 Pesticide application

All study areas were sprayed with an ultra low volume (ULV) formulation of fenitrothion (1.27 kg a.i./L). Undiluted technical pesticide was applied via Micronair AU5000 spray units mounted on the wings of aircraft flying at 160 to 200 km/h. Spray parameters include a flying height of 10 m, track spacing of 100 m and an application rate of 267 g a.i./ ha (Story and Cox 2001).

3.2.1.1 Sample Collection

The data reported in this study were collected from birds captured at three sites during pest control operational conditions. In February 2002, samples were collected approximately 200 km north-west of Quilpie, Queensland (S 26^{o} 3', 56.1; E 143^{o} 37', 12.8) where fenitrothion was applied to 330 hectares. In Tambo during December 2003 to January 2004, samples were collected from a property on the Landsborough

Hwy, Charleville, Queensland (S 25^{o} 3', 11.058; E 146^{o} 37', 29.660), before and after fenitrothion application to 824 hectares. Samples were again collected near Quilpie in February 2004 from a property on Diamantina Development Rd, Quilpie, Queensland (S 26^{o} 8', 21; E 143^{o} 41', 43), with application to approximately 707 hectares.

Due to the nature of locust outbreaks, time, weather and resources restricted our sampling regime to one area per spray event sampled on four days during the week before and on one, two, three, and five days after pesticide application. Birds were captured in mist-nets between 6 am and 11 am at all sites. All sites were open plain pasture areas comprising both native (e.g., but not restricted to, *Astrebla spp*., *Dicanthium sericum*, *Aristida latifolia*) and improved pasture (e.g. *Cenchrus ciliaris*). The Tambo site was gently sloping towards open sclerophyll forest dominated by *Eucalyptus populnea, Eucalyptus crebra*, and *Eucalyptus melaonphloia* with a grazed understory comprised of tussock grasses.

All birds were individually banded with Australian birds and bat banding scheme metal bands. Tarsus, wing lengths and body mass were measured upon capture and birds were scored for moult, fat and reproductive activity (e.g., presence of a brood patch, presence of fledglings or juveniles or evidence of reproductive behaviours). A blood sample (<1% of body wt) was taken from the brachial vein of adult birds by venipuncture with a 25-gauge needle and collected into heparinized microhematocrit tubes. Following centrifugation, plasma was extracted and frozen in liquid nitrogen. On return to the University of Wollongong the samples were stored in a –80^{o}C freezer until analysis. In total 94 plasma samples and three brain samples (collected

opportunistically from birds that died in the hand) taken from birds captured post spray were analyzed for activities and reactivation of ChE. Sufficient numbers of four species of birds were captured to allow plasma ChE characterisation, as well as the comparison of pre and post-spray blood samples.

3.2.2 ChE analysis

Brain samples were homogenized in ice-cold 0.05 M tris (pH 7.4) buffer (1:9,wt/vol) using an homogenizer (model basic T25, IKA Labortechnik, Staufen, Germany). Diluted plasma and brain samples were assayed using the Ellman et al. (1961) method as modified by Gard and Hooper (1993) for use in a 96-well spectrophotometric plate reader (Bio-Tek Instruments, Winooski, VT, USA) equipped with software for enzyme kinetic analysis (KC Junior, Bio-Tek). Assay and reactivation reagents were from Sigma (Castle Hill, NSW, Australia). All the samples were assayed in triplicate for total ChE and AChE activities at 25^{o} C for 3 min (readings taken at 13 s intervals). Assay components were acetylthiocholine iodide (AThCh, 10^{-4} M final concentration (FC); the ChE substrate), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) ($3.23x10^{-4}$ M FC), 0.05 M tris (pH 8.0) buffer and diluted enzyme with a total volume of 250 ml per microplate well. The assay was initiated by the addition of AThCh to all other components. Acetylcholinesterase was differentiated from BChE by preincubation (5 min, prior to AThCh addition) with the specific BChE inhibitor, tetraisopropyl pyrophosphoramide (iso-OMPA, 10^{-5} M FC prior to addition of AThCh). Butrylcholinesterase activity was calculated as the difference between total ChE and AChE activities. Horse serum, frozen in aliquots, provided a between-assay standard. Blank wells without enzyme quantified background color formation. A separate blank contained pyridine-2-aldoxime methochloride (2-PAM) ($1.2x10^{-4}$ M FC) to account for 2-PAM-induced color

formation in assays of 2-PAM-fortified enzyme sources. The increase in absorbance at 412 nm (ΔA/min), corrected for blank, was converted to μmoles AThCh hydrolysed/min/ml plasma (or /g brain tissue) using the molar extinction coefficient 13,600/cm/M.

3.2.3 Plasma ChE characterisation

Characterisation was conducted when blood samples from at least five different individuals of a species were available. The plasma collected was pooled, as the amount of plasma required for characterisation is in excess of that which can be taken from a small passerine bird. The appropriate plasma dilution factor, substrate and iso-OMPA concentrations were determined from the pooled plasma samples. The optimal plasma dilution was established so that total ChE activities achieved a value of 0.100 to 0.150 ΔA/min. Iso-ompa inhibition of BChE was determined over a range of iso-OMPA preincubation concentrations from 10^{-3} M to 10^{-13} M. The optimal concentration used in subsequent ChE determinations was that at which all BChE was inhibited, but at which AChE activity remained constant. Substrate affinity was determined by measuring AChE and BChE activities over a range of AThCh concentrations from 10^{-2} M to 10^{-6} M, using optimized iso-OMPA concentrations to separate ChEs.

3.2.4 Reactivation analysis

Reactivation of the OP-inhibited ChE was determined following the method described by Wilson et al. (1992) and modified by Parsons et al. (2000). Each diluted plasma or brain sample was divided into three 250 μl aliquots. One of the three aliquots was maintained on ice until assayed for ChE activity. The other two were used to test for ChE reactivation with 2-PAM. One of these aliquots was spiked with

2-PAM (FC = 10^{-4} M) and the other with an equal volume (5 µl) of deionized water. Both were incubated for 30 min in a 25°C water bath. All three aliquots were assayed together for ChE activity as previously described. An upper-tailed Student's *t*-test was used to compare the resulting ChE activities with 2-PAM to those without 2-PAM. When reactivation resulted in a greater than 5% increase in activity after 2-PAM treatment (i.e., > 5% reactivation), the sample was considered to contain OP-inhibited ChE and, thus, indicated fenitrothion exposure for those individuals.

Measured reactivation represents the minimum amount of inhibited ChE in a sample, as the incubation conditions do not lead to complete reactivation of all inhibited ChE. Further, that portion that has "aged" cannot be reactivated under any incubation conditions (Wilson et al. 1992). Percent reactivation was converted to percent inhibition using the equation:

(% reactivation / (100 + % reactivation)) * 100 = % inhibition

As inhibited, but unreactivated ChE activity cannot be accounted for using this method, the calculated percent inhibition represents a minimum level of inhibition, which would likely be greater were complete reactivation achieved and aged ChE accounted for.

3.2.5 Summary of statistical tests

Total ChE, AChE, and BChE activities in species caught both pre- and post spray, were assessed for normality using the Shapiro-Wilk test. When the data were not normally distributed the means before and after spraying were compared using the nonparametric Kruskal-Wallis analysis. When normally distributed they were

compared using Student's *t* test. In order to examine the variation in cholinesterase activity in the species caught both pre- and post spray, the post spray samples were further evaluated using diagnostic thresholds (DTs) as described by Hill (1988). Hill defined DTs as points two standard deviations (SDs) above and below the mean ChE activity of unexposed birds, incorporating 95% of all control values. Thus, within any species, plasma or brain samples collected after pesticide application that were two SDs below the mean ChE activity of birds collected before spraying were considered to fall below the DT and therefore considered to have been exposed to fenitrothion. The mean magnitude of reactivation was compared between days post spray using the Kruskal-Wallis test. Tests were conducted at $\alpha = 0.05$. Statistical procedures follow those outlined in Zar (1999).

3. 3 Results

3.3.1 Plasma ChE characterization

In plasma, a concentration-dependent decrease in ChE activity occurred as iso-OMPA concentration increased until a plateau of inhibition was reached. An iso-OMPA pre-incubation concentration of 10^{-5} M led to activities sufficiently within the plateau of BChE inhibition and was chosen for use in all species. Using the optimized iso-OMPA concentration, the AThCh concentration that led to maximum AChE activity was 1 mM in all studied species, with AChE activities declining at substrate concentrations below and above (via substrate inhibition) this level. As BChE activity increases throughout the entire substrate range (and beyond, limited only by AThCh solubility), the same concentration was used for both enzymes. All subsequent ChE analyses employed these optimum reagent concentrations.

3.3.2 Cholinesterase activity levels - inhibition, reactivation, and diagnostic thresholds

Average baseline AChE activity ranged from 0.35 μm/min/ml plasma in zebra finches (*Taeniopygia guttata*) to 1.06 μm/min/ml plasma in masked woodswallows (*Artamus personatus*) (Table 3.1). Average BChE activity ranged from 0.33 μm/min/ml plasma in the zebra finch to 1.1 μm/min/ml plasma in white-winged triller (*Lalage sueurii*) (Table 3.1) while total ChE activity was lowest in zebra finches, 0.69 μm/min/ml plasma and highest in white-winged triller, 2.11 μm/min/ml plasma (Table 3.1). Comparison of mean activities between pre- and post spray periods demonstrated significant differences in all three ChE activities for both the white-winged triller ($p < 0.02$, Kruskal-Wallis analysis) and the zebra finch ($p < 0.001$, Student's *t* test). Compared to pre-spray activities, post spray AChE activities in zebra finches and white-winged trillers were inhibited 75% and 57% respectively, while BChE was inhibited 78% and 70%, and total ChE was inhibited 71% and 64.5%. There were no significant differences between pre- and post spray ChE activities in white-browed woodswallows ($p > 0.5$, Kruskal-Wallis analysis) and masked woodswallows ($p > 0.1$, Kruskal-Wallis analysis).

Three of four of the post-spray plasma samples from white-winged trillers were below the diagnostic threshold (> two SDs below the mean of pre-spray plasma activity) for all AChE and BChE activity. Total ChE activity was below DT in two of the four individuals. All ChE activities in plasma samples from zebra finches collected post spray were below DT. Total and AChE activities in three of the six post spray plasma samples from white-browed woodswallows (*Artamus superciliosus*) were below DT values while BChE activity was lower in only one

plasma sample (Table 3.1). Total ChE and AChE activity in masked woodswallows was below the threshold in one individual caught post spray and BChE activity was not lower in any plasma samples for this species. There were no significant differences between mean body mass before and after spraying with fenitrothion in any species ($p > 0.100$).

Thirty-three different species were captured in mist nets at the three sites in 2002 and 2003. Twenty-two species showed evidence that they were undergoing their annual moult. In 16 species the presence of juveniles and brood patches showed evidence that breeding activities were underway (Table 3.2). Seventy-six samples, including three brain samples, were taken from birds at Tambo and Quilpie on days one, two, three and five-post spray and analyzed for ChE reactivation. In 19 of the 76 birds sampled cholinesterase was inhibited (Table 3.2). There was a range of reactivation levels that spanned from 5% to > 900%. Plasma ChE inhibition levels were highest in zebra finches (31.9%), masked woodswallows (35%), sacred kingfishers (*Todiramphus sanctus*) (28.5%), white-winged trillers (35.4%), and brown songlarks (*Cincloramphus cruralis*) (> 90%). Likewise total ChE inhibition in a white-winged triller brain that died in the hand was considerable at 29%, while two brains collected from birds that died in the hand showed no evidence of OP exposure. The number of samples exhibiting reactivation was highest on day one post spray (Table 3.3). However, beyond the initial decline in reactivating samples two days after pesticide application, the proportion of birds showing reactivation remained fairly constant and was no less five days post spray than at two days post spray (Table 3.3). Cholinesterase was reactivated from samples collected on all days post spray and the

difference between the magnitude of reactivation above 5% on any day was not significant for either total ChE or AChE (Table 3.3; $p > 0.1$).

