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**IMPACT OF FIPRONIL, A NEW GENERATION PESTICIDE, ON
AVIAN DEVELOPMENT AND HEALTH**

A thesis submitted in fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

by

MALSHA KITULAGODAGE BSc.

**SCHOOL OF HEALTH SCIENCES
2011**

CERTIFICATION

I, Malsha Michelle Kitulagodage, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Health 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

Fipronil is a new-generation pesticide aerially applied in semi-arid and agricultural areas of Australia to control locust outbreaks. Seasonal conditions that give rise to locust plagues are also ideal for breeding birds, with over 100 different avian species observed in areas of locust control operations. Despite the potential for exposure, there is very little research regarding the toxicological effects of fipronil in birds. Available avian toxicity information shows a high species-specific variability in fipronil sensitivity across the few species tested, making it extremely difficult to predict the toxicity of fipronil on unstudied species at high risk of exposure in the wild.

The aim of this thesis was to increase our understanding of the impact of fipronil on native birds at risk of exposure as a result of locust-control spraying. This was done firstly by examining the toxicity, effects, and duration of symptoms following exposure to fipronil and its major metabolite, fipronil-sulfone, in sensitive and non-sensitive avian species; secondly by evaluating the metabolism of fipronil in a selected bird species to gain insight into the mechanisms underlying variation in species sensitivity; and thirdly by examining whether exposure to fipronil at sub-lethal levels adversely affects exposed birds and their offspring.

Four previously unstudied species from three avian orders were examined in this study: two Passeriformes (zebra finch and house finch), one Psittaciformes (budgerigar), and one Galliformes (king quail). Three of these species are among the top 20 avian species predicted to be present at locust-control events.

Fipronil was moderately toxic to budgerigars and the two finch species tested, but was highly toxic to king quail, which is consistent with reported fipronil sensitivities for other galliforms. In contrast to earlier studies claiming biological metabolites of fipronil to have greater toxicity than the parent fipronil compound in vertebrates, fipronil-sulfone was equitoxic to king quail,

but significantly less toxic to zebra finch. Clear differences were also observed between the highly sensitive galliforms and the moderately sensitive passeriforms with respect to the onset and duration of signs of fipronil intoxication. During the course of this study we consequently discovered that the solvent used in the fipronil-based locust control formulation contributed significantly to the toxicity of the product.

Sublethal effects of fipronil were also examined in northern bobwhite quail, another galliform. In addition to being the standard US EPA avian test species for toxicity, bobwhites are also the most fipronil-sensitive species of those tested. Reduced feeding behaviour and progressive significant losses in body mass were observed across all fipronil-treated bobwhite quail over a 3-day period. This loss in body mass also correlated with an increase in fipronil-sulfone residue levels in brain, liver and adipose tissue over this period.

Because fipronil is highly lipophilic, it has great potential to be transferred into the lipid-rich yolk of eggs laid by pesticide-exposed female birds. The potential for maternal transfer of fipronil residues into eggs and consequent developmental effects was therefore examined in two experiments. The first examined the effect of fipronil exposure on breeding success in female Zebra Finches, and the second was an *in ovo* study using domestic fertile chicken eggs to examine fipronil effects on embryo development and survival. Behavioural and developmental abnormalities were observed in fipronil-exposed chicks that hatched from both of these studies, with a reduced hatchability rate observed for the zebra finch eggs. Domestic chicken hatchlings from fipronil-treated eggs showed reduced feeding behaviour, resulting in loss in body mass over the 48-hour period post hatch. Fipronil and fipronil-sulfone residues were detectable in brain and liver of fipronil-exposed chicks in both studies.

The substantially greater sensitivity of avian embryos to fipronil highlights the need to consider reproductive effects of pesticide exposure when evaluating the ecotoxicological risks in using them. This is especially important for those used to control insect outbreaks, as these are usually coincident with breeding events across many vertebrate taxa. The lipophilic nature of fipronil will potentially have far greater impact on many vertebrate populations, as developing young of reptiles and birds can be exposed through fipronil-contaminated yolk and developing mammals gain exposure through maternally derived milk lipids. This emphasises the need to evaluate pesticides across all life stages in species at risk.

THESIS FORMAT

In general, the chapters of this thesis are each in the format of a peer-reviewed paper and have either been published in a journal or in preparation to be submitted for publication. For this reason, there is a degree of overlap between the introduction, methods and discussion sections of each chapter. The collective references for each chapter have been listed at the end of the thesis.

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CHAPTER 1. INTRODUCTION

WHAT IS FIPRONIL?

Fipronil is a broad-spectrum insecticide belonging to a new class of pesticides: the phenylpyrazoles. It was developed by Rhone-Poulenc Agro in the late 1980s (Bobe *et al.* 1998a; Bobe *et al.* 1998b; Moffat 1993) and is the active ingredient in many animal health and pest control formulations currently available on the market. Commonly used products include Frontline® flea and tick treatments, Maxforce® cockroach bait and Termidor® termite control (NPTN 1997).

In Australia fipronil is registered for agricultural use as a locust-control pesticide under the trade name Adonis®. It was introduced in hope of replacing Fenitrothion, an organophosphate (OP) pesticide, due to rising concerns regarding the hazardous effects of OPs on non-target species and the environment (Lahr 1998; Sarikaya *et al.* 2004; van Barneveld 1999). The appeal of fipronil is that it provides effective control at significantly lower application rates than fenitrothion; between 0.25 and 1.0 g a.i./ha (grams of active ingredient per hectare) as compared to up to 267 g a.i./ha of fenitrothion required to have the same effect (APLC 2005). The Australian Plague Locust Commission (APLC), a federal agency that monitors and controls locust populations, has been spraying fipronil on large areas of arid zone habitat in Australia since 1997 (APLC 2005; APVMA 2003).

In the locust control context, fipronil is typically used at times when locusts are very abundant. Unfortunately this coincides with breeding seasons for many native vertebrates, such as birds and lizards; the conditions that promote locust plagues also enhance breeding success in vertebrates inhabiting semiarid regions of Australia. Further, because large numbers are readily available, flightless juvenile locusts are an attractive food source to many semi-nomadic avian species. In Australia, over one hundred different bird species have been observed in areas common to locust-control spraying events (Szabo 2005). Birds are

particularly susceptible as fipronil-contaminated locusts can remain alive for 7-10 days (EPA 2001) and feeding on these insects provides a major route of pesticide exposure. Granivorous birds are also at high risk of exposure; our lab has shown that high levels of fipronil and its metabolites are detectable in seeds after fipronil spraying events suggesting that ingestion of contaminated seeds is also a likely route of exposure (Szabo 2005). Dermal contact of body surfaces to chemical on vegetation and soil is also a potential route of exposure for non-target animals.

This chapter covers available information on fipronil with respect to insecticidal properties, environmental fate and toxicity data of both target and non-target species. It also highlights gaps in the current literature, particularly with regard to avian toxicology information. In this regard, there is no research explaining the species-specific variability among the few avian species tested for fipronil toxicity, nor are there data assessing the toxic effects of fipronil in species that are at high risk of exposure. Furthermore, studies examining behavioural and other sub-lethal effects of fipronil on avian species are even rarer. The research proposed aims to resolve this lack of essential information in two ways: Firstly to examine whether fipronil has identifiable sublethal effects in exposed birds and their offspring that compromise population health, and secondly to evaluate the metabolism of fipronil and its byproducts in selected bird species to gain insight into the mechanisms underlying variation in species sensitivity. Results from this research will provide critically needed information for understanding the ecotoxicological risks of using fipronil for locust control in Australia.

MODE OF ACTION OF FIPRONIL

Fipronil [(±)- 5-amino -1- (2, 6 -dichloro - α,α,α -trifluoro-*p*-tolyl) -4- trifluoromethylsulfinyl-pyrazole - 3-carbonitrile] is the first phenyl pyrazole insecticide to be introduced. It is an effective nerve agent that acts by blocking γ -aminobutyric acid (GABA) receptors, which are ligand-gated chloride channels prevalent in the nervous systems of insects, molluscs,

nematodes and across all vertebrate species (Bloomquist 2003; Hainzl and Casida 1996; Moffat 1993; North 1995). GABA is a major inhibitory neurotransmitter that regulates neural function and prevents over-stimulation of the synaptic pathways (Garrett and Grisham 1999). By interfering with the normal regulatory actions of GABA via its receptors, fipronil results in neural excitation and convulsions. Exposure of insects at sufficient doses causes severe paralysis and ultimately, death (Bobe *et al.* 1998a). Concentrations of fipronil required to inhibit 50% (IC₅₀) of GABA receptors were derived from radio-ligand binding studies, revealing that fipronil has higher affinity for the insect GABA receptors (composite IC₅₀ value of 7nM for housefly and fruit fly) than for vertebrate receptors (composite IC₅₀ value of 1103nM for six vertebrate species; (Hainzl *et al.* 1998). This higher relative toxicity for insects may be due to fipronil blocking glutamate-gated chloride channel (GluCl) receptors which are unique to the CNS of insects and nematodes (Bloomquist 2003; Zhao *et al.* 2003; Zhao *et al.* 2004).

FATE OF FIPRONIL

Technical grade fipronil (>98%) is a white solid at room temperature with a melting range of 200-201°C. It has low solubility in water (~2mg/L) but is highly soluble in acetone (>50%) and other organic solvents (Aventis 2001; Colliot *et al.* 1992). Fipronil is considered a moderately persistent pesticide due to its long half-life. The total toxic component of fipronil (which includes fipronil and all its degradates) is 188 days on average in field soil (Ying and Kookana 2002). The half-life of fipronil on treated surfaces is three to seven months, depending on surface conditions. Residues are still detectable in treated vegetation three weeks after application (Tingle *et al.* 2003). Fipronil degrades to various metabolites (Figure 1.1) depending on the conditions.

In the Environment

Exposure of fipronil to sunlight produces the major photoproduct, desulfinyl fipronil (MB46513) (Bobe *et al.* 1998a; Ying and Kookana 2002). In a study on pea and pear leaf surfaces, the desulfinyl photoproduct formed 45% of the total fipronil residues persistence (Hainzl and Casida 1996). In soil, degradation is dependent on the moisture content; residue analyses from soils with high moisture content revealed the sulfide derivative (MB45950) to be the main product via reduction processes, whereas in soils of low moisture content the sulfone degradate (MB46136) was the major product (Ying and Kookana 2002). Fipronil also hydrolyses in soil or water to form an amide degradate (RPA200766) (Bobe *et al.* 1998b).

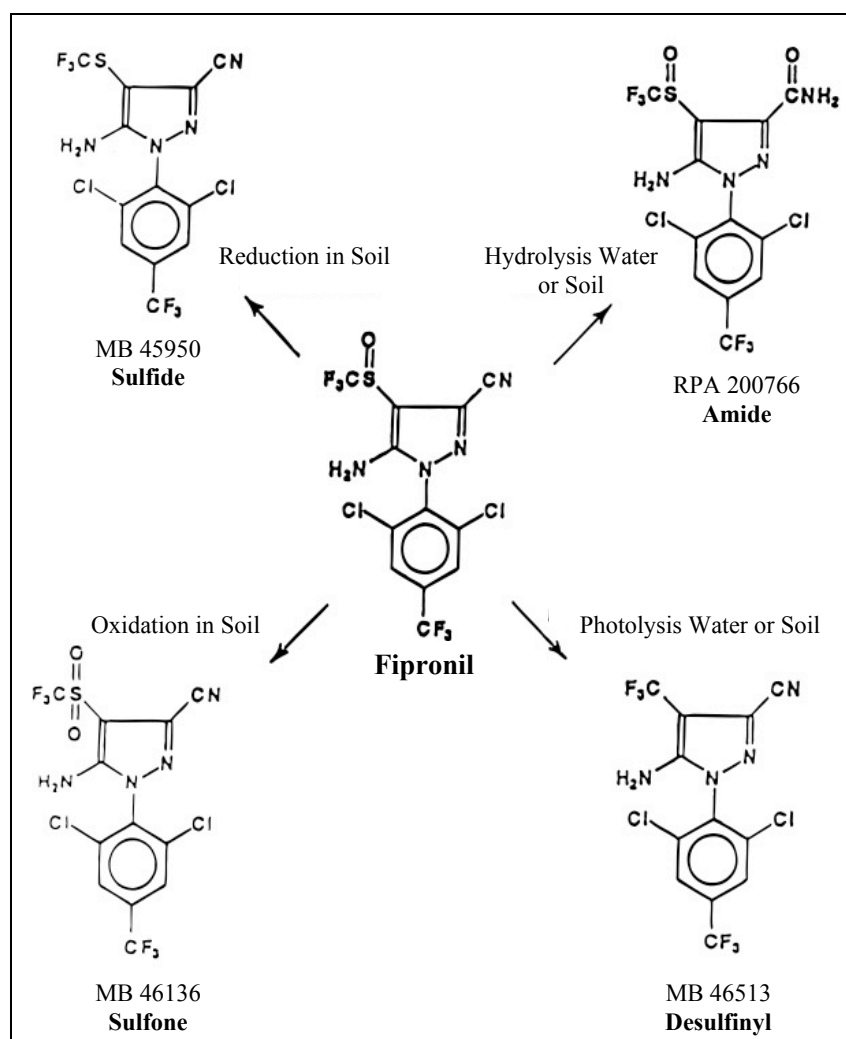


Figure 1.1 Fipronil degradation pathways (adapted from Bobe *et al.* 1998a)

In Plants

In plants the major fipronil metabolite formed is the sulfone (MB46136) via oxidation (Fenet *et al.* 2001). The amide metabolite (RPA200766) and very low levels of the sulfide (MB45950) are also detected (JMPR 2001). The desulfinyl photoproduct (MB46513) is not a detectable residue in plant tissue after fipronil exposure (JMPR 2001; Zhou *et al.* 2004).