Table 3-1 Plasma cholinesterase activity in species caught pre- and post-fenitrothion application (SD = standard deviation, *n* = sample size, DT = diagnostic threshold, * = significantly lower post-spray ChE activity than pre-spray samples).

Species	Treatment	Measures	Enzyme Activity (μmol/min/ml)		
			AChE	BChE	Total ChE
Masked Woodswallow	Pre-Spray	Mean	1.06	0.73	1.79
		SD	0.44	0.50	0.54
		n	3	3	3
		Lower DT	0.18	0.00	0.72
	Post-Spray	Mean	0.70	0.32	1.03
		SD	0.22	0.09	0.14
		n	7	7	7
		No. Below DT	1	0	1
White-Browed Woodswallow	Pre-Spray	Mean	0.82	0.38	1.20
		SD	0.11	0.14	0.18
		n	6	6	6
		Lower DT	0.60	0.10	0.84
	Post-Spray	Mean	0.63	0.34	0.96
		SD	0.19	0.20	0.31
		n	6	6	6
		No. Below DT	3	1	3
White-Winged Triller	Pre-Spray	Mean	0.98	1.11	2.11
		SD	0.35	0.35	0.67
		n	8	8	8
		Lower DT	0.29	0.40	0.77
	Post-Spray	Mean	0.42*	0.31*	0.73*
		SD	0.39	0.06	0.31
		n	4	4	4
		No. Below DT	3	3	2
Zebra Finch	Pre-Spray	Mean	0.35	0.33	0.69
		SD	0.07	0.08	0.09
		n	10	10	10
		Lower DT	0.21	0.17	0.51
	Post-Spray	Mean	0.13*	0.07*	0.21*
		SD	0.06	0.08	0.09
		n	4	4	4
		No. Below DT	4	4	4

Table 3-2 Summary of species caught with breeding and/or moulting status and reactivating ChE

Feeding Guild	Total # Species	# Species Breeding	# Species Moulting	# Birds caught pre-spray	# Post-spray samples with >5% reactivation			Mean % Inhibition		# Post-spray samples with <5% reactivation
					Total ChE (only)	AChE (only)	Both	Total ChE	AChE	
Birds of Prey	2	0	0	0	1	0	0	6.0%		1
Granivorous	5	2	4	26	2	2	0	33.1% (SD=1.7)	25.6% (SD=2.7)	21
Honeyeaters	6	4	4	17	0	2	1	36.1%	13.31% (SD=5.5)	12
Insectivorous	20	10	14	51	3	1	7	34.6% (SD=29.9, plasma) 29% (brain,n=1)	47.6% (SD=32.5, plasma) 23% (brain,n=1)	21 plasma; 2 brain
Total	33	16	22	94	6	5	8			57

Table 3-3 Frequency and magnitude of plasma ChE reactivation in avian species sampled during the first 5 days post-fenitrothion application

Days Post-spray	# Samples with > 5% reactivation	# Samples with < 5% reactivation	Mean % reactivation Total ChE (SD)	Mean % reactivation AChE (SD)
1	10	23	45.3 (35.4)	43.0 (33.7)
2	7	27	15.1 (10.3)	15.38 (10.4)
3	5	15	34.3 (21.1)	29.1 (15.5)
5	3	11	13.64 (6.84)	8.88 (4.4)

3. 4 Discussion

3.4.1 ChE inhibition and reactivation

This paper is the first attempt in Australia to establish whether pesticides, used in pest management practices such as locust control, place nontarget bird species at risk of pesticide exposure using ChE as a biomarker. Our findings confirm that Australian native birds are exposed to pesticide during APLC spray operations. Fenitrothion is the only ChE-inhibiting pesticide in use by the APLC and the sprayed plots and surrounding area (100 km^2) are located in grazing country. At the time of our investigations there was no chemical application in this region other than that used for locust control, by the APLC. It is therefore safe to make the assumption that birds caught during our investigations were only exposed to fenitrothion. All zebra finches, three of the four white-winged trillers and three of the six white-browed woodswallows caught after fenitrothion application, exhibited evidence of exposure to fenitrothion. Further, reactivated ChE in 19 birds of the 76 samples, collected from birds one, two, three and five days after the spray events, represents positive evidence that these animals were also exposed to ChE-inhibiting levels of fenitrothion. Not only the insectivorous locust-eaters were exposed, but also granivorous and honeyeater species showed reactivated ChE, indicating that a variety of exposure routes exist for birds during locust control (Table 3.2). In addition to plasma ChE inhibition, brain ChE in the white-winged triller collected 24 h after spraying at Tambo (2003) was inhibited by at least 29% (Table 3.2). In diagnostic work in wildlife toxicology brain cholinesterase inhibition greater than 50% is generally thought to be indicative of lethal OP or carbamate poisoning (Fairbrother et al. 1991). Considering that the reactivation method is conservative, and that not all

inhibited activity is reactivated, the data indicate that this individual had been exposed to OPs and it is possible that OP poisoning was the cause of its death.

Baseline plasma ChE in white-winged trillers fell within the range of control plasma ChE activities previously reported for passerines, while white-browed woodswallows and masked woodswallows were comparatively low (Burgess 1999). Baseline total ChE activity in zebra finches (0.69 μm/min/ml plasma, SD = 0.09; Table 3.1) in the present study was lower than previously reported values (1.054 μm/min/ml plasma, SD = 0.188; Holmes and Boag 1990), but within the range of ChE activity in captive zebra finches purchased from commercial suppliers (mean = 0.72 μm/min/ml plasma, SD = 0.24, n = 18; Karen Fildes, University of Wollongong, Wollongong, Australia, unpublished data). Diurnal and seasonal variation as well as diet may have contributed to this variability. These factors have been found to affect plasma ChE activity in some species (Thompson et al. 1988, Hill and Murray 1987).

Cholinesterase in plasma collected five days post spray was reactivated in three of eleven birds captured. This suggests that fenitrothion, and/or its metabolites, were still active for most of the sampling time period. Although both the proportion of birds with reactivating plasma and the magnitude of reactivation were larger on the first day following fenitrothion application, the difference between days was not significant and reactivation was still substantial on day five-post spray (Table 3.3). In previous studies maximum brain ChE inhibition occurred as far as seven and eleven days following spraying with fenitrothion and a longer sampling period was suggested by the authors (Hamilton et al. 1981). The present study however, targeted plasma ChE, which recovers more quickly than brain. Holmes and Boag (1990)

found that plasma ChE activity recovered to normal one to two days after zebra finches were dosed with 1.04 mg/kg and 3.80 mg/kg of fenitrothion. Despite using rapidly recovering plasma ChE as the biomarker and notwithstanding the low application rates used by the APLC, it would seem that a longer sampling timeframe is still required to establish the duration for which fenitrothion residues remain sufficiently high to affect birds.

It is important to note that these findings should be considered a minimum estimate of exposure. Depleting energy reserves caused by a reduction in food consumption, inactivity, or an inability to maintain flight as a result of fenitrothion exposure could cause birds to shelter if intoxicated (Grue et al. 1997, Fryday et al. 1995, Forsyth and Martin 1993). Sheltering by exposed individuals can lead to a bias in field sampling, as only birds well enough to be flying and feeding are likely to be caught in mist nets, while heavily exposed individuals would be poorly represented in the sample (Busby et al. 1991, Mineau and Peakall 1987). Further, our methods enabled us to catch only a cross section of the avian community that flock to locust outbreaks. For example, raptors are able to identify bands and follow locust movements from the air. Therefore locusts may represent an important component of their annual diet, and the extent of fenitrothion exposure in these species is yet to be investigated.

3.4.2 Significance of exposure

Fenitrothion exposure may have had significant detrimental impacts on native birds, since many were breeding and/or moulting during the locust control events (Table 3.2). Sublethal exposure to fenitrothion has been identified to be directly detrimental to breeding by contributing to disruption of breeding activity and reduction in number of young fledged (Varty 1980), territory abandonment, inability to defend

territory, disruption of normal incubation and clutch desertion (Busby et al. 1990). The authors of the latter study concluded that overall reproductive success was severely reduced when brain ChE activity was inhibited by 42% following a single application of fenitrothion, a level only 13% higher than the inhibition found in the white-wing triller brain at Tambo. Direct physiological effects from exposure to ChE-inhibiting chemicals during reproduction can include embryonic mortality and malformation, reduced egg production, reduced egg shell thickness, lower egg weight and decreased nestling weight (Mineau et al. 1994).

Fenitrothion is a broad-spectrum insecticide, its application causes an abrupt and dramatic reduction in insect numbers and, due to the consequent reduction in arthropod prey, bird numbers have been known to decrease (Carruthers et al. 1993, Mullie and Keith 1993). Locust population irruptions in Australia probably enhance breeding success for many local and invading avian species. The huge aggregations of flightless locust nymphs can provide a valuable nutritional resource for breeding and moulting birds. The late instar juvenile and adult female locusts are rich in protein (62% dry mass), fat (17% dry mass) and calcium (Defoliart 1975). In the present study, it was insectivorous species that exhibited the highest percentage of reactivated ChE (Table 3.2). Brown songlarks, white-winged trillers and masked woodswallows all showed evidence that a reproductive event was underway or had recently been completed. Fenitrothion application to locust bands could reduce avian reproductive output in these species through direct physiological effects, such as an inability to feed due to chemical intoxication, and indirectly through removal of the food supply in close proximity to nests (Morris 2005). Moulting birds would also be particularly vulnerable to a sudden and significant inability to feed, as avian moult is

a process requiring a substantial increase in energy expenditure and protein resources in most species (King and Murphy1990, Walsberg 1983).

This study has established that Australian native bird species were exposed to fenitrothion during APLC locust control operations. The effects of this exposure could be significant as many birds are in the process of reproducing and/or moulting, two of the most energetically expensive events in the avian life cycle. It could be argued then, that as part of the APLC's legislated environmental due diligence obligation (Story et al. 2005), additional study should be undertaken to provide sufficient information to inform a risk assessment. Research to evaluate the physiological and behavioural effects of sublethal exposure to fenitrothion in birds would be an invaluable. Further, whether or not such agricultural pest control operations have long-term implications for native bird populations could be evaluated through laboratory based reproductive studies along with field investigations into breeding success following locust control.

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4 THE EFFECT OF ACUTE FENITROTHION EXPOSURE ON A VARIETY OF PHYSIOLOGICAL INDICES, INCLUDING AVIAN AEROBIC METABOLISM DURING EXERCISE AND COLD EXPOSURE

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I conducted all dosing and blood sampling procedures and analysis of plasma samples for cholinesterase activity. I completed all physiological measurements, statistical analysis and drafted the manuscript. I received assistance from L. B. Astheimer and W. A. Buttemer in research design, logistics analysis and manuscript development.

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4. 1 Introduction

A number of studies have examined mortality as an endpoint of organophosphate (OP) exposure in birds (Mineau et al. 2006), but there is relatively little information on sublethal effects to predict other impacts that OP exposure might have on species of interest. Such information is particularly important when there is broad overlap of insect-control operations with high densities of non-target vertebrates. Such a scenario occurs in eastern Australia, where we have previously established that a variety of Australian native birds are exposed to fenitrothion during pesticide application for locust control (Fildes et al. 2006). Many exposed birds showed evidence of breeding and others were undergoing their annual moult, two of the most energetically expensive events in the avian annual cycle (King et al. 1990, Martin 1987).

Because OPs inhibit acetylcholinesterase (AChE), butylcholinesterase (BChE), and other esterase enzymes, they have the potential to act as neurotoxicants to both vertebrate and invertebrate species (Grue and Gilbert 1997). Even at sublethal levels, there is evidence that cholinesterase (ChE) inhibition can give rise to impairment of locomotor endurance and thermoregulatory ability in vertebrates (Grue et al. 1997). Such compromises in physical vigour and/or impaired thermoregulation in breeding and moulting birds could be detrimental to reproductive output, and the longer such pesticide effects persist, the less likely these birds will be capable of meeting their energetic requirements.

We therefore examined the extent and duration of sublethal effects of fenitrothion in selected species of birds: house sparrows (*Passer domesticus*), zebra finches

(*Taeniopygia guttata*), and king quails (*Coturnix chinensis*). We utilised several physiological parameters to assess impacts, including two ecologically relevant aerobic performance traits: flight and thermoregulatory capacity. Because peak aerobic metabolism requires the integrated function of enzymes, organelles, cells, tissues, organs, and organ systems (Chappell et al. 1999a), measuring peak metabolic rate (PMR) during flight provides a meaningful evaluation of an individual's overall physical vigour. Aerobic metabolism during cold exposure was measured as OPs can potentially affect thermoregulatory response to cold. This effect can occur either directly by perturbing hypothalamic neural circuitry (Grue et al. 1991, Hissa and Rautenberg 1975), or indirectly through inhibitory effects on shivering by skeletal muscles (Bicudo et al. 2001). Blood haemoglobin (Hb) content was considered a useful adjunct to metabolic measurements as changes in Hb, and therefore oxygen carrying capacity, have been shown to be influenced by changes in body condition, aerobic activity and aerobic endurance (Davey et al. 2000, Dufva 1996). Cholinesterase activity was measured as a biomarker to confirm that administered doses were biochemically effective in all species.

4. 2 Materials and Methods

4.2.1 Experimental animals

Free-living house sparrows were caught with mist-nets from March through to June 2006 in the Wollongong area and transferred to outdoor aviaries at the University of Wollongong. We obtained wild-type zebra finches and king quails from a commercial supplier. All birds were individually banded with metal bands, housed in outdoor aviaries (3,500 x 2,100 x 2,500 cm), and provided with food and water ad

libitum. Birds were allowed to adjust to caging conditions for at least two weeks before physiological measurements were taken.

4.2.2 Pesticide administration

Birds were placed in small holding cages the afternoon before dosing. Food was removed at 5 PM and pesticide administration began at 8 AM the following morning. Fenitrothion (Sumintomo Chemical Australia, Chatswood, NSW, Australia) was diluted in canola oil. The pesticide was administered via gavage to treated house sparrows and zebra finches in a 0.1 ml volume and in a 0.4 ml volume to treated king quail, using a curved gavage needle attached to 1 ml syringe. All pesticide treated birds were dosed simultaneously with control birds that received canola oil alone.

Blood samples were taken following all physiological measurements. However, it was not possible to take more than one blood sample every 10 d without potentially impacting animal health and thereby effecting experimental outcomes. Therefore to sample at close time intervals and gain an understanding of the duration of effects, more than one group of house sparrows, zebra finches and king quails were dosed per concentration of fenitrothion. Unless otherwise stated all dose groups consisted of eight birds.

House sparrows were used to fully evaluate the experimental protocol and gain some understanding of extent of fenitrothion effects on performance over a range of doses. House sparrows were divided into fenitrothion-dose groups; high (100 mg/kg fenitrothion, medium (60 mg/kg fenitrothion) and low (30 mg/kg fenitrothion). There were two groups of high dose house sparrows and two of medium dose house sparrows. The low dose was a true sublethal dose as there was no resultant mortality.

Low dose consisted of two groups of dosed birds, one of which was only used to obtain a blood sample at 6 h post-dose for ChE analysis.

Four groups of zebra finches were dosed with 3 mg/kg fenitrothion, one of which was only used to obtain a blood sample at 6 h post-dose for ChE analysis. This dose was chosen after conducting a dosing trial with 4 birds and with reference to Holmes and Boag (1990), who reported no detrimental effects of fenitrothion on zebra finches dosed at 1 mg/kg. In the present study one of two birds died after being dosed with 5 mg/kg, while two birds survived a dose of 3 mg/kg. Considering results reported by Holmes and Boag (1990) as well as these dose trials, a mid-range dose of 3 mg/kg was chosen as a reasonable estimate of a high sublethal dose.

Because fenitrothion sensitivity in king quails was also unknown, we followed the Organisation for Economic Co-operation and Development (OECD) guideline for testing of chemicals: the acute oral toxicity – up and down procedure (OECD 2001 Guidelines for the testing of chemicals. www.oeck.org) to establish a sublethal dose. We identified the lowest lethal dose of fenitrothion to an individual king quail, and used the next lowest dose on the OECD dosing schedule (slope six), thereby establishing the sublethal concentration of pesticide. Three groups of dosed king quails received 26 mg/kg fenitrothion, one of which was only used to obtain a blood sample at 6 h post-dose for ChE analysis.

4.2.3 Metabolism during cold exposure

All pre-dose measurements were taken two weeks before treated birds received pesticide or control birds received canola oil alone. House sparrows (low dose) and king quails were placed in 2 L respirometers fashioned from paint cans, which were

fitted with interior perches and inlet and outlet tubing. Respirometers were then transferred to a 25 °C refrigerated incubator. A gas mix of 79% helium and 21% oxygen (He-O_2) was provided to each respirometer at a flow rate of 1000 ml/min and controlled with mass-flow controllers (Tylan Model FC-280S, West Technology Systems, Yate, Bristol, UK). Because helium conducts heat four times faster than nitrogen, this mixture permits maximal rates of heat loss at higher temperatures than in air and, thus, reduces potential freeze-damage to tissues during cold exposure (Rosenmann and Morrison 1974). Respirometer exhaust gas passed though water and carbon dioxide absorbants (Drierite and soda lime, respectively) prior to gas analysis with an oxygen analyzer (Sable Systems Oxzilla, Sable Systems International, Las Vegas, NV, USA). Output signals from the oxygen analyser and mass flow meters were recorded on a Macintosh computer (Apple, Cupertino, CA, USA) fitted with an A-to-D converter and custom designed software (Warthog Systems, University of California, Riverside, CA, USA, www.warthog.ucr.edu). Once animals were in place for a 30 min adjustment period, the incubator was set to 2^{o}C and reached that temperature within 30 min, and due to the heliox the temperature was equivalent to below freezing. The birds were removed after remaining in the respirator for a further 60 min. Maximum metabolic rate (MMR) during cold exposure was selected as the highest oxygen consumption rate recorded during three consecutive minutes of cold exposure. The total oxygen consumed throughout the 60 min period was also calculated and is termed the integrated oxygen consumption. All oxygen consumption (VO_2) values are corrected to STP (standard temperature and pressure defined by International Union of Pure and Applied Chemistry as air at 0^{o}C and one standard atmospheric pressure) and volumes and the maximum VO_2 was computed

from the highest instantaneous VO_2 measured during a 3 min interval (Chappell et al. 1999b).

4.2.4 Metabolism during exercise

All pre-dose measurements were taken two weeks before treated birds received pesticide or control birds received canola oil alone. Birds were enclosed in a motorised wheel made of clear Perspex with an aluminium rim and carpet lining (flight wheel) through which an airflow of 5 L air/min was provided via a mass-flow controller (MKS Instruments, Cheshire, UK) as described in Chappell et al. (1999b). Oxygen content (+ 0.002%) of inlet and outlet air was measured using an oxygen analyzer (Sable Systems Oxzilla dual absolute and differential oxygen analyzer). Water and CO_2 were removed from sampled air prior to gas analysis using drierite and soda lime, respectively. Output air from the oxygen analyzer and flow meter were recorded on a Macintosh computer fitted with an A-to-D converter and custom designed software (Warthog Systems, www.warthog.ucr.edu). When oxygen analysis components were in place, birds were placed in the flight wheel, and after a 3 min settling period, the wheel rotation began. While the flight wheel was rotating birds were unable to perch and so were either constantly in flight, or running and performing flight take-offs thereby enforcing their intense exercise. Rotation speed began at a speed of approximately 0.3 m/s (at the rim) and increased to a rotation of up to 0.8 m/s. The wheel was stopped as soon as the animal showed signs of exhaustion. All oxygen VO_2 values are corrected to STP volumes and the PMR was computed from the highest instantaneous VO_2 measured during a 1 min interval (Chappell et al. 1999b).

4.2.5 Blood haemoglobin concentration and body mass

After metabolic measurements and before blood samples were taken birds were weighed to the nearest 0.1 g (Mettler Toledo Balance, Mettler Toledo, Port Melbourne, VIC, Australia). Blood samples were taken from a brachial vein following venipuncture with a 25-ga needle and collected into heparinised microhematocrit tubes. A portion of the whole blood collected in microcuvettes (Hemocue Australia, Tumbi Umbi, NSW) was used to measure haemoglobin content to the nearest 0.1 g/dl using a Hemocue haemoglobin analyzer (Hemocue Australia). The remaining blood was centrifuged and plasma extracted and stored in a –80^{o}C freezer until later ChE analysis.

4.2.6 ChE analysis

Diluted plasma samples were assayed using the Ellman et al. (1961) method as modified by Gard and Hooper (Gard and Hooper 1993) for use in a 96-well spectrophotometric plate reader (BioRad, Bio-tek, Winooski, Vermont, USA) equipped with software for enzyme kinetic analysis (KC Junior, Bio-tek, Winooski, Vermont, USA). All samples were run in triplicate at 25^{o} C and activity measured at 412 nm for 3 min (readings taken at 13 s intervals). The substrate acetylthiocholine iodide (AThCh, Sigma-Aldrich, Castle Hill, NSW, Australia), 5,5'-dithiobis (2-nitrobenzoic acid) (Sigma-Aldrich), tris 8.0 buffer and enzyme dilution were added to microplate wells. In house sparrows and zebra finches AChE was differentiated from BChE by preincubation with the specific BChE inhibitor, tetraisopropyl pyrophosphoramide (iso-OMPA, Sigma). Butyrylcholinesterase activity was then calculated as the difference between total ChE and AChE activities. Cholinesterase measurements in king quails are restricted to total ChE activity as no AChE was detected in an earlier characterisation of the plasma in this species (Fildes et al. 2008, University of Wollongong, Wollongong, NSW, Australia, unpublished data). Horse serum (Sigma) was used as a positive inter-assay control. Cholinesterase activity (optical density units/min) was converted to μM AThCh (substrate) hydrolyzed min/ml plasma using the extinction coefficient 13,600/cm/M.

Sample sizes vary between metabolic measurements and ChE analysis due to either mortality or individual blood samples obtained were unmeasurable.

4.2.7 Summary of statistical tests

Evaluations of fenitrothion effects on VO_2, MMR, and PMR in all birds were examined using paired-sample Student's *t*-tests. Values measured before pesticide ingestion in treated birds, and before canola oil ingestion in control birds, were compared with values measured post-ingestion (Zar 1999). Bonferroni sequential adjustment was applied when when multiple endpoints were measured on the same individual. The significance level was $p < 0.025$ (0.05/2) in king quail and low-dose house sparrows as metabolic rate was measured during exercise and then during cold exposure. The significance level was $p < 0.05$ in zebra finch, and high and medium-dose house sparrows as metabolic rate was measured during exercise only. Evaluations of ChE inhibition were examined using an analysis of variance comparing the mean ChE activity of birds after pesticide ingestion to the mean activity in birds unexposed to fenitrothion. The significance level was $p < 0.05$; results are expressed as mean ± standard error and all figures or figure legends include sample sizes (*n*). Analyses were performed using JMP statistical software (Ver. 5, SAS, Carey, NC, USA).