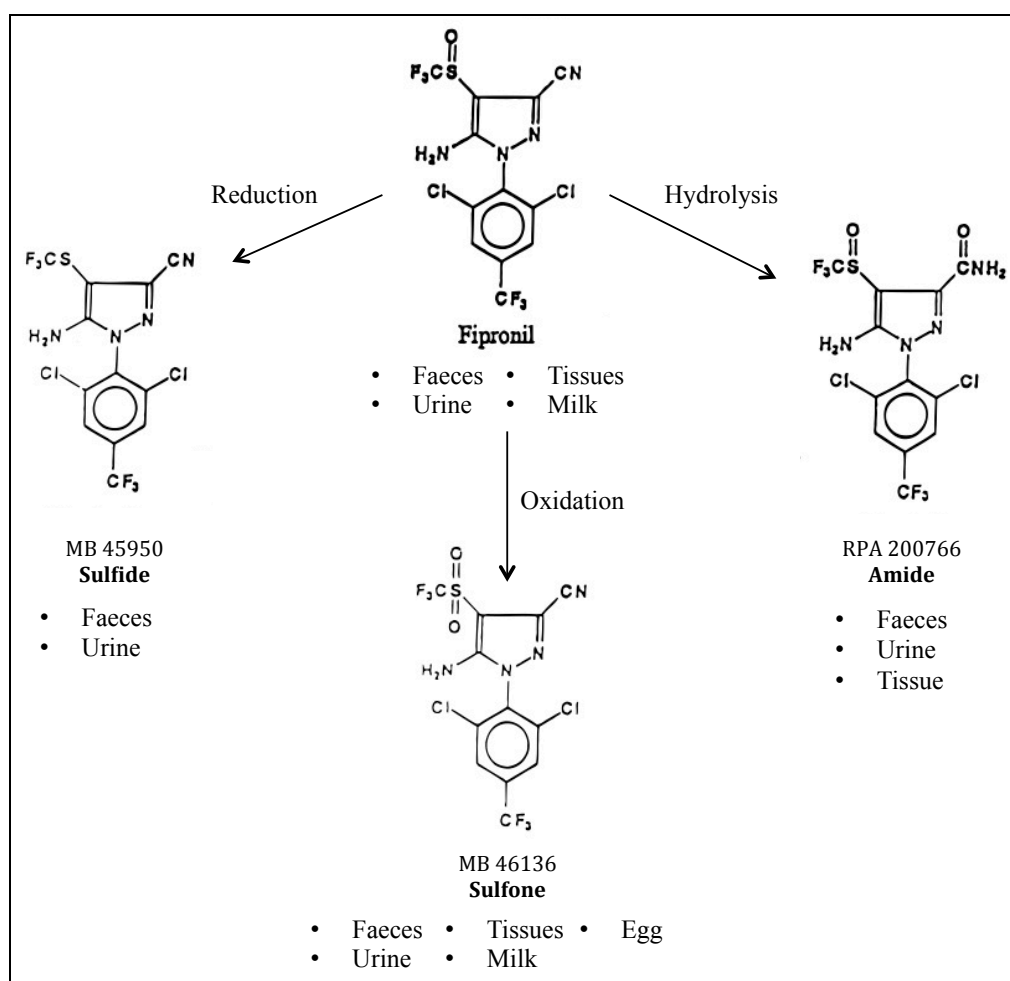


Figure 1.2. Metabolism of fipronil in animals (adapted from JMPR 1997)

In Animals

The Joint Meeting on Pesticide Residues (JMPR) published two toxicology assessment reports (1997 and 2001) reviewing all unpublished data and research on fipronil. This report indicated that the main route of fipronil and metabolite excretion in vertebrates is via defecation; 45-

75% of administered dose in rats; 18-64% for goats; and 28-42% for hens. The major metabolite detected in the tissues of treated animals was the sulfone (MB 46136) (JMPR 1997, 2001). The metabolic pathway of fipronil in animals was proposed from this data (Figure 1.2). In a published fipronil study on its metabolism in mice, the sulfone (MB46136) was the major fipronil residue detected in tissue, whereas the desulfinyl photoproduct (MB46513) was not detected (Hainzl and Casida 1996), hence confirming that fipronil-desulfinyl (MB46513) is not a by-product of metabolism.

DEGRADATE TOXICITY

Fipronil-sulfone (MB 46136), which is the major product formed during metabolism, has been reported as more toxic to both fish and bird species than the parent fipronil compound (USEPA 1996) and 6-fold greater binding affinity than fipronil at vertebrate GABA receptors (Hainzl *et al.* 1998). In studies on freshwater invertebrates the sulfide metabolite was reported to be 1.9 times more toxic than fipronil (USEPA 1996). Lethal dose studies have demonstrated that, despite not being a product of metabolism, the desulfinyl photoproduct is toxic to both houseflies and mice, with mean lethal dose (LD50) values approximately 2-fold those for fipronil. The authors also concluded from this study that the amide metabolite (RPA200766) is inactive due to its very low relative potency at GABA receptors (Hainzl and Casida 1996).

EXPOSURE RISKS TO NON -TARGET SPECIES

Fipronil is an effective insecticide, however it is evident that fipronil does not act exclusively on the GABA receptors of target insects. The range of studies that have assessed the effects of fipronil exposure on non-target species are summarised in Appendix 1. Exposure risks due to locust-control spray events are real; studies evaluating application levels demonstrated that fipronil, along with its sulfone, sulfide and desulfinyl metabolites do not migrate below the first 10cm of soil with residue levels remaining higher in the top 5cm (Bobe *et al.* 1998a)

therefore a wide range of terrestrial animals can easily come in contact with the compounds and become exposed.

As a result of its significant impact on bees and wasps (Appendix 1), fipronil was deregistered in France in February of 2004 due to the assumption that its use was responsible for the dramatic decline in honeybee populations and the associated economic losses (Hopquin 2004). Based on the toxicity data, the United States Environmental Protection Agency (USEPA) has classed fipronil as “very highly toxic” to aquatic organisms (Aventis 2001; USEPA 2005).

In summary, the appraisal of fipronil by the Joint Meeting of Pesticide Residues (JMPR) is that fipronil has similar metabolic fates in all species studied. Defecation was the main route of fipronil elimination. Of the amount absorbed, distribution in tissues is extensive, but relatively highest in tissues with higher lipid content. The main compound detected in egg yolk and tissues was the fipronil-sulfone (MB46136), whereas both the parent compound and the fipronil-sulfone were the major compounds detected in milk and fat (JMPR 2001).

The JMPR reviews a majority of the unpublished studies on fipronil that have been conducted in private labs associated with the compound’s development, manufacture and testing. However, much essential information and details are obscured due to confidentiality issues associated with commercial studies of pharmacologically active substances. A higher degree of transparency would allow greater understanding of the physiological and biochemical nature of the substance and possible adverse reactions (Woodward 2005a, 2005b).

AVIAN FIPRONIL ECOTOXICITY RISKS AS A RESULT OF LOCUST-CONTROL SPRAYING

Ecotoxicology has been described as the science of predicting the effects of potential toxic agents on natural ecosystems and non-target species (Hoffman *et al.* 2003). Pesticides have the potential to cause a significant ecological impact. Rather than specifically attempting to define

the toxicological mechanisms, ecotoxicological research attempts to evaluate the cause-and-effect relationships impacting on population and ecosystem levels. Avian toxicity data shown in Table 1.1 demonstrates that there is high species-specific variability in fipronil sensitivity in the few species studied; it is highly toxic to galliforms yet practically non-toxic to mallard ducks (USEPA 2005).

Table 1.1 Reported avian fipronil lethal doses (LD50s)

Species	Reported LD50	Reference
Northern bobwhite quail <i>Colinus virginianus</i>	11.3 mg/kg	Aventis 2001
Ring-necked pheasants <i>Phasianus colchicus</i>	31 mg/kg	
Red-legged partridge <i>Afectonis rufa</i>	34 mg/kg	
Field sparrows <i>Spizella pusilla</i>	1120 mg/kg	Avery et al. 1998
Pigeons <i>Columba livia</i>	>2000 mg/kg	
Mallard ducks <i>Anas platyhynchos</i>	2150	Aventis 2001

Although LD50 evaluations are a good measure of acute lethality, knowledge of effects of sublethal doses are equally important for assessing the biological effects of chemicals, especially if the compound has carcinogenic or teratogenic effects on parents or their offspring at doses considered non-hazardous by acute toxicity study standards. Surprisingly, there is no published research regarding physiological or behavioural sub-lethal effects of fipronil on avian species. This knowledge is essential for understanding the potential risks of exposing free-living vertebrates to fipronil.

CHAPTER 2. FIPRONIL AND FIPRONIL-SULFONE TOXICITY IN AVIAN SPECIES AT RISK OF EXPOSURE

This chapter is in preparation for *Toxicology Letters*

M. Kitulagodage, W.A. Buttemer, M.J. Hooper and L.B. Astheimer. Fipronil and fipronil-sulfone toxicity in selected avian species

M. J. Hooper assisted with experimental design and dosing of house finch.

ABSTRACT

Fipronil, a phenyl pyrazole pesticide, is used in Australia to control locust outbreaks. Locust populations build to plague proportions when rainfall occurs in late winter and spring, promoting early vegetation growth. These conditions also attract breeding birds, with over 100 species identified at locust control operations. Avian exposure to fipronil and its main metabolite fipronil sulfone occurs via direct contact and by ingesting contaminated insects or seeds. Available avian toxicity information shows high species-specific variability in fipronil sensitivity for the few species tested. We established fipronil LD50 estimates (Up-and-Down protocol; OCED 2003) for two Passeriformes: house finch and zebra finch; a Psittaciformes: budgerigar; and a Galliformes, king quail (670 mg/kg, 310 mg/kg, 148 mg/kg and 23 mg/kg, respectively). We also determined fipronil sulfone LD50 estimates for zebra finch and king quail (>820 and 30 mg/kg, respectively). The onset and duration of signs of fipronil intoxication differed conspicuously between orders. Both passerines showed signs of intoxication within 20 minutes of treatment, but only at lethal doses. Budgerigars displayed intoxication signs as early as 10 minutes after fipronil exposure and up to 48 hours after ingesting sublethal and lethal doses. By contrast, the galliforms did not show any definite signs of intoxication until at least 2 days after treatment, but birds did not eat or drink during this period. Fipronil and fipronil sulfone affects galliforms differently from other orders tested, which may contribute to their greater sensitivity.