4. 3 Results

4.3.1 Dosing toxicity

Seven of 16 house sparrows (high) and three of 16 house sparrows (medium) died within 3 d of ingesting fenitrothion. House sparrows (low) were behaviourally asymptomatic, with no visual signs of muscle tremors, reduced activity, or ptiloerection and no mortality resulted from this dose. Zebra finches were very sensitive to fenitrothion with 25% mortality at the relatively low dose of 3 mg/kg, whereas king quail suffered no mortality after ingesting 26 mg/kg fenitrothion.

4.3.2 Metabolism during cold exposure

All control birds had statistically indistinguishable MMR and integrated VO_2 before, and at any time after dosing with canola oil ($p > 0.2$). The mean MMR during cold exposure before pesticide ingestion was 7.3 (± 0.2) ml/min in sparrows (low-dose) and 8.0 (± 0.2) ml/min in king quail. Fenitrothion treatment had no effect on MMR in house sparrows ($p > 0.3$) or king quail ($p > 0.19$, Fig. 4.1.) at any time post-pesticide ingestion. Fenitrothion also had no effect on integrated VO_2 in king quail ($p > 0.07$) or house sparrows ($p > 0.1$, Fig. 4.1.).

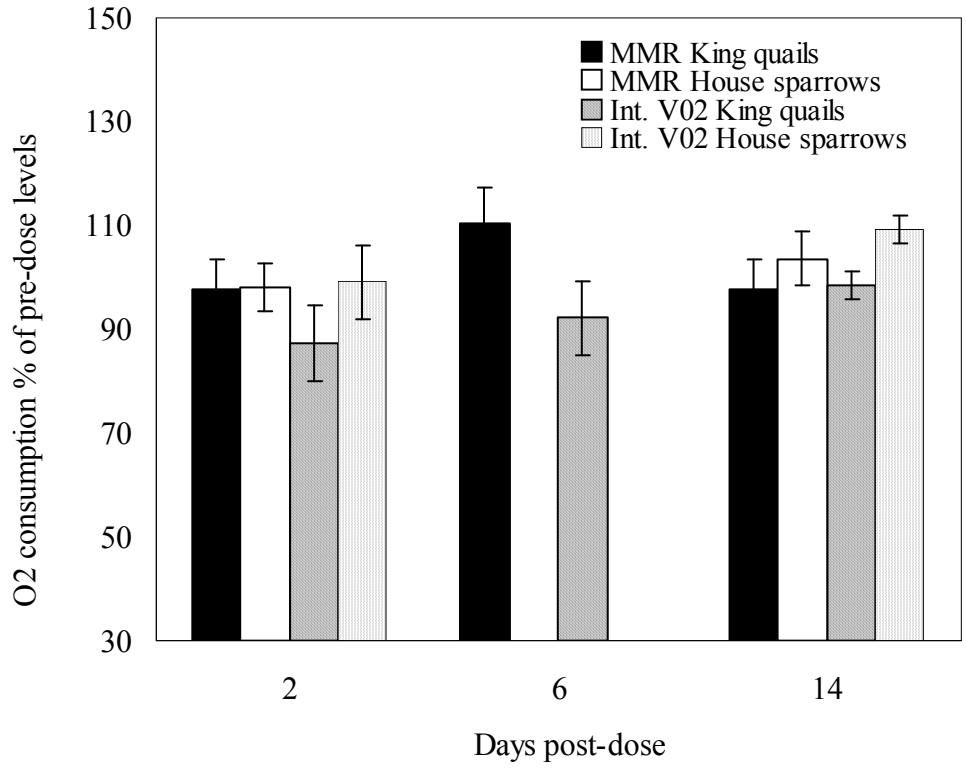

Figure 4-1 Mean maximum metabolic rate (MMR) and integrated (Int.) oxygen consumption (VO_2) as a percentage of pre-dose levels during cold exposure after dosing with 30 mg/kg fenitrothion in house sparrows and 26

mg/kg fenitrothion in king quails. (± 1 standard error; number of individuals sampled = eight treated and eight control birds for both species)

4.3.3 Metabolism during exercise

All control birds had statistically indistinguishable PMR before and any time after dosing with canola oil ($p > 0.2$). Pre-treatment PMR measurements in house sparrows averaged 12.3 (± 0.3) ml/min. In zebra finches pre-treatment PMR averaged 7.6 (± 0.5) ml/min and the pre-treatment average oxygen consumption in king quail was 6.5 (± 0.8) ml/min. While in the flight chamber, exercise in house sparrows and zebra finches took the form of a rapidly repeated series of flight/landing/flight take-offs, but the king quail mostly ran with occasional flying/flapping. The PMR in house sparrows dosed with 100 mg/kg fenitrothion was significantly reduced from pre-dose levels. In these birds, aerobic performance 48 h post-dose was 42% of pre-dose measurements and remained substantially impaired for up to 14 d (Fig 4.2). The low and medium dose of fenitrothion also significantly reduced PMR in house sparrows. Exercise performance was significantly reduced by 20% in sparrows dosed with 30 mg/kg ($p < 0.02$) fenitrothion and by 18% in sparrows dosed with 60 mg/kg ($p < 0.03$, Fig 4.3). Peak metabolic rate during exercise was reduced 2 d post-dose by 58% in the high-dose group of house sparrows ($p < 0.02$; Fig 4.2). By 14 d post-fenitrothion ingestion, flight capacity had recovered to pre-dose measurements in the medium-dose group, however aerobic performance was still significantly impaired in the high-dose group ($p < 0.01$) and did not return to pre-dose capacity until 21 d post-dose (Fig 4.2).

Aerobic performance was significantly reduced in zebra finches two hours after fenitrothion ingestion ($p < 0.01$), and remained significantly reduced at 72 h post-dose ($p < 0.03$; Fig 4.3). The aerobic capacity of king quail was significantly reduced by 23% 2 d after pesticide administration ($p < 0.01$), however treated birds had recovered to pre-dose capacity by 6 d post-dose ($p > 0.5$; Fig 4.4).

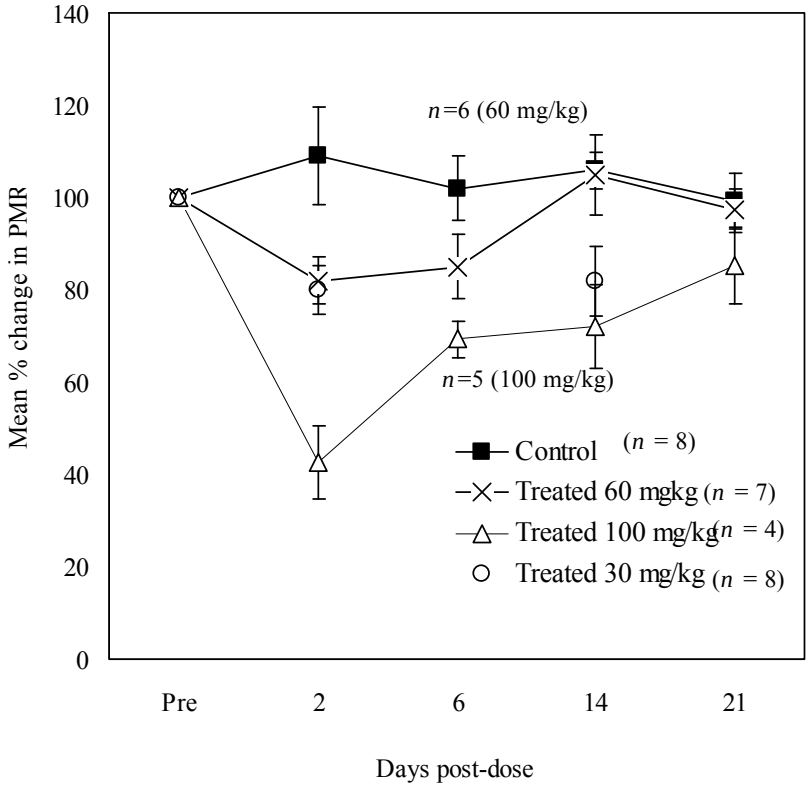

Figure 4-2 Mean peak metabolic rate as a percentage of pre-dose levels during exercise in house sparrows before and after dosing with fenitrothion in pesticide-exposed and unexposed birds. (± 1 standard error; number of individuals sampled = eight treated and eight control birds, except where specified as (*n*) beside data-points).

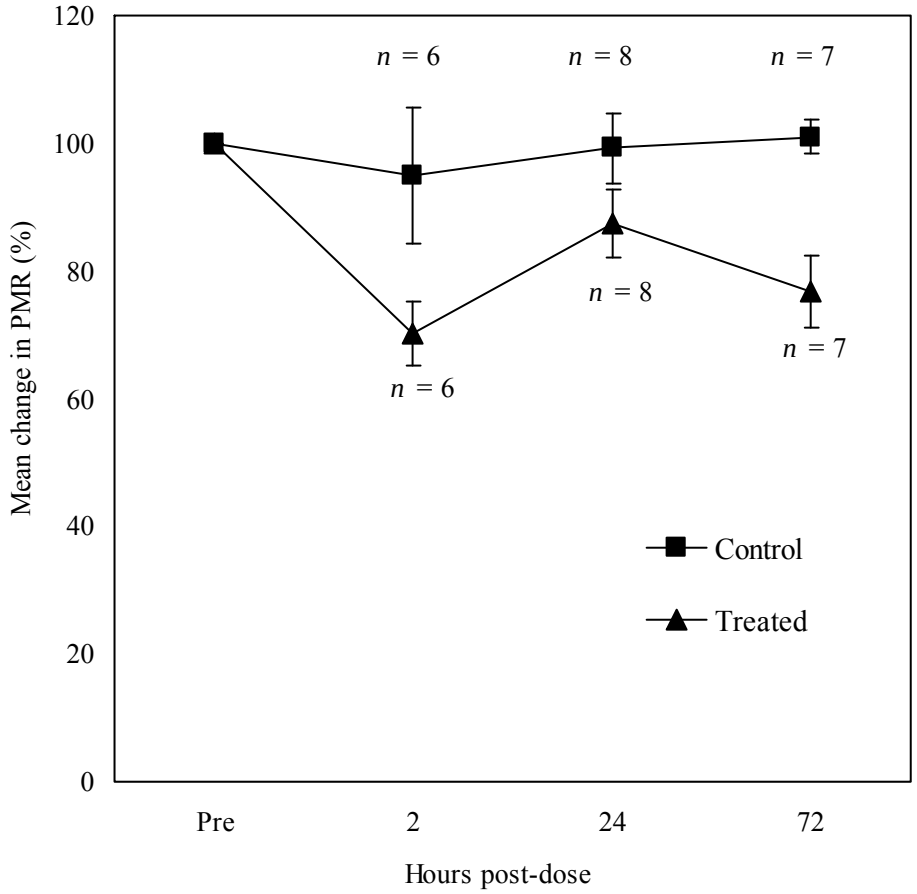

Figure 4-3 Mean percentage change in peak metabolic rate during exercise in zebra finches before and after dosing with fenitrothion in exposed and unexposed birds (± 1 standard error; *n* = number of individuals).

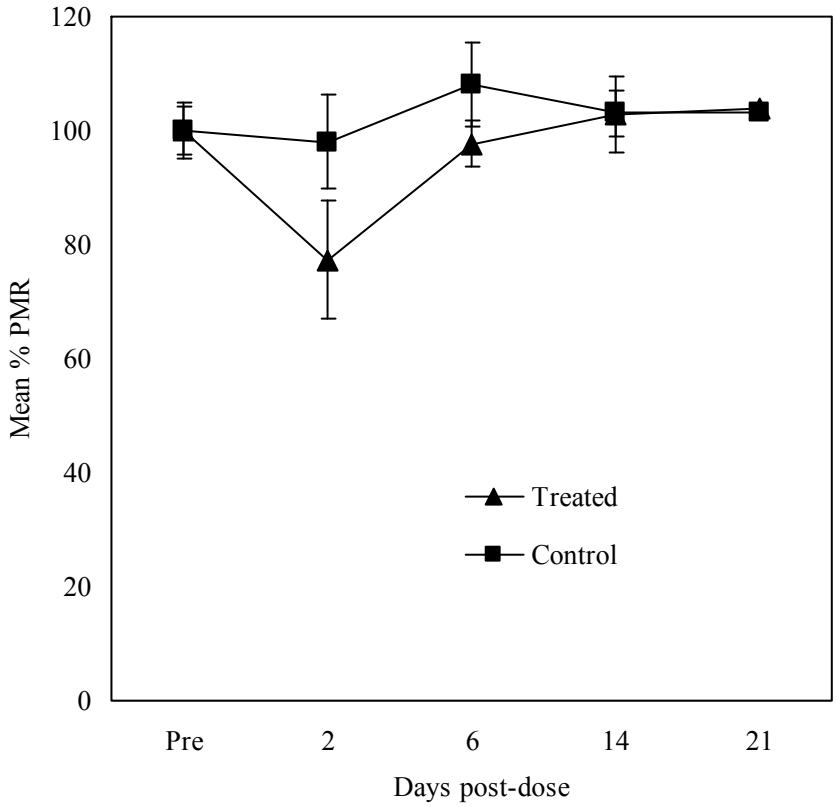

Figure 4-4 Mean percentage change in peak metabolic rate during exercise in king quails before and after dosing with fenitrothion in exposed and unexposed birds. (± 1 standard error; number individuals sampled = eight treated and eight control birds)

4.3.4 Blood haemoglobin and body mass

House sparrows ranged in body mass from 21.6 to 27.8 g, zebra finches from 10.5 to 14.3 g, and king quails ranged in body mass (Mb) from 35.4 to 52.6 g. There was no change in mean Mb in control or treated zebra finches, medium dose or low dose house sparrows at any time post fenitrothion ingestion ($p > 0.2$). Body mass in house sparrows in the high-dose group was significantly lower than pre-dose weight for up to 48 h post-dose ($p < 0.006$). In treated king quails Mb was significantly lower than

pre-dose at 48 h post-dose ($p < 0.01$) and although mean weight remained lower at 6 d post-dose, the difference was not significant ($p > 0.055$).

Whole-blood haemoglobin content in house sparrows ranged from 12.5 to 20.2 g/dl, in zebra finches from 15.7 to 21.1 g/dl and from 11.9 to 20.3 g/dl in king quails. There was no significant difference between pre-dose and post-dose haemoglobin content in control or treated birds at any time, in any species ($p > 0.1$).

4.3.5 Cholinesterase activity

Fenitrothion-treated house sparrows from the high and medium-dose groups had total ChE and AChE activities inhibited by over 90% 2 d after fenitrothion ingestion compared to ChE activity in unexposed birds. Total ChE activity in sparrows from the high and medium dose groups had a longer recovery time than did AChE. In the high-dose group, total ChE activity remained significantly inhibited for up to 14 d post-dose, and for up to 6 d in the medium-dose group ($p < 0.005$, Table 4.1). In low-dose sparrows, total ChE was significantly inhibited by 88%, and AChE activity by 75% 6 h after fenitrothion ingestion, however there was no significant inhibition at 2 d post-dose when compared to unexposed birds ($p > 0.44$, Table 4.1).

Total ChE and AChE activity in zebra finches was at maximum inhibition 6 h post-dose (Table 4.1). Total ChE was inhibited by 66% and AChE by 78% ($p < 0.03$, Table 4.1). Cholinesterase activity was not significantly inhibited 24 h post-dose ($p > 0.1$) and had completely recovered to within the range of control birds by 3 d post dose ($p > 0.4$). The lowest measure of ChE activity in king quails also occurred at 6 h post-dose, with ChE activity in treated birds inhibited by 94% ($p < 0.0001$, Table

4.1). However, by 48 h post-dose, plasma activities in king quails were not significantly lower than activities in unexposed individuals ($p > 0.11$; Table 4.1).

Table 4-1 Mean plasma cholinesterase activity in birds exposed and unexposed to fenitrothion (* = significantly lower activity compared to unexposed samples)

Total Cholinesterase activity (μmols/min/ml plasma)		6 h	24 h	48 h	72 h	6 d	14 d	21 d	Unexposed Birds
Zebra finch	Mean	0.264*	0.442		0.680				0.631
	SD	0.20	0.22		0.28				0.17
	n	7	6		5				7
King quail	Mean	0.08 *		1.200		1.640			1.850
	SD	0.02*		0.58		0.65			0.72
	n	7		6		6			10
House sparrow	Mean	0.087*		0.420			0.644		0.626
(30 mg/kg)	SD	0.04		0.14			0.17		0.23
	n	8		8			7		8
House sparrow	Mean			0.033*		0.283*	0.443	0.560	0.603
(60 mg/kg)	SD			0.02		0.10	0.12	0.10	0.12
	n			6		5	5	6	8
House sparrow	Mean			0.011*		0.14*	0.405*	0.516	0.607
(100 mg/kg)	SD			0.01		0.09	0.04	0.10	0.11
	n			4		4	5	4	8
Acetylcholinesterase activity (μmols/min/ml plasma)									
Zebra finch	Mean	0.102*	0.246		0.550				0.440
	SD	0.10	0.15		0.23				0.16
	n	7	6		5				7
House sparrow	Mean	0.050*		0.137			0.169		0.199
(30 mg/kg)	SD	0.03		0.03			0.06		0.07
	n	8		8			7		8
House sparrow	Mean			0.03*		0.087	0.154	0.183	0.151
(60 mg/kg)	SD			0.02		0.02	0.03	0.05	0.03
	n			6		5	5	6	8
House sparrow	Mean			0.005*		0.097	0.130	0.131	0.150
(100 mg/kg)	SD			0.01		0.06	0.04	0.10	0.04
	n			4		4	4	4	8

4. 4 Discussion

4.4.1 Metabolism during cold exposure

We found that fenitrothion had no effect on the ability of king quail and house sparrows (low) to respond metabolically to decreasing temperature. Our

measurements of MMR during cold exposure in both treated and control king quails and house sparrows are more than six times the basal rates recorded in both species (Chappell et al. 1999a, Hinds et al. 1993) which is consistent with maximal rates of cold-induced thermogenesis reported in birds (Hinds et al. 1993). Although body temperature was not measured in the present study, these results suggest that the birds exposed to fenitrothion did not experience adverse affects on their thermoregulatory control centre or on effector mechanisms of heat generation. The absence of OP effects was also found by Bain et al. (2004) who measured preferred body temperature and standard metabolic rate in the central bearded dragon (*Pogona vitticeps*) and found that fenitrothion had no effect on either physiological variable.

These results contrast with the reported impairment of thermoregulation in birds exposed to cholinesterase-inhibiting pesticides (Grue et al. 1997). Exposure to cold has been demonstrated to increase the toxicity of OPs in northern bobwhite quail (*Colinus virginianus*), American kestrels (*Falco sparverius*) (Rattner and Becker 1987, Rattner and Franson 1984) and mallard ducklings (*Anas platyrhynchos*) (Fleming et al. 1985). Our results may reflect the fact that birds in the current study only experienced cold temperatures for very limited periods, whereas birds chronically exposed to cold may have a different response to OPs at the same dose.

4.4.2 Blood haemoglobin and body mass

The reduced Mb in treated king quails, and house sparrows (high), was expected and likely to be due to reduced food consumption caused by nausea from pesticide exposure. Reduced feeding is well documented and referred to as pesticide-induced anorexia (Grue 1982). However the lack of effect of fenitrothion on Mb in house sparrows (medium and low), and in zebra finches was surprising, especially

considering the resultant zebra finch and house sparrow (medium) mortality. Given the size of these passerines, any reduction in feeding is easily detectable by fluctuations inMb. The cause of mortality therefore, was not likely to be due to a secondary effect of intoxication, such as suppressed feeding, but rather to the direct toxic action of fenitrothion. This together with the lack of effect on Hb and on metabolism during cold exposure does provide some evidence that the observed impact of fenitrothion on performance is not due to an effect on energy and oxygen supply.

4.4.3 Metabolism during exercise

Peak aerobic exercise, as shown by previous studies, elicits a greater increase in oxygen consumption rate than does shivering in response to cold exposure (Hinds et la. 1993). In the present study, unexposed house sparrows showed a 12-fold increase (Buttemer et al. 2008a); unexposed zebra finches an 11-fold (Hinds et al. 1993) and unexposed king quails a 5-fold increase in oxygen consumption during exercise over their reported basal metabolic rate (Roberts and Baudinette 1986). From a mechanistic viewpoint such locomotor and aerobic performance requires the effective and coordinated functioning of the total body system. The repeatability of measurements assessing these performance indicators has been confirmed in the present study, as control birds remained invariant throughout the course of the experiment, and our measurements revealed compromised physical vigour and recovery from exposure when other indicators showed no evidence of pesticide effects.

Despite being asymptomatic with regard to all other measured variables, aerobic capacity in zebra finches remained impaired by 17% in this species 3 d after dosing

(Fig 4.3). The adverse effect of fenitrothion on locomotor performance was also evident in king quails and house sparrows. At 48 h post-pesticide ingestion, treated sparrows (low) were behaviourally asymptomatic, Mb was invariant and ChE activity was not significantly different to unexposed birds (Table 4.1) whereas locomotory aerobic capacity remained significantly impaired (Fig 4.2). The extent and duration of PMR depression was even greater in the high-dose group. Aerobic performance in sparrows dosed with 100 mg/kg was 58% lower than pre-dose measurements 2 d after pesticide ingestion (Fig 4.2), and it remained significantly impaired for up to 14 d, by which time ChE activity was restored (Table 4.1).

Locomotor performance, due to its association with survival and fitness, has recently been identified as a useful toxicological endpoint by several investigators (Holem et al. 2006, Hopkins et al. 2005). The findings presented here are consistent with the recent investigations into the effect of ChE-inhibiting pesticides on locomotor performance. In studies with the western fence lizard (*Sceloporus occidentalis*), malathion was found to have no effect on sprint velocity (Holem et al. 2006), whereas carbaryl slowed sprint speed and impaired arboreal performance (DuRant et al. 2007). Carbaryl also slowed swimming velocity in swamp snakes (*Seminatrix pygaea*) and diamondback water snakes (*Nerodia rhombifer*) (Durant et al. 2007). Similarly, experiments in our laboratory found that fenitrothion reduced running endurance in dunnarts (*Sminthopsis crassicaudata*) (Buttemer et al. 2008b) and Punzo (2003) found that carbyl resulted in a slower running speed in the meadow jumping mouse (*Zapus hudsonius*). Our results together with the previous findings provide evidence that impaired locomotor performance may be a generalised result of OP exposure in vertebrates.

A large aerobic capacity is particularly important for birds because of the high power output needed to gain lift and sustain flight (Loli and Bicudo 2005). Flight requires increases of approximately eight times resting energy expenditure (Marsh and Dawson 1989). Constraints on maintaining this level of output limit a bird's ability to engage in activity and thus have significant impacts on free-living birds, especially those breeding and moulting during locust control (Fildes et al. 2006). Success in breeding through activities such as courtship, aggressive displays, competing for nesting sites and nest defence, depend to a great extent on aerobic endurance (Marsh and Dawson 1989, Chappell et al. 1999b). Impaired aerobic performance could negatively impact on any or all of these activities, as well as an animal's ability to supply adequate food to themselves and/or their young, capture prey or escape from predators.