INTRODUCTION

Fipronil is a new generation, phenyl pyrazole insecticide, registered for animal health and pest control use worldwide (APVMA 2003, BASF 2005). Fipronil and its sulfone metabolite are both effective neurotoxins that act by targeting gamma-amino butyric acid (GABA) receptors (Hainzl et al. 1996); these receptors are prevalent throughout the central nervous system of both vertebrates and insects. Fipronil has shown to have a much higher affinity for insect than for vertebrate GABA receptors (Hainzl et al. 1998) (hence its effectiveness as an insecticide), however there is little information regarding toxicological effects of fipronil or any of its metabolites on vertebrates and, as it is a new generation pesticide, there is even less information on signs of intoxication.

When fipronil was reviewed in the United States in 1994, the USEPA guidelines for pesticide registration only required acute oral avian toxicity data for one upland game and one waterfowl species (Hoffman *et al.* 1995). Species tested from these groups differ in their sensitivity to this pesticide by nearly 2 orders of magnitude; fipronil is highly toxic to three galliform species studied with LD₅₀ values less than 34 mg.kg⁻¹ (Goodyear 1994a, Stavola 1994a, Stavola 1994b), yet it is practically non-toxic to the mallard duck *Anas platyrhynchos*, with an LD₅₀ greater than 2150 mg.kg⁻¹ (Goodyear 1994b, USEPA 1996). Fipronil toxicity studies have also been undertaken for additional species including house sparrow *Passer domesticus*, a passerine, with an LD₅₀ of 1000 mg.kg⁻¹ (Goodyear 1994c), and rock pigeon *Columba livia*, a columbiform, with an LD₅₀ greater than 500 mg.kg⁻¹ (Stavola 1994c) demonstrating that avian fipronil sensitivity is highly variable across the few species tested.

There is also considerable variation in vertebrate toxicity associated with the sulfone metabolite of fipronil (fipronil-sulfone), and often at variance with fipronil. Fipronil-sulfone has been reported as being just as toxic as fipronil in mice (Hainzl *et al.* 1998), or more toxic than fipronil in avian species and freshwater fish (USEPA 1996). Hainzl *et al.* (1998) showed

that the fipronil-sulfone has an average 6-fold greater binding affinity than fipronil for GABA receptors in vertebrate brains (human, dog, mouse, chicken, quail and salmon) studied *in vitro*. However, the high variability in the effects of fipronil and the lack of a reliable fipronil biomarker makes it difficult to extrapolate existing toxicity data to unstudied species at risk of exposure.

In Australia fipronil is registered for agricultural use as a locust-control pesticide (APVMA 2009; APLC 2007). The Australian Plague Locust Commission (APLC) conducts locust management in eastern Australia through aerial application of several insecticides, including fipronil, on locust bands and swarms. The rainy conditions that give rise to locust aggregations, also promotes plant growth and insect abundance, which attract many native vertebrates and stimulate their breeding. In Australia, over one hundred avian species have been observed in areas sprayed with fipronil for locust control (Szabo 2005).

A risk assessment of locust-control impacts on Australian arid-zone species, based on a current atlas of Australian bird distribution and APLC GIS data for locust control locations, identified twenty avian species expected to co-occur with locust outbreaks. These include the zebra finch, *Taeniopygia guttata*, budgerigar *Melopsittacus undulatus* and button quail *Turnix varia* (Szabo 2005). In this study we examined fipronil toxicity in four previously unstudied granivorous species: zebra finch, budgerigar, house finch *Carpodacus mexicanus* and king quail *Coturnix chinensis*, the latter chosen as a surrogate species for button quail. We also examined the sensitivity of zebra finch and king quail to fipronil-sulfone. In addition to determining the pesticide sensitivity in these species, we made careful observations of clinical signs of toxicity, which have not been documented previously for avian species.

MATERIALS AND METHODS

Chemicals

Fipronil (C₁₂H₄Cl₂F₆N₄O₂S or (±)-5-amino-1-(2,6-dichloro- α,α,α -trifluoro-*p*-tolyl)-4-trifluoromethylsulphonylpyrazole-3-carbonitrile), CAS No. 120068-37-3, 97% purity; fipronil-sulfone (C₁₂H₄Cl₂F₆N₄O₂S or 1-(2,6-Dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethanesulfonyl-5-aminopyrazole), CAS No. 120068-36-2, 98.2% purity. Both fipronil and fipronil-sulfone were obtained from Chem Services, Inc., USA.

Experimental Animals

Adult house finches were obtained from the wild at The Institute of Environmental and Human Health (Lubbock, Texas, USA) and were housed in individual cages (45 x 30 x 40 cm). Adult zebra finches were obtained from a breeding colony at the University of Wollongong (Wollongong, NSW, Australia). Adult king quails and adult native bush budgies were purchased from aviculturists in Australia. The zebra finch, quail and budgies were housed in individual cages (39 x 44 x 34 cm). All species were held for 14 days prior to treatment to allow environmental adjustment. Commercial seed mix, water and grit were provided *ad libitum*.

Preparation of test substances

Due to the low water solubility of technical grade fipronil (Sigma-Aldrich 2006), fipronil and fipronil-sulfone solutions were prepared using a minimum amount of acetone solvent (approx. 60 μ l acetone per 20 mg fipronil compound). Acetone was chosen as the solvent due to the high solubility of fipronil in acetone (545.9 g/L; BASF 2005). Solutions were then further diluted in canola oil to achieve the desired dosing concentration. Canola oil is the carrier used in the commercial fipronil formulation. Control solutions consisted of acetone and canola oil alone.

Up-and-Down estimation of LD50

Acute oral toxicity test procedures employed in this study follow those outlined in the Organisation for Economic Co-operation and Development (OECD) guidelines for testing of chemicals (OECD 2003). This protocol was introduced in 1998 to minimize the number of animals required to estimate acute oral toxicity to a chemical. In summary, the first animal is given an estimated sublethal dose as determined from available literature (175 mg.kg⁻¹ body weight (bw) is the recommended default starting dose); if this animal survives, the dose administered to the next animal is increased by a factor identified from the OECD dose progression table (default 3.2; OECD 2003); if the first animal dies, the dose is decreased by the same factor. Birds were tested individually and observed routinely for at least 48 h after dose administration. Each administered dose and the respective survival outcome were entered progressively into the statistical analysis program provided with the OECD guideline documentation (Acute Oral Toxicity [Guideline 425] Statistical Program Version 1.0, Westat 2001), from which calculations were made to determine when a confidence interval had been reached. From this established confidence interval, the estimated LD50 (*e*LD50) value was then calculated using the maximum likelihood method. This calculated *e*LD50 allows for the substance to be ranked and classified according to the Globally Harmonised System for classifying chemicals that cause acute toxicity (OECD 1998).

Test procedure

Birds were fasted overnight prior to testing and weighed on the day of dosing. Test solutions were prepared fresh on the day of dosing and either fipronil, fipronil-sulfone or control solution was administered as a single oral dose via gavage in accordance with the OECD Acute Oral Toxicity Testing guideline recommendations to limit maximum liquid dose volumes to no more than 2ml/100g bw (OECD 2003). Doses were based on guidelines from the OECD Up-and-Down dose progression schedule (OECD 2003). Food was returned 30 minutes after dosing and birds were video-recorded for the first three hours following

treatment, and checked and weighed periodically during daylight hours over the duration of their involvement with the experiment.

RESULTS

Fipronil Toxicity and Clinical Signs

The house finch eLD_{50} value for fipronil of 679.7 mg.kg^{-1} was calculated from progressive doses of 55, 550 and 820 mg.kg^{-1} bw, with mortalities occurring at 820 mg.kg^{-1} . For zebra finch the eLD_{50} value of 311.8 mg.kg^{-1} was calculated from doses of 17.5, 26, 37.5, 55, 82, 175, 260, 375, and 550 mg.kg^{-1} bw, with mortalities occurring at the three highest doses. For budgies the eLD_{50} of 147.5 mg.kg^{-1} was calculated from doses of 69, 81, 120, 175, 260 and 375 mg.kg^{-1} bw, with mortalities occurring at the three highest doses. The fipronil eLD_{50} results are summarised in Table 2.1. Signs of fipronil intoxication observed in house finches, zebra finches and budgerigars included involuntary and rapid wing flapping. These signs were observed only at lethal doses of fipronil in both finch species, with onset observed as early as 20 min after treatment, mortality occurring within two to four hours after treatment. Signs of fipronil intoxication were observed in budgerigars at both lethal and sublethal doses as early as 10 min after treatment; mortality occurring as early as 20 min up to 48 hours after treatment. In budgies administered with a sublethal dose of fipronil, duration of these signs of fipronil intoxication, including involuntary movements and convulsions, persisted for up to 48 h post treatment.

The king quail eLD_{50} for fipronil of 22.8 mg.kg^{-1} was calculated from doses of 17.5, 37.5, 81 and 175 mg.kg^{-1} bw with mortalities occurring at the three higher doses. Signs of fipronil intoxication in quail were only observed at lethal doses. Initial signs included cessation of feeding and inactivity; birds usually huddled in a corner of the cage, resting on their sternum (sternal recumbancy), eyes closing. These signs were observed as early as 20 min after

treatment. Gular fluttering was also a common observation. It was not until 2 days after treatment that signs of intoxication such as fanned tail feathers, and involuntary and rapid wing flapping were observed. Mortality occurred from as early as 24 h after treatment and continued up to 3 days after treatment.

Table 2.1. Acute fipronil toxicity in House Finch, Zebra Finch, King Quail and Budgerigar
Average body weight of house finch = 19.1 ± 0.4 g; zebra finch = 12.6 ± 0.4 g; king quail = 40.3 ± 0.4 g and budgerigar = 29.3 ± 1.0 g. Control solutions were non-toxic to all individuals tested.

Species	NOAEL ^a (mg.kg ⁻¹)	eLD50 (mg.kg ⁻¹)	95% CI ^b (mg.kg ⁻¹)	n
House Finch <i>Carpodacus mexicanus</i>	550	679.7	550 – 820	7
Zebra Finch <i>Taeniopygia guttata</i>	175	311.8	178.7 – 649	10
King Quail <i>Coturnix chinensis</i>	17.5	22.8	17.5 – 37.5	8
Budgerigar <i>Melopsittacus undulatus</i>	69	147.5	85.4 – 343	9

^a No Observable Adverse Effects Level (NOAEL)

^b Confidence Interval (CI)

Fipronil-sulfone Toxicity in Zebra Finch and King Quail

The zebra finch eLD50 for fipronil-sulfone was determined as being greater than 820 mg.kg⁻¹ from progressive doses of 375, 550 and 820 mg.kg⁻¹ bw; only one mortality occurred at 820 mg.kg⁻¹ from four birds administered at this highest dose. The fipronil-sulfone eLD50 results are summarised in Table 2.2. The signs of fipronil-sulfone intoxication observed in zebra finch were identical to those of fipronil intoxication and were only observed at the highest fipronil-sulfone dose 820 mg.kg⁻¹. Signs of fipronil-sulfone intoxication were also observed in the three finches that survived and the single finch that did not survive this dose. Onset of signs were observed as early as 20 min after treatment in the finch that did not survive, with mortality occurring within two to four hours after treatment. For the finches that survived, complete remission was observed by 24 h post dose.

Table 2.2. Acute fipronil-sulfone toxicity in King Quail and Zebra Finch

Average body weight of zebra finches used for fipronil-sulfone dosing study = 12.4 ± 0.3 g; king quail = 47.9 ± 1.1 g.

Species	NOAEL (mg.kg ⁻¹)	eLD50 (mg.kg ⁻¹)	95% CI (mg.kg ⁻¹)	n
King Quail <i>Coturnix chinensis</i>	5.5	29.9	0 to > 20,000	11
Zebra Finch <i>Taeniopygia guttata</i>	375	> 820	-	8

The king quail eLD50 value for fipronil-sulfone, of 29.9 mg.kg⁻¹, was calculated from progressive doses of 5.5, 17.5, 37.5, 55, 82 and 240 mg.kg⁻¹; the only dose at which mortalities did not occur was 5.5 mg.kg⁻¹. Signs of fipronil-sulfone intoxication in quail were identical to signs of fipronil intoxication and were observed at both lethal and sublethal doses. For birds that survived the administered dose of fipronil-sulfone, signs persisted for up to 48 h after treatment.

For all four species tested, mortalities from either fipronil or fipronil-sulfone followed violent whole-body convulsions. Posture assumed on death was the head arched back slightly, legs extended, wings dropped ventrally and tail feathers fanned out, with rigor mortis following almost immediately. The no observable adverse effect limit (NOAEL) for each tested species is summarised in Tables 1 and 2 for fipronil and fipronil-sulfone respectively. Excessive preening and ptiloerection was observed in all treated birds at all doses; recovery was based on return to normal activity, behaviour and feeding. No signs of intoxication or abnormal behaviours were observed in birds given the canola oil control.

Body Weight & Feeding Behaviour

In king quail, exposure to a lethal dose of either fipronil or fipronil-sulfone, resulted in significant and progressive loss in body mass (Figure 2.1) until death ensued. These individuals did not appear to feed or drink after treatment. This was also observed in a number of king quail exposed to a sublethal dose of fipronil-sulfone, however recovery, based on gain

in body mass and return to normal feeding behaviour, varied from 48 hours to up to four days post dose. King quail administered with a sublethal dose of fipronil, however, did not show mass loss. Although these birds did not feed or drink on the first day of treatment, a return to normal feeding behaviour was observed within 24 hours. No mass losses or abnormal feeding behaviour were observed in either of the finch species or budgies; budgies were even observed feeding in the periods between convulsive episodes.

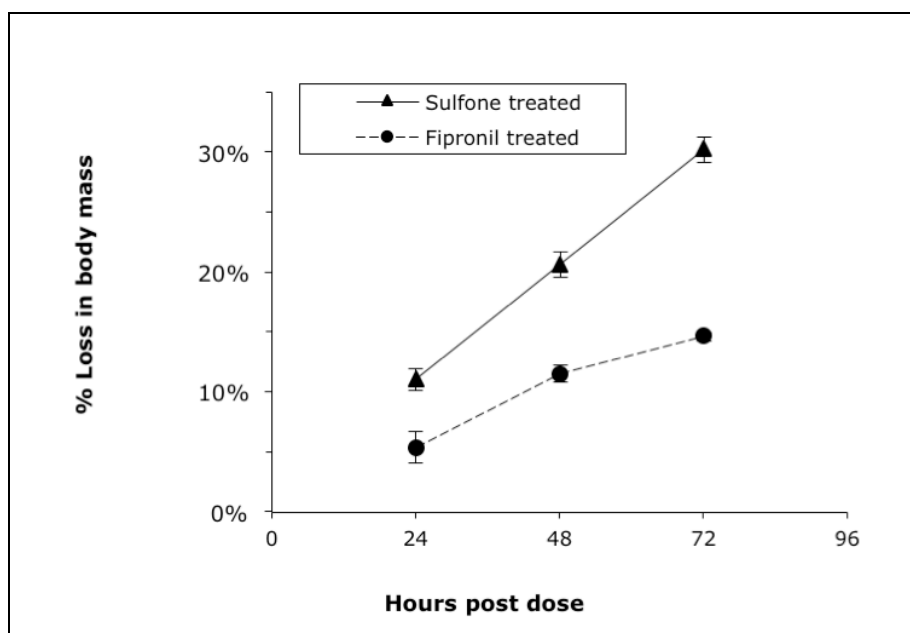


Figure 2.1. Measured loss in king quail body mass as a percentage of initial pre-dose body mass after single lethal oral doses of fipronil (mean data for birds dosed with $37.5 - 175 \text{ mg.kg}^{-1}$) or fipronil sulfone (mean data for birds dosed at $17.5-82 \text{ mg.kg}^{-1}$). Bars indicate standard error. $n = 4,2,3$ for fipronil data points and $n = 5,3,2$ for fipronil-sulfone data points at 24, 48 and 72 hour time-points respectively.

DISCUSSION

Although fipronil sensitivity is highly variable across the few avian species studied (USEPA 1996), when these data are combined with results from this study there appears to be pattern at the ordinal level. The passeriforms tested appear to be moderately sensitive to fipronil; house finch, zebra finch (this study) and house sparrow (Goodyear 1994c), as did the budgerigar, a psittaciform species. By contrast, galliform species are highly sensitive to fipronil; the eLD_{50} determined from this study for king quail is consistent with fipronil sensitivity observed in

bobwhite quail (Kitulagodage et. al unpublished data) and those reported for two other galliform species (Goodyear 1994a, Stavola 1994a, Stavola 1994b). This is the first study to examine fipronil toxicity in a psittaciform species.

There have been varying reports on the relative toxicity of vertebrates to fipronil-sulfone (USEPA 1996) in relation to the parent fipronil compound. Hainzl et al. (1998) demonstrated *in vitro* that sulfone had almost a 6-fold greater binding affinity to mouse brain GABA-receptors than fipronil, however reported mouse LD50s of 41 and 50 mg/kg for fipronil and fipronil-sulfone, respectively. When comparing the toxicity of fipronil-sulfone to fipronil in this study we can see that they are relatively equitoxic in king quail. In zebra finch however, fipronil-sulfone is at least 2.5-fold less toxic than fipronil.

The signs of fipronil intoxication we observed in treated birds including involuntary limb movement, tremors and convulsions have been previously stated for fipronil-treated rats, mice and beagles and for fipronil-sulfone treated rats in unpublished reports submitted for pesticide registration purposes by Rhone Poulenc Agro, the original manufacturer of fipronil (JMPR 1997). With respect to the onset and duration of signs of fipronil intoxication, clear differences were observed between the galliform and passeriform species. Signs were identical for both Passeriformes (zebra finch and house finch), with rapid onset and complete remission by 24 h post treatment. For the Galliformes (king quail), expected signs of fipronil intoxication were not observed until at least 2 days after treatment, and lasted up to 72 h post-dosing. At lethal doses of fipronil, quail did not eat or drink after treatment, reflected by a progressive loss in body mass. This treatment-related reduced feeding behaviour and weight loss has also been observed in fipronil-treated bobwhite quail, another galliform, in addition to identical onset and duration of signs of intoxication (Kitulagodage et. al unpublished data). Although signs of fipronil intoxication persisted up to 48 h post treatment in the psittaciform species tested (budgerigar), no such effects on feeding behaviour or weight loss was observed. It is

interesting to note that identical signs of intoxication observed in fipronil-treated king quail including reduced feeding behaviour and weight loss were also observed in fipronil-sulfone treated king quail. A study on fipronil treated bobwhite quail demonstrated a significant correlation between weight loss and fipronil-sulfone residue levels in brain, liver and adipose tissue (Kitulagodage et. al unpublished data). This suggests that the sulfone metabolite of fipronil may be responsible for the observed reduction in feeding behaviour and progressive weight loss in both quail species. Such reductions in feeding may exacerbate the toxic effects of pesticides and contribute to the greater fipronil sensitivity in Galliformes.

This study suggests fipronil sensitivity varies more across the avian orders than among species within a given order. Further research is needed, however, to elucidate the influence of the sulfone metabolite on the toxicity of fipronil across the different avian orders. This information would improve prediction of likely ecotoxicological impacts of fipronil on unstudied avian species at risk of exposure.

CHAPTER 3. FORMULATION TOXICITY: SOLVENT USED CONTRIBUTES TO TOXICITY OF FORMULATED PRODUCT

This chapter has been published as

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ABSTRACT

Fipronil, a phenyl pyrazole pesticide, is aerially applied in eastern Australia to control locust outbreaks, usually as “Adonis 3UL Insecticide®” (BASF), an ultra low (UL) volume formulation containing 0.3% active pesticide. We tested the toxicities of technical-grade fipronil, the Adonis 3UL formulation and its components in zebra finch, a native bird at risk of exposure in locust control regions. We estimated oral-dose LD50 by the Up-and-Down method. Under laboratory conditions, we identified unexpectedly high toxicities due exclusively to diacetone alcohol (DAA), a solvent making up 12.5% of the Adonis 3UL formulation. In contrast, finches were asymptomatic when exposed to 0.3% technical grade fipronil dissolved in a minimum amount of acetone. Depending upon the behaviour and persistence of DAA under field conditions, this formulation of Adonis 3UL may pose a far greater threat to the health of small birds and possibly other vertebrates than expected for fipronil alone.

INTRODUCTION

Many pesticide studies have demonstrated significant differences between the toxicities of formulation components and the active ingredient (Marc *et al.* 2001; Paul *et al.* 2005; Peixoto 2005; Braconi *et al.* 2006; Skandrani *et al.* 2006). For example the presence of surfactants increase the toxicity of Roundup® (Mann *et al.* 1999) and Arsenal 250NA® herbicides (Grisolia *et al.* 2004) while additives are known to increase the toxicity of 5 insecticide formulations; Dicarzol 200®, Lannate 20®, Pirimor G®, Kiros EV® and Talstar® (Skandrani *et al.* 2006). These findings demonstrate the importance of including appropriate formulation blank controls when assessing pesticide formulation toxicity.

Adonis 3UL insecticide®, a commercial fipronil-based pesticide formulation registered for use in Australia, is used to control locusts in semi-arid and agricultural areas (APLC 2007). There is no toxicological information specific to this formulation, which contains 0.3% fipronil (active ingredient, by mass) and 12.5% diacetone alcohol (DAA, by mass; BASF 2003). Furthermore, although DAA is a commonly used solvent listed as an “inert of unknown toxicity” by the Environmental Protection Agency in the United States (USEPA 2004), its toxicological effects on vertebrates have not been examined. Fipronil is an effective neurotoxin targeting gamma-amino butyric acid (GABA) receptors (Hainzl *et al.* 1996), and, despite the abundance of these receptors in vertebrate brains, there is still little available information regarding toxicological effects of fipronil in vertebrates. Available information however, demonstrates there is high species-specific variability in fipronil sensitivity across the few avian species studied; fipronil is highly toxic to the two galliform species tested, yet considered non-toxic to the mallard duck (USEPA 1996). This variability makes it extremely difficult to predict the toxicity of fipronil on unstudied species.

In the course of evaluating the sensitivity of fipronil in zebra finches (*Taeniopygia guttata*), a

native Australian bird species, we identified the toxicity of the commercial Adonis 3UL formulation to be greater than that of formulations using technical-grade fipronil (Kitulagodage *et al.* unpubl. data). Consequently, the objective of this study was to examine the toxicity of Adonis 3UL and its ingredients technical-grade fipronil, a lab-made formulation mimicking the Adonis 3UL mixture, and DAA.

MATERIALS AND METHODS

Chemicals

Fipronil (C₁₂H₄Cl₂F₆N₄O₂ or (±)-5-amino-1-(2,6-dichloro-a,a,a-trifluoro-p-tolyl)-4-trifluoromethylsulphonylpyrazole-3-carbonitrile), CAS No. 120068-37-3, 97% purity was obtained from Chem Services, Inc., USA (Cat. No. PS-2136). Adonis 3UL Insecticide® was obtained from BASF Australia Ltd. and contained 0.3% fipronil, 12.5% DAA in the carrier canola oil. Diacetone alcohol (DAA; C₆H₁₂O₂ or 4-Hydroxy- 4-methyl-2-pentanone), CAS No. 123-42-2, purity 99% was obtained from Sigma- Aldrich Pty. Ltd., USA (Cat. No. H41544).

Experimental Animals

Adult male zebra finches were obtained from a breeding colony at the University of Wollongong. Birds were housed two per cage (38 x 44 x 34 cm) for at least 14 days prior to treatment to allow environmental adjustment. Commercial finch seed mix, water and grit were provided *ad libitum*.

Preparation of test substances

We prepared a lab version of the Adonis 3UL fipronil formulation, “Lab-Adonis”, containing 0.3% technical grade fipronil and 12.5% DAA (15 mg fipronil dissolved in 625 mg DAA) prepared to a total volume of 5 ml using canola oil (the carrier used in Adonis 3UL). A 0.3%

fipronil formulation, “Fipronil stock”, was prepared using 15 mg technical-grade fipronil dissolved in 60ml of acetone, then prepared to a total volume of 5 ml using canola oil. Acetone was chosen as the solvent due to the high solubility of fipronil in acetone (545.9 g/L; BASF 2005). A 12.5% DAA formulation, “DAA stock”, was prepared using 125 mg DAA prepared in 1 ml of canola oil. In addition we used fresh commercial Adonis 3UL and a canola oil control. The composition of substances tested is summarised in Table 3.1.