In conclusion, we have found that fenitrothion has the potential to compromise an animal's physiological vigour for a significant length of time post-exposure. Further, exercise intensity is a useful indicator of pesticide effects even at sublethal doses, when exposure is undetectable using other variables. The pesticide-induced lethality resulting from locomotor impairment in free-living birds would be under-estimated by the standard measure for expressing and comparing toxicity of chemicals, the LD50 (lethal dose that kills half the population of animals tested) established under laboratory conditions. This highlights the importance of using performance-based measures of pesticide effects when examining ecologically relevant risk factors in a given species.

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5 THE EFFECT OF AN ACUTE SUBLETHAL EXPOSURE TO FIPRONIL-BASED ADONIS 3UL® INSECTICIDE (BASF) ON AVIAN AEROBIC METABOLISM DURING FLIGHT AND COLD EXPOSURE

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I completed all dosing proceedures, physiological measurements, statistical analysis and drafted the manuscript. I received assistance from L. B. Astheimer and W. A. Buttemer in research design, logistics analysis and manuscript development.

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5. 1 Introduction

Fipronil [(±)-5-amino-1 (2,6-dichloro-alpha, alpha, alpha-trifluoro-p-tolyl)-4-trifluoromethyl-sulfinylpyrazole-3-carbonitrile] a phenol pyrazole, is a relatively new class of pesticide that elicits neurotoxicity by blocking the $\tilde{\gamma}$-aminobutyric acid (GABA)-gated chloride channels in the central nervous system (Hainzl et al. 1998). Fipronil controls a broad spectrum of insects, is effective at low application rates, but because it has a relatively long half-life and a lipophilic character, it can bioaccumulate following application (Gunasekara et al. 2007, Chaton et al. 2001, Balanca et al. 1997). The degradation products of fipronil have been shown to have similar toxic potential (Schlenk et al. 2001, Hainzl et al. 1998) and to be more environmentally stable than the parent compound (Hainzl and Cassida 1996). Fipronil is applied in Australia to control locust outbreaks as Adonis 3UL ® Insecticide (BASF), a formulation containing 0.3% fipronil as active pesticide. Kitulagodage et al. (2007) found that toxicity varied significantly with formulation. For example, the LD50 of technical grade fipronil in zebra finches was 310.2 mg/kg while Adonis 3UL was 45.41 mg/kg. We therefore clarify that it is the Adonis 3 UL ® Insecticide formulation of fipronil we will be referring to throughout this study.

Locust control in Australia and resultant Adonis 3 UL ® application generally occurs in the spring and summer months when favourable conditions have stimulated locust outbreaks (Hunter et al. 2001). The same conditions can also stimulate breeding in Australian native bird species in the arid and semi-arid zone (Ford 1989). Three possible routes of exposure to fipronil in birds during locust control have been identified (Szabo 2005). Residues found on seeds, vegetation and locusts have been assessed and the risk of adverse effects of fipronil in avian species has been

predicted. Szabo (2005) using reported food consumption rates in various species and pesticide residue analysis, estimated that 5% of exposures would lead to LD_{50} level mortality in the most sensitive species. Based on potential for exposure Szabo (2005) reasoned that species likely to be at the greatest risk of fipronil effects included obligate granivorous species, especially finches, while insectivorous species and birds of prey are also at risk to pesticide exposure.

There is limited information on the effects of fipronil in non-target vertebrate species. However, the pesticide has been shown to impair the development of spinal locomotor pathways in fish (Stehr et al. 2006); to have adverse reproductive effects in rats, to result in alterations in thyroid hormones, neuronal activity and behavioural activity in rats (Szegedi et al. 2005, Ohi et al. 2004, Hurley et al. 1998); and to be detrimental to several species of lizards (Peveling and Demba 2003). Similar studies on avian species are lacking despite avian toxicity to technical grade fipronil being highly variable. The LD50 of northern bobwhite quails (*Colinus virginianus*), for example is as low as 11.3 mg/kg, while it is 1120 mg/kg in field sparrows (*Spizella pusilla*) and is greater than 2150 mg/kg in mallard ducks (*Anas platyrhynchos*) (Tingle et al. 2003). While assessments of toxicity with mortality as an endpoint are invaluable, their usefulness in risk assessment is limited as they ignore sublethal effects. Sublethal pesticide effects on birds are wide and varied and include reproductive effects, impairment to locomotory performance and impacts on thermoregulatory ability (DuRant et al. 2007, Grue et al. 1997, Mineau et al. 1994). Given the limited information on fipronil, coupled with the variability in its toxicity, its potential for environmental persistence, and the added toxicity of its metabolites, investigations into sublethal effects on avian species are clearly mandated.

The aim of this investigation therefore, was to assess sublethal effects of fipronil on metabolic performance in two native granivorous birds. For our study species we targeted a passerine, the zebra finch (*Taeniopygia guttata*) and a non-passerine species, the king quail (*Coturnix chinensis*). We examined the extent and duration of effects by utilizing aerobic metabolism (oxygen consumption) as a measure of physiological performance. Metabolic measurements were taken before and after pesticide ingestion, during exercise (flight and flight take-off), in both species. The measurement of peak metabolic rate (PMR) during exercise in birds has proven to be repeatable over time (Chappell et al. 1996) and aerobic performance provides a meaningful evaluation of an individual's overall physical vigour (Chappell et al. 1999). The maximum metabolic rate (MMR) and integrated oxygen consumption (VO_2) in king quail was measured during cold exposure before and after pesticide ingestion to enable the evaluation of pesticide effects on an animal's metabolic response to cold and resultant thermoregulatory abilities.

5. 2 Materials and Methods

5.2.1 Experimental animals

We obtained wild-type zebra finches and king quails from a commercial supplier. All birds were individually banded with metal bands, housed in outdoor aviaries (3,500 x 2,100 x 2500 cm), and provided with food and water ad libitum at the University of Wollongong. Birds were allowed to adjust to caging conditions for at least two weeks before physiological measurements were taken.

5.2.2 Experimental protocols

Metabolic rate during flight take-off and cold exposure were measured in all birds two weeks before they were dosed with pesticide. King quail, were then measured 2 d, 6 d and 14 d post-fipronil exposure. Zebra finches were measured 1 d, 2 d, 10 d and 20 d post-pesticide ingestion. All post-dose metabolic measurements were analysed and compared with values from pre-dose measurements.

5.2.3 Pesticide administration

To establish a sublethal dose we followed the "OECD guideline for testing of chemicals: the acute oral toxicity – up and down procedure" (OECD 2001, www.oecd.org.). The intention was to find the lowest dose lethal to an individual bird, and use the next dose lower on the dosing schedule (slope 6), thereby establishing the sublethal dose. Zebra finch, ranging in body mass from 10 to 14.7 g, received 17.5 mg/kg fipronil and king quail weighing from 35.2 to 51.1 g were dosed with 30 mg/kg fipronil.

Birds were placed in small holding cages the night before dosing. Food was removed at 5 PM and pesticide administration began at 8 AM the following morning. The Adonis 3UL ® (supplied by the Australian Plague Locust Commission) was diluted in canola oil. The pesticide was administered to zebra finch in a 0.1 ml volume and a 0.4 ml volume to treated king quail, by gavage using a curved gavage needle attached to a 1 ml syringe. Control birds received canola oil alone.

5.2.4 Metabolism during exercise

Birds were enclosed in a motorised wheel made of clear Perspex with an aluminium rim and carpet lining (flight wheel) through which an airflow of 5 L air/min was

provided via a mass-flow controller (MKS Instruments, Cheshire, UK) as described in Chappell et al. (1999). Oxygen content (± 0.002%) of inlet and outlet air was measured using a Sable systems oxygen analyser (Sable systems Oxilla, Sable Systems International, Las Vegas, NV, USA). Water and CO_2 were removed from sampled air prior to gas analysis using drierite and soda lime, respectively. Output air from the oxygen analyser and flow meter were recorded on a Macintosh computer fitted with an A-to-D converter and custom designed software (Warthog Systems, University of California, Riverside, CA, USA www.warthog.ucr.edu). When oxygen analysis components were in place, birds were placed in the flight wheel, and after a 3 min settling period, the wheel rotation began. While the flight wheel was rotating birds were unable to perch and so were either constantly in flight, or running and performing flight take-offs thereby enforcing their intense exercise. The wheel was stopped as soon as the animal showed signs of exhaustion. All oxygen consumption (VO_2) values are corrected to STP (standard temperature and pressure defined by International Union of Pure and Applied Chemistry as air at 0^oC and one standard atmospheric pressure) volumes and the MMR was computed from the highest instantaneous VO_2 measured during a 1 min interval (Chappell et al. 1999).

5.2.5 Metabolism during cold exposure

Birds were placed in 2 L respirometers, fashioned from paint cans, fitted with interior perches and inlet and outlet tubing. Respirometers with the birds inside were transferred to a 25°C refrigerated incubator. A gas mix of 79% helium and 21% oxygen (He-O_2) was provided to each respirometer at a flow rate of 1000 ml/min and controlled with mass-flow controllers (Tylan Model FC-280S, West Technology Systems, Yate, Bristol, UK). Because helium conducts heat four-times faster than nitrogen, this mixture permits maximal rates of heat loss at higher temperatures than

in air and, thus, reduces potential freeze-damage to tissues during cold exposure (Rosenmann and Morrison 1974). Respirometer exhaust gas passed though water and carbon dioxide absorbants (Drierite and soda lime, respectively) prior to gas analysis with Sable Systems Systems FC-1 oxygen analyser (Sable Systems Oxzilla). Output signals from the oxygen analyser and mass flow meters were recorded on a Macintosh computer (Apple, Cupertino, CA, USA) fitted with an A-to-D converter and custom designed software (Warthog Systems, University of California, Riverside, CA, USA, www.warthog.ucr.edu). Once animals were in place for a 30 min equilibration period, the incubator was set to 2°C and reached that temperature within 30 min and due to HeO_2 the temperature was equivalent to below freezing (Rosenmann and Morrison 1974). The birds were removed after remaining in the respirometre for a further 60 min. Maximum metabolic rate during cold exposure was selected as the highest oxygen consumption rate recorded during a 3 min period of cold exposure. The total oxygen consumed throughout the period from when the incubator was set to $2^{o}C$ was also calculated and is termed the integrated oxygen consumption. All VO_2 values are corrected to STP volumes and the maximum VO_2 was computed from the highest instantaneous VO_2 measured during a 3 min interval (Chappell et al. 1999).

5.2.6 Blood haemoglobin concentration and body mass

After metabolic measurements and before a blood sample was taken birds were weighed to the nearest 0.1 g (Mettler Toledo balance). A blood sample was taken from a brachial vein following venipuncture with a 25-ga needle and a portion of the whole blood was collected to measure haemoglobin content to the nearest 0.1 g/dl using a Hemocue haemoglobin analyser (Hemocue Australia Pty. Ltd., Tumbi Umbi, NSW, Australia).

5.2.7 Summary of statistical tests

Evaluations of fipronil effects on MMR and integrated oxygen consumption during cold exposure and PMR during exercise were tested for normality. When the data were normally distributed we examined results using paired-sample Student's *t*-tests of values measured for treated and control birds before and after pesticide ingestion (Zar 1999). When the data were not normally distributed, the matched pairs of means before and after pesticide exposure were compared using the nonparametric Wilcoxon analysis. A Bonferroni sequential adjustment was applied as metabolic rate and cold exposure were measured in the same individuals, hence the significance level was $p < 0.025$ (0.05/2). Analyses were performed using JMP statistical software (Ver. 5)

5. 3 Results

The dose of fipronil that resulted in the first zebra finch mortality was 26 mg/kg and 36 mg/kg resulted in the first king quail death. There was no mortality throughout the course of the experiment in king quail dosed with 30 mg/kg fipronil, or in zebra finch dosed with 17 mg/kg fipronil. Control birds remained invariant throughout the course of the experiments ($p > 0.2$).