Table 3.1 Proportion of Fipronil and DAA per test substance.

Test Substance	Fipronil (w/v)%	DAA (w/v)%
Adonis 3UL	0.3	12.5
Lab-Adonis	0.3	12.5
DAA stock	0	12.5
Fipronil stock	0.3	0
Control	0	0

Up-and-Down estimation of LD50

Acute oral toxicity test procedures employed in this study follow those outlined in the Organisation for Economic Co-operation and Development (OECD) guidelines for testing of chemicals (OECD 2003). This protocol was introduced in 1998 to minimize the number of animals required to estimate acute oral toxicity to a chemical. In summary, the first animal is administered an estimated sublethal dose as determined from available literature (175 mg/kg is the recommended default starting dose); if this animal survives, the dose administered to the next animal is increased by a factor identified from the OECD dose progression table (default 3.2; OECD 2003); if it dies, the dose is decreased by the same factor. Birds were tested individually and observed routinely, during the immediate 48 h after dose administration. The “stopping criteria” to determine when a confidence range has been reached is as follows: if three animals survive at the highest dose in the progression, the chemical is considered to be of low acute toxicity hazard to the species; if five reversals are observed in any six consecutive animals tested (a reversal is a change in survival outcome) then a “confidence interval” of

lethality between those two dosing concentrations has been reached. From this established confidence interval, the estimated LD50 (*e*LD50) value was calculated statistically using the maximum likelihood method as per OECD guidelines (OECD 2003). This *e*LD50 allows for the substance to be ranked and classified according to the Globally Harmonised System for the classification of chemicals, which cause acute toxicity (OECD 1998).

Test procedure

Birds were fasted overnight prior to testing and weighed on the day of dosing (average zebra finch weight of 13g). Adonis 3UL, Lab-Adonis, the DAA stock, the fipronil stock, or the canola oil control were administered as a single oral dose via gavage. Dosing volumes followed OECD Up-and-Down guideline recommendations of maximum liquid dose volumes of 2ml/100g body weight (OECD 2003). Food was returned 30 minutes after dosing and birds were video-recorded for the first three hours following treatment and checked periodically over the next 48 h. Adonis 3UL was administered at progressive doses of 26, 37.5 and 55 mg fipronil/kg body weight (bw) based on a 3.2 factor according to the OECD dose progression schedule (OECD 2003). To compare toxicity levels, doses of Adonis 3UL, the Lab-Adonis, the fipronil stock, the DAA stock, and the canola oil control were administered on a ml/kg bw basis at progressive doses of 8.7, 12.5 and 18.3 ml solution/kg bw (equivalent to 26, 37.5 and 55 mg fipronil/kg for solutions containing fipronil).

Vaporisation of DAA in test solutions

The vapour pressure of DAA is 0.97mm Hg at 20° C and it has a slow relative evaporation rate of 0.14 (nBuAc = 1.0; Celanese 2000). To investigate whether significant amounts of DAA might be evaporated after aerial application, thus reducing likely avian exposure, we weighed 200ml aliquots of each formulation (DAA alone, Lab-Adonis, Adonis 3UL, and canola oil) at 2-min intervals over 20 minutes. This was measured in triplicate under standard lab conditions with constant room temperature of 21° C.

RESULTS

Adonis 3UL toxicity

The *eLD50* for technical-grade fipronil (OECD up-down method) was established in a previous study (Table 6.2; Kitulagodage *et al.* unpubl. data). In the present study a lethal dose confidence interval of fipronil in Adonis 3UL was established in 5 reversals (*i.e.*, 6 individuals) and thus an *eLD50* value of 45.41 mg fipronil/kg. This is equivalent to an *eLD50* of 15.14 ml Adonis 3UL/kg (Table 3.2).

Table 3.2 Acute toxicity of Adonis 3UL and Technical-grade Fipronil in Zebra Finch

Toxicity Units	<i>eLD50</i>	Confidence interval
Adonis 3UL	45.41 mg fipronil/kg	37.5 – 55 mg fipronil/kg ^(a)
	15.14 ml Adonis 3UL/kg	12.5-18.3 ml Adonis 3UL/kg ^(a)
Technical Fipronil	310.2 mg/kg	175 – 550 mg/kg ^(b)

^(a) *Present study*

^(b) *Kitulagodage et al. unpubl. data*

Formulation component toxicity

Using the OECD methods, identical *eLD50* values of 15.14 ml/kg were calculated for Adonis 3UL, Lab-Adonis and the DAA stock (Table 3.3). This was based on established identical toxicity confidence intervals, lying between 12.5 and 18.3 ml/kg. Both the fipronil stock and the control solutions were non-toxic to all birds at the doses administered (8.7, 12.5 and 18.3 ml/kg); despite the fipronil stock containing the same concentration of fipronil as Adonis 3UL and the Lab-Adonis solutions.

The comparative vaporisation test demonstrated all the test solutions had slow rates of evaporation. The total evaporative loss over 20 min as a percentage of initial mass was 0.4% (± 0.02) for DAA, 1.4% for both 1 Canola oil (± 0.02) and the DAA formulation (± 0.04), and

2.3% (± 0.03) for Adonis. The resulting evaporation rates were 2.0, 6.6 and 10.4 mg/h respectively.

Table 3.3 Acute toxicities of test solutions administered to Zebra Finch

Test Solution	eLD50 (ml/kg)	Confidence interval (ml/kg)	n
Adonis 3UL	15.14	12.5 – 18.3	7
Lab-Adonis	15.14	12.5 – 18.3	7
DAA stock	15.14	12.5 – 18.3	7
Fipronil stock	Non-toxic	Non-toxic	5
Control	Non-toxic	Non-toxic	5

Signs of Intoxication

Identical signs of intoxication were observed in all birds dosed with both lethal and sublethal amounts of Adonis 3UL, lab-Adonis, and the DAA formulation. These included ataxia, wing drop, fanned tail feathers, diarrhoea and loss of righting reflex. Signs were observed as early as 1 min after treatment. When birds died, mortality occurred overnight with no specific posture on death. Of those birds that survived, recovery, based on return to normal activity, behaviour and feeding, was observed 24-h post treatment. No signs of intoxication were observed in birds given the fipronil stock or the canola oil control.

DISCUSSION

These results clearly demonstrate that the presence of DAA render fipronil formulations far more toxic to birds than would be predicted from evaluations of fipronil alone. This is shown by Adonis 3UL, Lab-Adonis and the DAA stock having identically toxic effects on zebra finches. By contrast, fipronil administered in a formulation lacking DAA (fipronil stock) was not toxic at the same administered doses. Therefore, the toxicity observed appears to be directly attributable to DAA exposure. The presence of DAA in the formulations is responsible

for the 7-fold difference between the eLD50s in zebra finch for Adonis 3UL (45.41 mg fipronil/kg) and technical fipronil (310.2 mg fipronil/kg; Kitulagodage *et al.* unpubl. data). Importantly, all test substances containing DAA provoked identical signs of intoxication in zebra finches, but very distinct to those observed in birds dosed with higher amounts of technical-grade fipronil without DAA (Kitulagodage *et al.* unpubl. data). Fipronil acts by targeting gamma-aminobutyric acid (GABA) receptors, resulting in neural excitation and convulsions (Cole *et al.* 1993). Signs observed in zebra finches dosed with high amounts of technical-grade fipronil included involuntary wing flapping and convulsions; consistent with expected fipronil toxicity effects. Such indicators of fipronil toxicity were not observed in zebra finches in the current study, further demonstrating that DAA, not fipronil, was responsible for the observed toxicity in zebra finches dosed with our formulations.

Little is known about the ecotoxicity of DAA to terrestrial species, although it is known to have low toxicity to a few aquatic organisms tested (toxicity values greater than 100 mg/L; OECD 2000). To accurately assess the ecotoxicological impact of Adonis 3UL as a locust-control method, a better understanding of the behaviour of Adonis under field conditions is required. Considering that Adonis 3UL is applied as an aerial spray (APLC 2007), the rate of vaporisation of the formulation and its components should be assessed. Available physical and chemical data (Celanese 2000) and results of our simple comparative test, however, indicate that DAA has a slow evaporation rate. When released in air, DAA is expected to have a half-life between 10 and 30 days (JSL 2005). Furthermore, about half of airborne DAA may accumulate in water, where it is stable indefinitely at pH 7 (OECD 2000; JSL 2005). With respect to the release of Adonis 3UL into the environment via aerial spraying, a significant proportion of the DAA released is likely to accumulate in surface water where it will remain as DAA for periods of weeks or months. In Australia, Adonis 3UL is used to control locusts in large areas of arid zone habitat (APLC 2007). Although pesticide spraying in the vicinity of water bodies is strictly prohibited in operational guidelines for locust control, small pools of

water, and subsequent rainfall cannot be easily avoided and these sources of water will be highly attractive to terrestrial animals in these arid areas. Thus, the use of Adonis UL formulations with DAA represents a significant exposure risk for birds and other terrestrial vertebrates using water sources on or adjoining sprayed areas.

In addition to being an ingredient in the Adonis 3UL formulation, DAA has been used in an 8.5UL (8.5 g fipronil/L; pers. comm. APLC 2007) and a 10UL (10 g fipronil/L) Adonis formulation (Peveling *et al.* 2003) which are all Ultra Low Volume (ULV) liquid formulations. ULV formulations are typically dispersed as fine droplet particles at very low application rates (Micronair 2006), therefore reducing the amount of active ingredient and solution needed to achieve insect control compared to high volume methods (AFPM 1999). There is no public information regarding the function of DAA in the Adonis formulation. However, since its most common use is in printing inks to achieve favourable flow and levelling characteristics (Celanese 2000), it is likely that DAA is an effective dispersant in Adonis. Clearly, DAA in Adonis formulations should be replaced with an alternative solvent.

CONCLUSIONS

It is clear that DAA toxicity may pose a greater risk to birds than fipronil in relation to the Adonis 3UL formulation used in locust control. Care and due diligence are essential in developing new formulations. This study demonstrates that assessment of the active ingredient toxicity alone, in this case fipronil, is inadequate in evaluating the toxicity of pesticide formulations.

CHAPTER 4. REDUCED FEEDING BEHAVIOUR AND WEIGHT LOSS IN FIPRONIL TREATED BOBWHITE QUAIL

This chapter has been published as

Malsha Kitulagodage, John Isanhart, William A. Buttemer, Michael J. Hooper, Lee B.

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M. J. Hooper assisted with experimental design and J. Isanhart assisted with sample collection.

ABSTRACT

Fipronil is a phenyl pyrazole insecticide registered for agricultural use in many countries.

Avian exposure to fipronil occurs mainly by ingesting contaminated insects or seeds. There is little information regarding the toxicological effects of fipronil in avian species and even less research documenting avian behavioural responses to fipronil ingestion. We examined the effects of a single oral dose of fipronil in northern bobwhite quail, the most fipronil-sensitive species tested to date, in respect to signs of intoxication and the metabolic fate of fipronil.

Fipronil-treated birds did not eat or drink following pesticide administration, and as a result lost a significant amount of body mass. Treated birds also appeared withdrawn and did not respond to disturbance within the first hour after treatment. Identifiable signs of fipronil toxicity were not observed until at least 2 days after treatment. Chemical analyses indicated a difference between fipronil and fipronil-sulfone residue distribution and bioaccumulation, with significantly higher (30- to 1000-fold) tissue concentrations of the sulfone detected at all time points from 8 to 96h post-dose in brain, liver and adipose tissues. Tissue sulfone concentrations increased significantly in fipronil-treated birds, peaking at 72h post-dose. Body mass decreased at all time points in dosed birds. The coincidence of the particular intoxication symptoms with the time course of rise in brain sulfone levels after fipronil dosing gives insight into possible mechanisms of toxicity in this highly sensitive species.

INTRODUCTION

Fipronil is a phenyl-pyrazole pesticide developed by Rhone-Poulenc Agro in the late 1980s (Bobe *et al.*, 1998a, 1998b; Moffat 1993). It is aerially applied as an agricultural insecticide in many countries including Russia, South Africa and Australia (APLC, 2007; BASF 2005). It is typically used at times when insect pests are abundant; these seasonal conditions also attract many vertebrates, such as birds and lizards, presenting a high fipronil exposure risk to these species. In Australia over one hundred different bird species have been observed in areas common to fipronil spraying events for locust control (Szabo, 2009), with ingestion of contaminated seeds and insects a likely route of exposure for these birds and indeed many other vertebrates.