In king quail the mean MMR during cold exposure before pesticide ingestion was 8.44 ml/min (± SD = 1.07; $n = 14$) and integrated oxygen consumption was 6.22 ml/min (± SD = 0.82; $n = 14$). Maximum metabolic rate did not significantly change at any at any time post-dose ($p > 0.61$; Fig 5.1), nor was there any difference in integrated VO_2 before and at any time after pesticide ingestion ($p > 0.6$; Fig 5.2).

Peak metabolic rate during exercise averaged 6.41 ml/min (± SD = 1.00; n = 15) before fipronil ingestion in zebra finch and king quail averaged a PMR of 6.50 ml/min (± SD = 0.97; n = 14) before dosing. While in the flight chamber, exercise in zebra finch took the form of a series of flight/flights. By contrast, king quail ran more often than attempting flight, which resulted in a comparably lower VO_2 for its size. There was no difference between PMR measurements taken during exercise before fipronil ingestion and those taken after in zebra finch ($p > 0.2$; Fig 5.3), or in king quail ($p > 0.2$; Fig 5.4). Aerobic performance was not affected by pesticide exposure at anytime post-dose.

5.3.1 Blood haemoglobin and body mass

Zebra finches ranged in body mass from 10.2 to 14.3 g and king quails ranged in body mass from 35.4 to 52.6 g. There was no change in mean body mass in control or treated zebra finches ($p > 0.2$) or king quails ($p > 0.055$) at any time post-dose. Whole-blood haemoglobin content in zebra finches ranged from 16.9 to 22.2 g/dl and from 11.9 to 20.3 g/dl in king quails. There was no significant difference between pre-dose and post-dose haemoglobin content in control or treated birds at any time, in any species ($p > 0.07$).

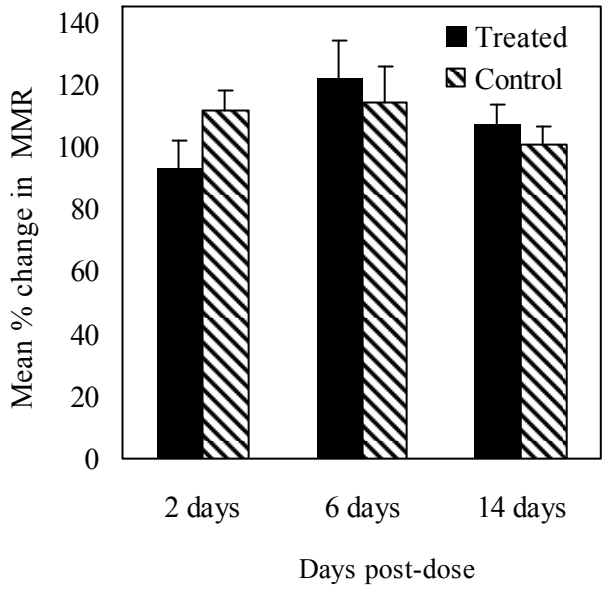

Figure 5-1 Mean percentage change (± standard error) in maximum metabolic rate (MMR) during cold exposure after king quail received 30 mg/kg fipronil (number of treated birds = 7; number of control birds = 8)

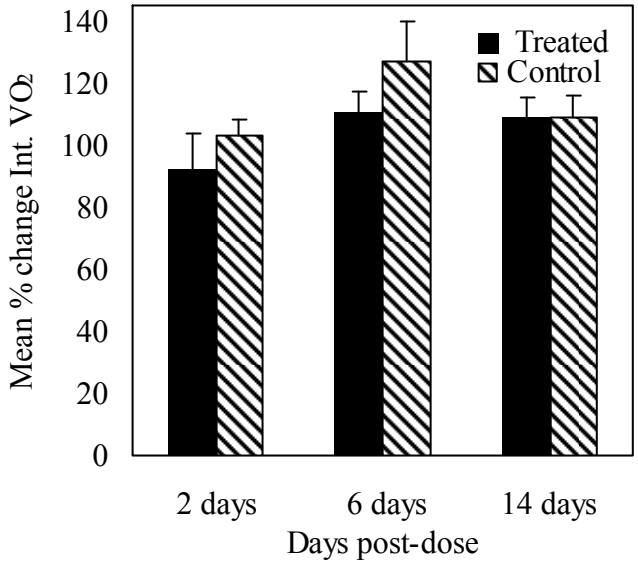

Figure 5-2 Mean percentage change (± standard error) in integrated oxygen consumption (VO_2) during cold exposure after king quail received 30 mg/kg fipronil (number of treated birds = 7; number of control birds = 8)

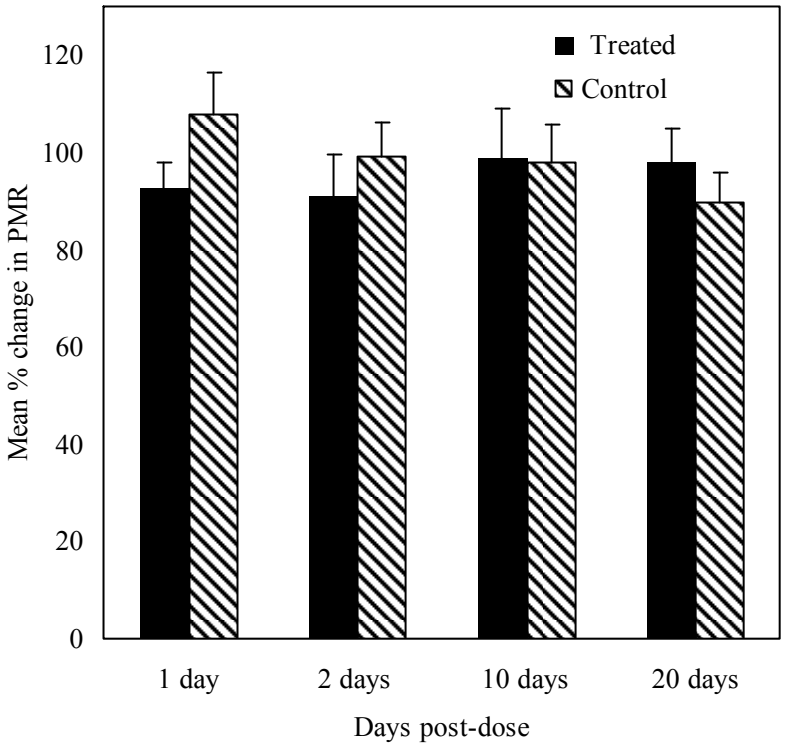

Figure 5-3 Mean percentage change (± standard error) in peak metabolic rate (PMR) during exercise after zebra finch received 17.5 mg/kg fipronil (number of treated birds = 7; number of control birds = 8)

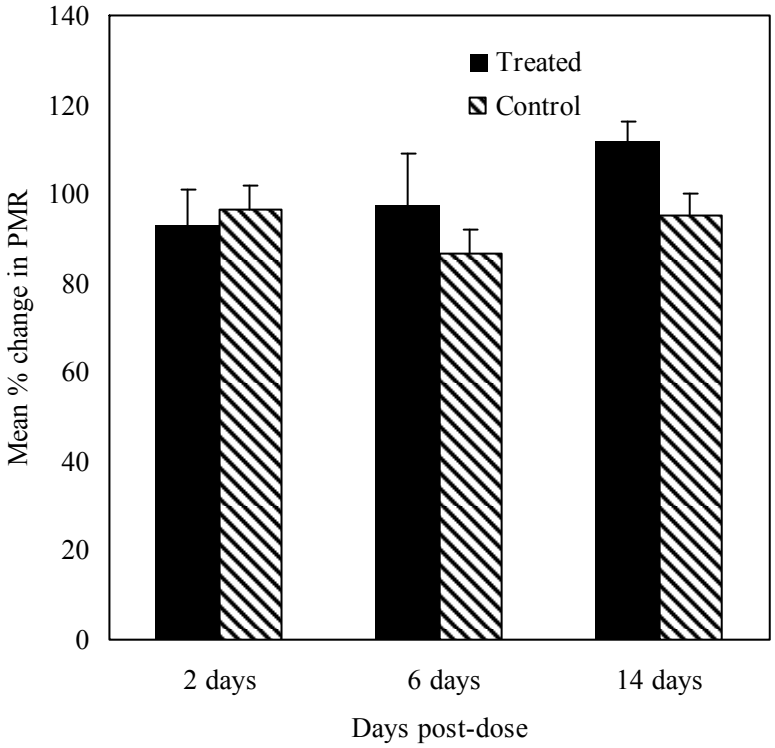

Figure 5-4 Mean percentage change (± standard error) in peak metabolic rate (PMR) during exercise after king quail received 30 mg/kg fipronil (number of treated birds = 7; number of control birds = 7)

5. 4 Discussion

5.4.1 Metabolism during cold exposure

The maximum metabolic rate during cold exposure in king quails was 8.44 ml O_2/min which was an increase of more than six times the basal rates previously recorded in this species (Hinds et al. 1993) (Fig 5.1). These results are consistent with the metabolic increases associated with maximal rates of cold-induced thermogenesis in birds (Roberts and Baudinette 1986). Thus the metabolic measurements appear to represent peak levels of shivering thermogenesis.

Fipronil had no effect on metabolism during cold exposure in king quails. Maximum metabolic rate in king quails did not significantly change at any time post-exposure, nor did integrated VO_2 (Fig 5.1 & 5.2). Maintenance of a stable body temperature requires a dynamic balance between heat production and heat loss and is regulated by hypothalamic control centres that effect shivering during exposure to cold (Gordon 1994). Previous studies concerning pesticide effects on avian thermoregulatory abilities have shown that some compounds can potentially affect thermoregulatory responses to the cold directly via hypothalamic neural circuitry, or indirectly through inhibitory effects on shivering by skeletal muscles (Bicudo et al. 2001, Grue et al. 1997). Although fipronil has been shown to interfere with thyroid function (Hurley et al. 1998), the present study found that fipronil had no effect on the ability of king quails to respond to decreasing temperature. There was no mortality under cold stress, no impairment in the delivery of O_2 via respiratory /circulatory mechanisms and no effect on shivering thermogenesis in treated birds at any time post-fipronil ingestion. Similar results have been found in our laboratory when metabolism was measured during cold exposure in king quails, house sparrows and a dasyurid marsupial, the fat-tailed dunnart (*Sminthopsis crassicaudata*) exposed to sublethal doses of fenitrothion (Buttemer et al. 2008, Fildes et al. 2008).

5.4.2 Metabolism during exercise

From a mechanistic viewpoint locomotor and aerobic traits require the effective and coordinated functioning of all body systems and as such have the potential to be good indicators of toxicity. The repeatability of measurements assessing these performance indicators have been confirmed in red jungle fowl and house sparrows and in previous experiments conducted in our laboratory (Chappell et al. 1999, Chappell et al. 1996). The present study confirmed that aerobic performance

measurements are a reliable integrative metric of physiological quality, and the PMR of control birds remained invariant throughout the course of the experiment confirming repeatability.

Metabolic rate during exercise, as shown by previous studies of birds, can increase up to 12 times resting metabolic rate (Chappell et al. 1999). In the present study average pre-dose PMR in zebra finches was 6.41 ml/min approximately 10 times the reported minimum metabolic rate of 0.68 ml/min (Hinds et al. 1993). Average pre-dose PMR in king quails was 6.50 ml/min (0.26) and increased approximately five times the previously recorded minimum metabolic rate of 1.2 ml/min reported for this species (Hinds et al. 1993). The comparably smaller increase in quail was due to the tendency for these birds to run more than fly. Nevertheless, all king quail were exercised intensely during PMR determination and these measurements are thus a good index of physical vigour.

Contrary to our predictions, there is no evidence that an acute sublethal exposure to fipronil causes compromised physical performance. Treated birds were indistinguishable from control birds with no behavioural signs of toxicity. Previous findings have suggested that performance can serve as an ecologically relevant response to contaminant exposure in reptiles, birds and mammals (Buttemer et al. 2008, Holem et al. 2006, Punzo 2003). Swimming velocity was significantly slower in snakes after exposure to 2.5 mg/kg and 5 mg/kg carbaryl (Hopkins et al. 2005) and agility was impaired in the western fence lizard (*Sceloporus occidentalis*) after exposure to the same chemical (Durant et al. 2007). Studies in our laboratory on house sparrows (*Passer domesticus*), zebra finch and king quail have all shown

impaired aerobic performance following acute sublethal exposure to fenitrothion (Fildes et al. 2008) When Holem et al. (2005) examined sublethal effects on sprint performance in lizards exposed to the neurotoxic metal lead, they found no difference between exposed animals and controls. Likewise Fryday and colleagues (1995) found that although there was a significant reduction in activity in European starlings (*Sturnus vulgaris*) exposed to chlorfenvinpos, flying performance was not significantly different between control and dosed birds.

The lack of effect of fipronil on PMR during exercise and cold exposure in the present study indicates that acute sublethal fipronil exposure does not adversely effect physical vigour in either zebra finches or king quails under laboratory conditions. Peveling and Demba (2003) however, found that locomotor activity, prey consumption and body mass in the lizard *Acanthodactylus dumerili* remained significantly lower than controls in this species and mortality gradually increased during the weeks after treatment, with an LD50 estimated to be as low as 30 mg/kg fipronil. Conversely, Avery et al. (1988) found that red-winged blackbirds (*Agelaius phoeniceus*), brown-headed cowbirds (*Molothrus ater*), and boat-tailed grackles (*Quiscalus major*) showed no visual signs of toxicity and no evidence of ill effects when fed fipronil treated seed at 325 and 500 mg/kg. Given the persistence of fipronil in the environment, future experiments should examine the effects of chronic exposure to sublethal doses of fipronil.

5. 5 Conclusion

Contrary to our predictions, fipronil did not compromise physical vigour in zebra finches or king quails for up to three weeks post-exposure. Although these results suggest that fipronil has no ill-effects on locomotory performance and thermoregulatory abilities they should be interpreted with caution. The exposure to a single acute sublethal dose of fipronil, while precise and informative may not be representative of exposure, or its effects on physiological compromise, in the field. Based on potential for exposure, residue analysis and reported food consumption rates, Szabo (2005) reasoned that 5% of exposures would lead to LD_{50} level mortality in the most sensitive granivorous species. Birds in the wild will endure longer exposures due to the slow breakdown rate of fipronil, its tendency to bioaccumulate and its degradation into various metabolites. Furthermore, given the lipophilic nature of fipronil, there is strong likelihood that breeding females will transfer fipronil to yolk which, in turn, will result in embryonic exposure to this pesticide. Given these factors further study is necessary to fully evaluate the potential impact of fipronil on health and breeding potential of free-living birds.

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6 SUMMARY OF MAJOR FINDINGS AND DIRECTIONS FOR FUTURE RESEARCH.

The main aims of this study were to establish through field investigations whether non-target native Australian birds are exposed to fenitrothion during locust control; to investigate through captive studies the sublethal effects of fenitrothion exposure on various physiological endpoints in three species of birds; and finally, utilising the same physiological endpoints, to examine the sublethal effects of fipronil in two avian species. The major findings of each chapter are outlined below.

Because there is little information on cholinesterase (ChE) activities for Australian birds, an important component of this study was to characterise plasma ChE activity in a variety of avian species to establish assay conditions for this and future pesticide studies. There was considerable interspecific variability in avian plasma ChE activity. Plasma acetylcholinesterase (AChE) ranged from 0.089 μmols/min/ml in budgerigars to 0.860 μmols min/ml in clamorous reed-warblers, while king quail had no measurable AChE activity. This variability underscores the need to characterise AChE before using this enzyme in OP-exposure monitoring schemes.

There was no short-term diurnal variation in plasma ChE activity in captive zebra finches and budgerigars purchased from commercial suppliers. This finding allows for greater temporal flexibility in sampling regimes in captive studies. There was however, a significant difference in plasma ChE between seasons in captive zebra finches, which highlights the need for contemporaneous characterisation of ChE activities in unexposed birds at the time of organophosphate or carbamate

application. Interestingly, commercially purchased zebra finches and free-living zebra finches had different plasma ChE activities. Such temporal and spatial variation in intraspecific plasma ChE activity emphasises the need for baseline data to be obtained from neighbouring, unexposed conspecifics when investigating exposure to anti-ChE compounds.

Baseline measurements of ChE activity in Australian native bird species are virtually absent in the literature, therefore the present contribution provides critically needed information. Further field studies to collect and analyse plasma ChE activities in a wider variety of species would aid biological monitoring of non-target avian wildlife by providing reference values for birds at risk of exposure to anti-ChE compounds. It is also recommended that further examination of interpopulation variation in plasma ChE be determined to establish the effectiveness of relying on non-local reference data for monitoring anti-ChE exposure.

Aggregations of flightless locust nymphs are a very visible and attractive food source for native bird species of the semi-arid and arid regions of eastern Australia. Hence a wide variety of species flock to locust outbreaks. Field investigations using ChE inhibition and reactivation as biomarkers of fenitrothion exposure show that Australian native bird species are exposed to this pesticide during Australian Plague Locust Commission (APLC) locust control operations. Avian species exhibiting ChE depression included granivorous species feeding on grasses, insectivorous species feeding on locusts and other insects, and birds of prey feeding on locusts and birds. Many of these birds were in the process of breeding and/or moulting during pesticide application. Zebra finches, white-winged trillers, and white-browed woodswallows

exhibited significant depressions of ChE activity following fenitrothion spraying . Examination of ChE reactivation in plasma samples revealed that fenitrothion exposure was not restricted to insectivorous species, as grass finch and honeyeater species also showed reactivated ChE. This suggests that a variety of pesticide exposure routes exist for birds during locust control. A further group of birds not focused upon in the current study but which are arguably at risk of pesticide exposure are the avian predators. Birds of prey feed on sprayed locusts and other insects as well as other vertebrates that may have been exposed to pesticide. Therefore further study targeting birds of this feeding guild would give a better understanding of pesticide exposure in non-target avian species.

To complement these studies of fenitrothion-exposure in birds, captive studies were undertaken to investigate the effect of such exposure on selected physiological endpoints. King quails and house sparrows receiving high fenitrothion doses lost weight following fenitrothion exposure. This is consistent with reports of pesticide-induced anorexia in other bird species (Grue et al 1997). Conversely, fenitrothion had no effect on body mass in zebra finches and house sparrows (medium and low-dose). Given the size of these passerines and the mortality they experienced at these doses, this indicates that the cause of mortality was a direct toxic action of fenitrothion, and not a consequence of suppressed feeding.

Although impaired thermoregulation following organophosphate exposure has been reported for a number of bird species (REFS), none of the species from this study showed reduced thermoregulatory response following fenitrothion ingestion. This

indicates that fenitrothion did not adversely affect the thermoregulatory control centre or effector mechanisms of heat generation in king quail or house sparrows.

Fenitrothion did have a pronounced effect on avian locomotory performance. This ecologically relevant activity was impaired for a significant length of time post-exposure. Despite being asymptomatic with regard to all other measured variables, zebra finches had a 17% reduction in aerobic capacity at three days after dosing. Similar reductions in locomotor performance were also evident in king quails and house sparrows following fenitrothion exposure. Treated birds were behaviourally asymptomatic, whereas locomotory aerobic capacity remained significantly impaired, even after plasma ChE levels were restored to pre-dose levels. The extent and duration of peak metabolic rate depression was even greater in sparrows dosed with 100 mg/kg. Aerobic performance was 58% lower than pre-dose measurements two days after pesticide ingestion and remained significantly impaired for up to 14 days, by which time ChE activity was restored. Therefore performance-based toxicological endpoints can provide valuable information when examining risk factors in a given species, revealing sublethal pesticide effects when exposure is undetectable using other measures. These reductions in aerobic performance are not a consequence of reduced capacity to circulate oxygen, as haemoglobin (Hb) levels in plasma were unaffected by fenitrothion treatment in captive birds.

Through a combination of laboratory and field investigations that integrated biochemical and physiological endpoints, a better understanding of the ecological consequences of fenitrothion exposure in birds has been gained. The field component revealed that free-living birds are exposed to fenitrothion during locust control while

many of these birds are in the process of reproducing and/or moulting, two of the most energetically expensive events in the avian life cycle. The captive component of this investigation found that reduced exercise capacity resulted from pesticide exposure, even at sublethal doses. Such constraints on maintaining high-level aerobic performance during breeding or moulting could limit a bird's ability to sustain activities at normal levels. Impaired locomotory capacity could negatively impact on an animal's ability to obtain adequate food for themselves and/or for their young, or to escape from predators. Furthermore, impaired aerobic performance could have deleterious effects on reproduction by interfering with breeding activities such as courtship, aggressive displays, mate guarding, competing for nesting sites, and nest defence.

Finally, although exposure to fipronil following APLC spray operations was not evaluated in free-living birds in the current study, the evidence of fenitrothion exposure demonstrates that birds are also at risk of exposure to fipronil when this pesticide is applied for locust control. Future field investigations to establish the extent of avian exposure to fipronil following APLC spray events would be invaluable. This study aimed to assess sublethal effects of fipronil in the insecticide formulation Adonis 3 UL ® utilising the physiological endpoints measured in captive birds exposed to fenitrothion. Fipronil had no effect on metabolic performance, plasma Hb levels, or body mass at any time post-exposure in king quails or zebra finches. Although fipronil has no ill effects on locomotory performance or thermoregulatory abilities, they are not fully indicative of pesticide-exposure in free-living birds. Birds in the wild will endure longer exposures than birds in captive studies due to the slow breakdown rate of fipronil, its tendency to bioaccumulate and

its degradation into various metabolites. It is recommended that further study to fully evaluate the potential impact of fipronil in free-living birds could use performance-based measures, particularly those related to reproduction, using formulations of fipronil as used by the APLC (Adonis 3 UL ®) as well as using higher sublethal doses of technical grade fipronil. Such investigations would contribute to gaining a more comprehensive understanding of the ecological consequences of fipronil exposure following APLC locust control in free-living species.

The major findings from this study contribute to the field of environmental toxicology by providing information that can be utilised to monitor exposure to anti-ChE compounds in Australian avifauna. Further, the research has identified that sublethal doses of fenitrothion consistently reduce locomotor performance of birds for extended periods. Such reductions in performance may have lethal consequences under free-living conditions and must be considered in future risk assessment appraisals of anti-ChE chemicals. Because of the coincidence of bird breeding and locust abundance, decision makers must take care when deciding which chemicals to use for pesticide control and the timing of application. Given the widespread occurrence of fenitrothion exposure among free-living birds following locust control and its persistent effect on their locomotor capacity, alternatives to fenitrothion application should be strongly considered. It is recommended that a thorough risk assessment of fenitrothion use be completed as part of the APLC's legislated environmental due diligence obligation.

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