Publicly available toxicity studies by Rhone-Poulenc identify that reduced feeding behaviour was observed in fipronil-treated northern bobwhite quail (Goodyear, 1994a), ring-necked pheasant (Stavola, 1994a), and in the red-legged partridge (Stavola, 1994b) at various dosage levels. This reduction in food consumption also resulted in significant losses in body mass, most significantly in the bobwhite quail, with birds reported as severely emaciated. These observations have not been reported in peer-reviewed literature, however they are of potential consequence as fipronil has also been shown to be highly toxic to these galliform species, with LD₅₀ values of 11.3, 31 and 34 mg.kg⁻¹ reported for bobwhite quail (Goodyear, 1994a) ring-necked pheasant (Stavola, 1994a) and red-legged partridge (Stavola, 1994b), respectively.

Fipronil is an effective neurotoxicant targeting gamma-amino butyric acid (GABA) receptors (Hainzl *et al.*, 1996), and despite the abundance of these receptors in vertebrate brains, there is still little available information regarding toxicological effects of fipronil in vertebrates.

Available information demonstrates there is high species-specific variability in fipronil sensitivity across the few avian species studied, ranging from highly toxic to Galliformes, as mentioned above, to practically non-toxic to the mallard duck (LD₅₀ greater than 2150 mg.kg⁻¹;

Goodyear, 1994b; USEPA, 1996). This variability makes it extremely difficult to predict the toxicity of fipronil in unstudied species.

In this study we examined fipronil toxicity in bobwhite quail, the most fipronil-sensitive species, at the cited LD₅₀ of 11.3 mg.kg⁻¹. In addition to detailed behavioural observations, the metabolic fate of fipronil in these birds was assessed in order to gain further insight into underlying mechanisms responsible for their high sensitivity to fipronil.

MATERIALS AND METHODS

Chemicals

Fipronil (C₁₂H₄Cl₂F₆N₄O₅ or (±)-5-amino-1-(2,6-dichloro- α,α,α -trifluoro-p-tolyl)-4-trifluoromethylsulphonylpyrazole-3-carbonitrile), CAS No. 120068-37-3, of 97% purity was obtained from Chem Services, Inc., USA.

Experimental Animals

Northern bobwhite quail (*Colinus virginianus*) adult males (44 weeks old) were obtained from Diamond H Ranch (Bandera, Texas, USA). Birds were housed indoors in individual cages (25 x 25 x 61cm) for 14 days prior to treatment to allow environmental adjustment. Room temperature and humidity were maintained at 23.0 ± 2.0°C and 50.0 ± 5.0% respectively throughout the study. Commercial poultry feed (game bird flight conditioner, Purina Mills, St. Louis, MO, USA) and tap water were provided *ad libitum*.

Preparation of test substances

Due to the low water solubility of technical grade fipronil (Tingle *et al.*, 2003), the fipronil solution was prepared using a minimum amount of acetone solvent (approx. 60 µl acetone per 20 mg fipronil). Acetone was chosen as the solvent due to the high solubility of fipronil in

acetone (545.9 g.L⁻¹; BASF, 2005). The solution was then diluted in canola oil to the required dose concentration of 11.3 mg ml⁻¹ (confirmed by AgriSolutions Pty. Ltd., Brisbane, Queensland, Australia). Canola oil is the vehicle used in common fipronil formulations for locust-control (e.g. Adonis®; BASF, 2003). Control solutions consisted of acetone and canola oil, only.

Test procedure

Birds were fasted overnight prior to testing and weighed on the day of dosing (mean \pm SE, bobwhite quail weight of 193 \pm 11g). Test solutions were prepared fresh on the day of dosing and administered using a gavage needle as a single oral dose. Treatment group quail (25 birds) were administered with fipronil solution at 1ml/200g body weight to achieve a dose of 11.3 mg.kg⁻¹; control group quail (15 birds) were administered with a volume-based equivalent dose of control solution. Dose volumes followed the Organization for Economic Co-operation and Development (OECD) guidelines for Acute Oral Toxicity Testing (OECD, 2003) and were adjusted according to body weight. Food was returned 30 minutes after dosing and birds were observed constantly for the first six hours following treatment, and then monitored for 20 minutes each hour during daylight hours over the 96 hour duration of the study. Behavioural observations were documented throughout the study. Birds were weighed and euthanised in groups at 8, 24, 48, 72 and 96 hours post-dose; brain, liver and adipose tissue were collected for fipronil residue analysis.

Chemical Extraction and Analysis

Tissue samples collected for analysis of fipronil and fipronil derivatives were extracted and analysed at AgriSolutions Pty. Ltd. (Brisbane, Queensland, Australia). Samples were homogenized, extracted in hexane and then filtered and liquid-liquid partitioned with acetonitrile. Residues were then isolated and purified via activated carbon and reverse phase C18 solid phase extraction chromatography. The final purified extracts were brought up in

acetonitrile, and passed through a 0.45 μm polytetrafluoroethylene filter prior to quantitative analysis via gas chromatography tandem mass spectrometry (GC/MS/MS). The level of parent fipronil and fipronil metabolite for each sample were expressed as mg compound per kg of tissue sample.

Statistical Analysis

Comparative fipronil and fipronil-sulfone residue levels in brain, liver and adipose tissue for each time-point (eight, 24, 48, 72 and 96h) were analysed using a *t*-test. The effect of time as a factor on fipronil-sulfone levels were examined using one-way analysis of variance (1-way ANOVA) with Tukey's post-hoc tests. Correlation plots were used to assess the relationship between tissue residue concentrations and percent body mass loss; fipronil and fipronil-sulfone residue levels detected in brain tissue ($n=24$), liver tissue ($n=23$) and adipose tissue ($n=16$) from each fipronil-treated quail were plotted against its respective percentage loss in body mass. This percentage loss in body mass was measured as (pre-treatment weight - final weight when euthanized) / pre-treatment weight. Pearson correlation coefficients (*r*) were calculated for each data set, i.e. loss in body mass (%) vs. (a) brain fipronil, (b) brain sulfone, (c) liver fipronil, (d) liver sulfone, (e) adipose fipronil and (f) adipose sulfone levels (mg.kg^{-1}). Analysis was performed using GraphPad Prism software (Version 5.02, GraphPad Software Inc., CA, USA).

RESULTS

Signs of Intoxication

Signs observed immediately after treatment included: ptiloerection, sternal recumbancy, withdrawal, eyes closing, and lack of response to disturbance. It was not until at least 48h after treatment that involuntary movements including tremors and convulsions, typical signs of fipronil toxicity (JMPR, 1997), were observed (Figure 4.1).

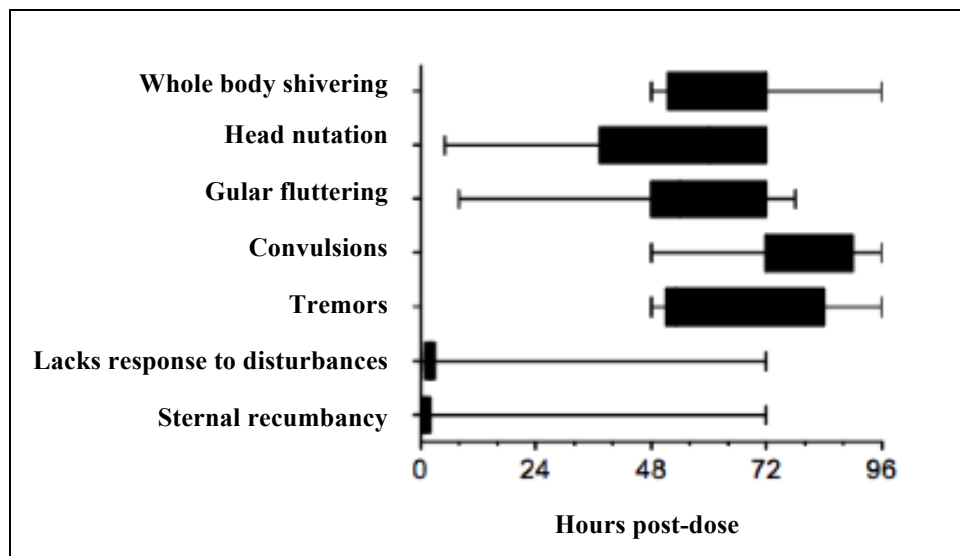


Figure 4.1. Onset of signs of intoxication observed in fipronil treated bobwhite quail after administration of an 11mg kg⁻¹ single oral dose of fipronil. Bars indicate earliest and latest onset of signs observed.

Other observations at this stage included: gular fluttering, head nutation and whole body shivering. Percentages of treated birds exhibiting signs observed are presented in Table 4.1. No treated birds appeared to eat or drink for the duration of the study after dosing. A single mortality of a dosed bird occurred, this individual was found dead on the morning of the third day after treatment. Posture assumed was the head arched back slightly, legs extended, wings dropped ventrally and tail feathers fanned out; brain liver and adipose tissue samples were collected. No signs of intoxication or behavioural abnormalities were observed in birds given the control solution.

Table 4.1 Percentage of treated birds displaying observed signs of intoxication at 8, 24, 48, 72 and 96h post-dose after administration of a single 11.3 mg kg⁻¹ oral dose of fipronil. Control group birds did not display any signs of intoxication throughout the study.

Time post-dose	Treatment group birds (%) displaying signs of intoxication observed						
	Sternal recumbancy	Lack of response to disturbances	Tremors	Convulsions	Gular fluttering	Head nutation	Whole body shivers
8h	84%	80%	0%	0%	4%	4%	0%
24h	84%	80%	0%	0%	4%	4%	0%
48h	92%	80%	60%	4%	16%	12%	20%
72h	100%	100%	76%	36%	28%	24%	56%
96h	100%	100%	100%	48%	28%	24%	64%

Feeding Behaviour & Body Mass

Once dosed, absence of feeding behaviour was only observed in fipronil-treated birds, this was not observed in any control group birds. Birds from both groups had lost approximately 2.5 percent of their initial body mass 8h after dosing (Table 4.2). The control group birds regained and exceeded their pre-dose body mass by 40h post-dose and maintained these weights throughout the remainder of the study. In contrast, fipronil-dosed birds continued to lose weight throughout the study and by 96h post-dose had lost approximately 15% of their pre-dose body mass. The significant progressive weight loss in fipronil-treated quail limited the study duration to 96h for ethical reasons as outlined in the OECD Humane Endpoint Guidance Document (OECD, 2000).

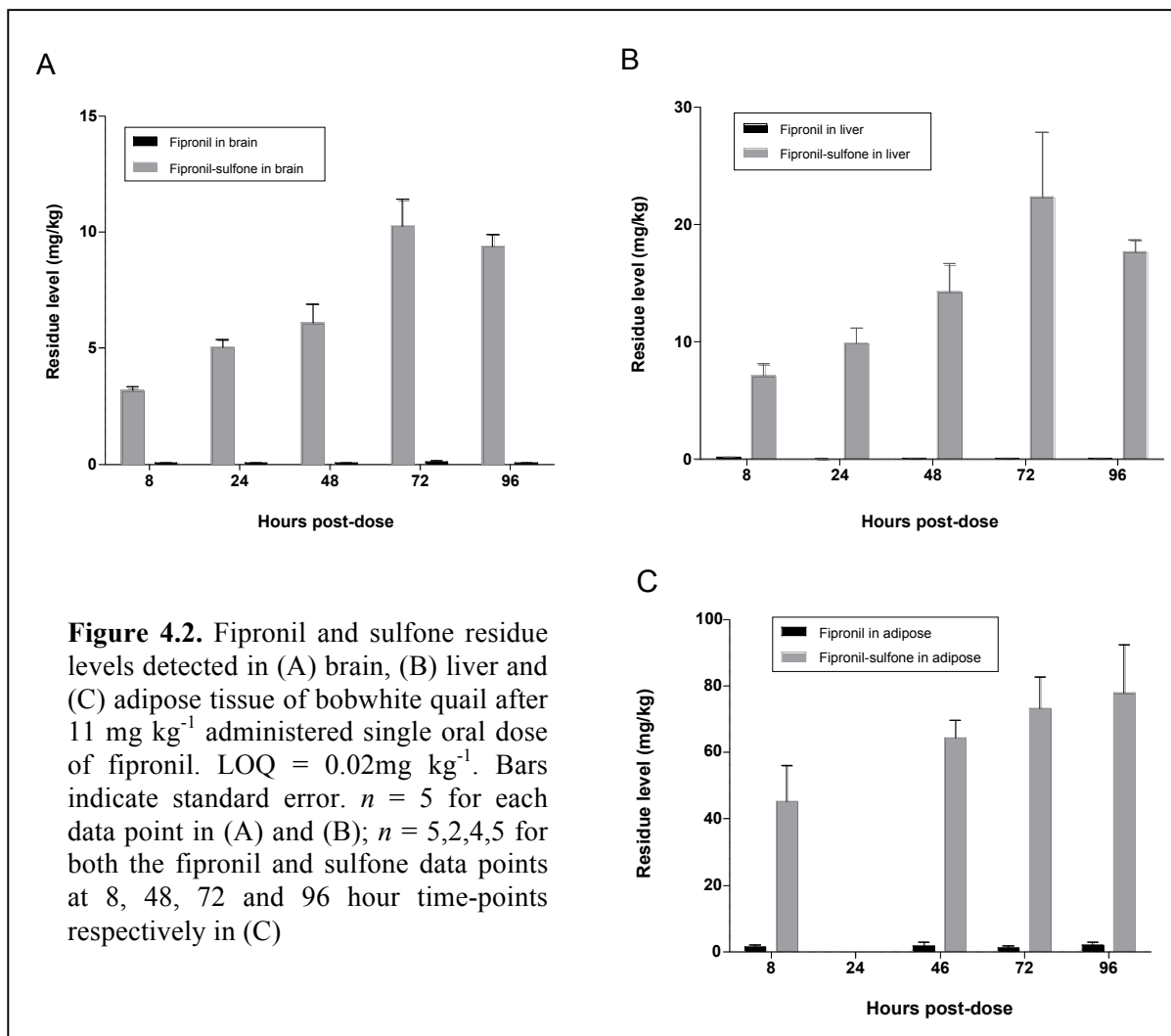
Table 4.2 Progressive change (Δ) in body mass of fipronil-treated bobwhite quail compared with control group calculated as % of pre-dose weight are represented as Means \pm SE. Treatment group birds were administered a single oral dose of fipronil at 11.3 mg kg⁻¹. No control group birds were weighed at 24h post-dose.

Time post-dose	Treatment Group		Control Group	
	Δ body mass (%)	n	Δ body mass (%)	n
8h	-2.48% \pm 0.004	5	-2.52 \pm 0.005	5
24h	-5.05% \pm 0.005	5	-	-
48h	-9.34% \pm 0.012	15	3.78 \pm 0.004	10
72h	-13.95% \pm 0.018	10	3.39 \pm 0.006	5
96h	-14.85% \pm 0.015	5	3.26 \pm 0.006	5

Fipronil Disposition

Fipronil and its sulfone metabolite were the only detectable residues in all three tissues above the Limit of Quantification (LOQ) of 0.02 mg kg⁻¹. No fipronil or fipronil metabolites were detected in tissue samples collected from control birds. Sulfone metabolite concentrations were significantly greater than those of fipronil in brain ($p < 0.003$), liver ($p < 0.0049$) and adipose tissue ($p < 0.0155$) in fipronil-treated birds at all times following dosing (Figure 4.2). Sulfone concentrations increased significantly over the first 72 hours in brain ($p < 0.001$) and liver ($p <$

0.0061) then dropped slightly between 72 and 96 hours in both (not statistically significant; Figure 4.2A). Although mean sulfone concentrations increased in adipose tissue at each time point, this was not statistically significant (Figure 4.2C).



Comparing the three tissue types, at 8h post-dose, sulfone concentrations in adipose tissue were significantly higher than in brain (15-fold, $p < 0.0162$) and 6-fold higher than in liver ($p < 0.0196$). The concentrations of both sulfone and fipronil residues remained significantly higher ($p < 0.001$) in adipose tissue than those in brain and liver throughout the post-dose sampling period.

Fipronil and sulfone concentrations detected in tissue of the quail found dead 72 hours post-

dose (Table 4.3) did not differ more than one standard deviation from the mean of levels detected in the 72 hour time point group in all cases apart from sulfone levels detected in adipose tissue which differed by two standard deviations.

Table 4.3 Fipronil and sulfone residue levels in brain, liver and adipose tissue of lone bobwhite quail found dead 72 hours post-dose and of quail euthanised 72 hours post-dose (represented as Means \pm SD). All birds were administered a single oral dose of fipronil at 11.3 mg kg⁻¹. Tissues were analysed for their fipronil and fipronil-derived sulfone content.

		72h mortality	72h group	
		Residue level (mg kg ⁻¹)	Mean residue level (mg kg ⁻¹)	n
Fipronil	Brain	0.02	0.07 \pm 0.09	4
	Liver	0.00	0.03 \pm 0.06	4
	Adipose	1.86	1.07 \pm 0.99	3
Sulfone	Brain	11.14	10.25 \pm 2.25	4
	Liver	24.96	22.25 \pm 9.61	3
	Adipose	98.53	73.07 \pm 16.35	3

Relationship between weight loss and residue levels

There was a strong positive relationship between loss in body mass and sulfone levels across all three tissues ($p < 0.0063$, all comparisons; Figure 4.3), with the strongest correlation found in brain tissue ($p < 0.0001$; Figure 4.3B). No significant relationship was observed between weight loss and fipronil residue levels for any of the three tissue types sampled.

DISCUSSION

Feeding behaviour

The absence of feeding behaviour and consequent weight loss observed in bobwhite quail treated with fipronil in this study are consistent with reports on three fipronil-sensitive galliform species: bobwhite quail (Goodyear, 1994a), ring-necked pheasant (Stavola, 1994a) and red-legged partridge (Stavola, 1994b). It should be noted that not all methodological details and specific results are provided in these non-peer-reviewed reports as they were submitted for pesticide registration purposes by Rhone Poulenc Agro, therefore subject to

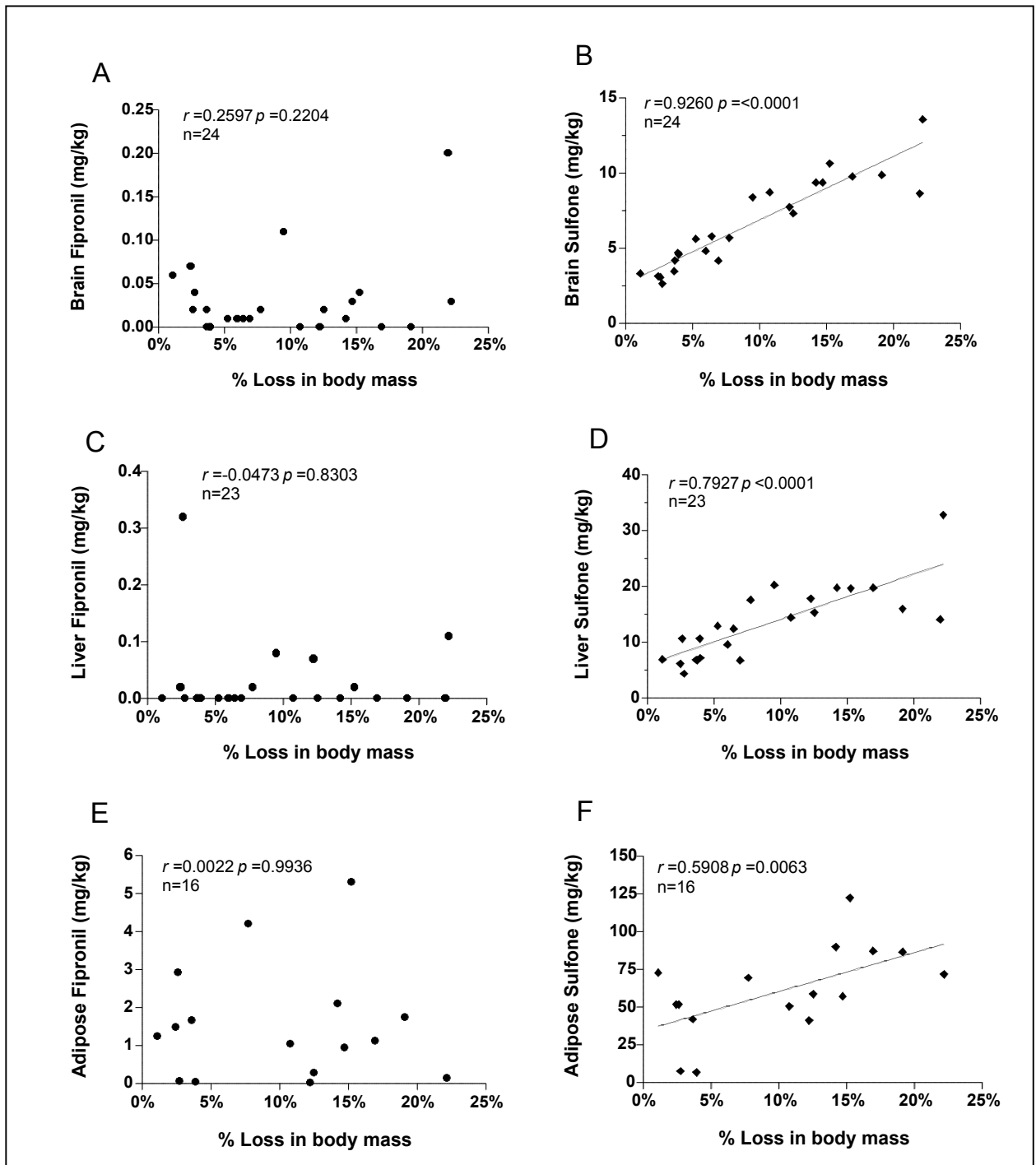


Figure 4.3. Relationship between percentage loss in body mass and detected residue levels of (A) fipronil in brain, (B) sulfone in brain, (C) fipronil in liver (D) sulfone in liver, (E) fipronil in adipose, and (F) sulfone in adipose tissue of bobwhite quail after 11 mg kg^{-1} administered single oral dose of fipronil. Percentage loss in body mass is the measured as difference between pre-treatment weight and final weight at time individual was euthanised for tissue sample collection.

confidentiality requirements. In the peer-reviewed literature, Avery *et al.* (1998) examined the selective feeding response of red-winged blackbirds (*Agelaius phoeniceus*) to fipronil-treated rice seeds and noted no adverse toxic effects over a 3-hour period of visual observation. However, birds demonstrated a significant avoidance of the treated rice, which had been dyed blue. The fact that red-winged blackbirds have demonstrated a strong colour avoidance response to food dyed blue in other studies (Avery *et al.*, 1999; Werner *et al.*, 2008) suggests a bias in the study design negates the authors' conclusion that fipronil had little effect on the blackbirds.

The absence of feeding behaviour observed in our fipronil treated quail has also been reported in common grackles (*Quiscalus quiscula*) exposed to four different organophosphate insecticides and discussed as 'pesticide-induced anorexia' (Grue, 1982), resulting in a loss of over 25% of bird body mass before death. In respect to fipronil, ingestion clearly results in cessation of feeding in bobwhite quail, to the extent that the resulting loss in body mass itself is potentially lethal. In addition to this, galliform species are highly sensitive to fipronil, with LD₅₀s ranging between 11.3 mg kg⁻¹ to 34 mg kg⁻¹ (Tingle, *et al.* 2003). This toxicity of fipronil to galliform species is on par with that of fenthion, which is commonly used as an avicide (pheasants LD₅₀ of 17.8 mg kg⁻¹; ANC, 2005). However, fipronil is not registered or used as an avicide and the high sensitivity and observed effect on feeding behaviour poses a significant risk to non-target organisms with similar sensitivity to fipronil as bobwhite quail.

Consequences for the cited LD₅₀ value

The treatment dose of fipronil administered to bobwhite quail in this study was 11.3 mg kg⁻¹; the cited fipronil LD₅₀ for this species (Goodyear, 1994). By day 3 post-dose, all treatment group birds were in a prolonged state of lack of feeding and rapid weight loss, two attributes identified in the Humane Endpoint Guidance Document (OECD, 2000) as being associated with an animal in a moribund state. By these toxicity testing standards, OECD recommends

that such conditions be defined as an experimental endpoint requiring euthanasia. The summary report (Goodyear, 1994) in which the LD₅₀ value of 11.3 mg kg⁻¹ is cited does not provide study details and does not refer to weight loss over the 21 day study.

The behavioural effects observed in our fipronil-treated quail (Figure 3.1), consistent with previously reported observations for this species and two other Galliformes (Goodyear, 1994a; Stavola, 1994a; Stavola, 1994b) are of significant consequence to these fipronil-sensitive species in the wild. Their lack of response to disturbance would make them considerably more vulnerable to predation, also prolonged period without feeding and significant weight loss would have a negative impact on energy reserves for thermoregulation and general immune system requirements. Under free-living conditions, all dosed quail would likely die of starvation (thus making this dose effectively an LD₁₀₀). These factors not only have a significant impact on survival of individuals, but also then have a flow-on effect at the population level (Walker, 2003).

Metabolic fate of Fipronil

The increase in sulfone levels compared to fipronil levels, as measured in brain, liver and adipose tissue from this study (Figure 4.2), extend previous work, particularly in relation to our longer time course. Hainzl *et al.* (1998) demonstrated that after exposure to fipronil, sulfone levels in mouse brain increased and exceeded levels of fipronil over the 2h study period. The authors mention this also occurred in the liver, with sulfone levels increasing 2-fold faster than the rate observed in brain. The conversion of fipronil to its sulfone metabolite is an oxidative process carried out by cytochrome P450 enzymes in both vertebrates and insects (Durham, 2002; Tang, 2004). Administration of piperonyl butoxide, a cytochrome P450 inhibitor, blocked sulfone formation in mice (Hainzl *et al.*, 1998) and thus provides further support regarding fipronil metabolism.

Influence of weight loss on sulfone levels

Our results clearly demonstrate a strong correlation between loss in body mass and increasing sulfone residue levels in brain, liver and adipose tissue (Figure 4.3). Similar results have been reported for DDT exposed birds where starvation-induced weight loss resulted in a significant increase in brains levels of the major metabolite, DDE (Ecobicho *et al.*, 1969; Noakes *et al.*, 1965). These increased metabolite levels appear to be the result of mobilization of lipophilic residues from adipose depots during weight loss; these compounds are then redistributed to other target tissues (Hue, 2006; Villeneuve, 1975). Weight loss-induced mobilization of the high levels of sulfone stored in adipose tissue (Figure 4.2C) appears to be occurring in fipronil-treated bobwhite quail, leading to redistribution of sulfone to brain (Figure 4.3B) and liver (Figure 4.3D). Although there is still a positive correlation between weight loss and sulfone levels in adipose tissue, it is weaker than for brain and liver tissue. This is most likely a consequence of fat stores being depleted at a faster rate than residues are being eliminated, a finding supported by Chevrier *et al.* (2000), where weight loss significantly increased adipose concentrations of 8 lipophilic toxic residues due to decreased adipose tissue storage capacity. The lack of correlation between weight loss and levels of fipronil residue in brain (Figure 4.3A), liver (Figure 4.3B) and adipose (Figure 4.3C) tissue is most likely due to its rapid metabolism to the sulfone as shown in Figure 4.2.

Relationship between residue levels and intoxication

Fipronil acts as an effective neurotoxicant by interfering with the normal regulatory actions of GABA via its receptors, resulting in neural excitation and convulsions (Bobe *et al.*, 1998a). Exposure to fipronil at sufficient doses causes severe paralysis and ultimately death. Signs consistent with exposure to this neurotoxicant were observed in treated bobwhite quail after 48h post-dose in this study (Figure 4.1). These signs, which included involuntary wing flapping and convulsions, have previously been observed in zebra finch (*Taeniopygia guttata*) and house finch (*Carpodacus mexicanus*) treated with lethal doses of fipronil (Kitulagodage *et*

al., unpublished data). However, onset occurred much earlier in these passerine birds, as early as 4h post-dose, and did not continue beyond 24h. The onset of these signs of fipronil neurotoxicity in bobwhite quail coincided with the most rapid increase in brain levels of fipronil and sulfone between 48h and 72h post-dose (Figure 4.2A). As the brain has a high concentration of GABA receptors (Enna and Mohler, 2007), it is possible that the onset of signs observed in quail was due to the rise of both fipronil and sulfone levels in brain affecting sufficient GABA receptors to cause neural excitation. This relationship between brain residue levels and intoxication was also observed in brown-headed cowbirds (*Molothrus ater*) treated with DDT (Velzen *et al.*, 1972); weight loss-induced mobilization of DDT and its metabolites resulted in accumulation of lethal levels in brain, causing tremors and other signs of DDT intoxication, and ultimately resulting in death.

Why are bobwhite quail so sensitive to fipronil?

Fipronil is significantly more toxic to the three Galliformes (bobwhite quail, ring-necked pheasant, red-legged partridge) tested compared with other avian species, including the house sparrow (*Passer domesticus*), LD50 of 1000 mg.kg⁻¹ (Goodyear, 1994c) and the mallard duck (*Anas platyrhynchos*), LD50 < 2150 mg.kg⁻¹ (Goodyear, 1994b). The absence of feeding behaviour and weight loss observed in fipronil treated galliform species were not reported in the less sensitive house sparrow or mallard duck. Zebra finch (*Taeniopygia guttata*) and house finch (*Carpodacus mexicanus*) were both shown to be less sensitive to fipronil than bobwhite quail (Kitulagodage *et al.*, unpublished data), and like other non-galliform species, did not exhibit reduced feeding behaviour or weight loss. The absence of feeding observed after exposure to fipronil therefore, only appears to occur in the highly sensitive species, suggesting that the resulting weight loss enhances the effectiveness of the pesticide. The significance of weight loss with regard to pesticide toxicity was similarly demonstrated with DDT in brown-headed cowbirds (*Molothrus ater*). A sublethal dose of DDT in combination with non-lethal

food restriction resulted in mortality, whereas individually, these factors had little effect on the health of the birds (Velzen *et al.*, 1972).

The strong correlation between increasing sulfone levels and weight loss also indicates that the conversion of fipronil to sulfone may enhance the effect on feeding behaviour observed. An issue of confusion in the literature however, is the reported relative toxicity of the sulfone compared with the parent compound, with the sulfone variously reported as being just as toxic (Hainzl *et al.*, 1998), or more toxic (*in vivo*) than fipronil itself (USEPA, 1996). Hainzl *et al.* (1998) also demonstrated *in vitro* that the sulfone metabolite has a 6-fold greater binding affinity than fipronil for GABA receptors in vertebrate brains (composite data from six species). Considering that the sulfone is potentially more toxic than fipronil in vertebrates, high tissue sulfone levels, most importantly concentrations accumulated in the brain, may account in part for the high fipronil sensitivity in bobwhite quail. Our findings suggest however, that the treatment-induced absence of feeding and resulting weight loss, in combination with the high accumulation of sulfone metabolite in brain tissue, is responsible for the low fipronil LD50 in bobwhite quail.

CHAPTER 5. MATERNAL TRANSFER OF FIPRONIL TO EGGS ADVERSELY AFFECTS CHICKS IN ZEBRA FINCHES AND CHICKENS

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ABSTRACT

Two studies were carried out to examine the impact of maternal fipronil exposure on embryonic and offspring development. In the first study, breeding female zebra finches were orally dosed with single sublethal levels of fipronil (1, 5, and 10 mg/kg body weight) to determine behavioural and developmental consequences on chicks following maternal pesticide exposure. Significant levels of fipronil and fipronil-sulfone residues were detected in eggs laid by females in all dosed groups, however these were undetectable in eggs laid 13 days after treatment. The level of sulfone detected in eggs was consistently higher than that of the parent fipronil compound. Of the seven eggs laid in the treatment groups, only one (14%) chick hatched and this was from the lowest dose group. This chick was severely underdeveloped at 10 days of age in comparison to control chicks and fiproles were detected in brain, liver and adipose tissues collected following euthanasia of this individual. In contrast, there was 100% hatchability of control group eggs and all chicks fledged nests on schedule. In the second study, domestic chicken eggs were injected with 5.5, 17.5 and 37.5 mg/kg egg weight of fipronil directly into the yolk sac on day 12 of incubation. Treatment did not affect hatching success, however, behavioural and developmental abnormalities were observed in hatchlings from the highest dose group. These chicks also demonstrated reduced feeding rates,

as indicated by reduced body mass at 48-h period post hatch. Both fipronil and fipronil-sulfone residues were detected in brain and liver tissue of hatchlings at all pesticide dose levels tested.

INTRODUCTION

Fipronil is a new generation, broad-spectrum pesticide commonly used to control locust outbreaks in a number of countries including Australia and others within Africa. Seasonal conditions that promote increases in locust populations, such as rainfall and consequent plant growth, often stimulate breeding activities for a variety of terrestrial vertebrates including birds and lizards. This unfortunately results in locust-control spray events often being coincident with peak breeding periods for many non-target animals. In Australia, over one hundred different bird species are known to frequent areas that occasionally receive aerial application of fipronil for locust control (Szabo 2009). While little is known of the effects of fipronil on free-living birds there are many studies that have examined the impact of other pesticides on avian reproduction and development (DeWitt 1955, Fernie et al. 2009, Lundholm 1997, Wiemeyer and Porter 1970). Results from these studies have demonstrated the importance of understanding the effects of pesticides on reproduction for better predicting their effects on avian populations.

Despite the widespread use and obvious exposure risks of fipronil, there is very little known about the impact of fipronil exposure in breeding birds. Insectivorous birds are particularly susceptible as fipronil-contaminated locusts can remain alive for 7-10 days (EPA 2001) and feeding on these and sympatric insects, provides a major route of pesticide exposure.

Granivorous birds are also at high risk of exposure as fipronil and its metabolites are detectable in seeds after fipronil spraying events (JMPR 2001), suggesting that ingestion of contaminated seeds is a likely route of exposure (Szabo 2005). Contact of dermal and plumage surfaces to chemical residues on vegetation and soil are also potential routes of exposure for non-target birds. Residue on plumage and nesting material may result in indirect exposure of

eggs to the pesticide, while direct exposure can occur in areas of spray operations (Hoffman et al. 2003).

Fipronil acts by targeting gamma-amino butyric acid (GABA) receptors (Hainzl et al. 1998). These receptors are prevalent throughout the central nervous system of both vertebrates and insects, however fipronil has a much higher affinity for insect than for vertebrate GABA receptors hence its effectiveness as an insecticide. When fipronil was reviewed in the United States in 1994, the USEPA approved its registration for use with the provision that studies examining its chronic effects on avian reproductive be undertaken on two species: bobwhite quail (*Colinus virginianus*), a highly fipronil-sensitive species, and mallard duck (*Anas platyrhynchos*), a relatively low fipronil-sensitive species (Bryceland 1994a, 1994b). Both studies found no adverse reproductive effects on either species. However, these were non peer-reviewed reports submitted for pesticide registration purposes, therefore were subject to confidentiality requirements and lack the details needed to scrutinize their methods and results. Following this, the Joint Meeting on Pesticide Residues (JMPR) published two toxicology assessment reports (1997 and 2001) reviewing unpublished data and research on fipronil. The only avian study reviewed was a fipronil dosing study on laying hens (*Gallus domesticus*); the highest levels of fipronil residues were reportedly detected in the yolk fraction of eggs laid by dosed hens (JMPR 1997, 2001), consistent with fipronil's lipophilic nature. A study by Russ (2005) also found detectable levels of fipronil residue in the yolk of eggs laid by breeding female zebra (*Taeniopygia guttata*) finch administered with a single oral dose of fipronil (1.13×10^{-1} mg/kg). However, the study measured total fiproles (fipronil and its derivatives) and did not distinguish between levels of the parent fipronil compound and its metabolites.

Yolk lipids are the primary source of energy and nutrients for the developing embryo (Speake et al. 1998), a process requiring substantial amounts of energy to fuel metabolism and growth. Thus, any contaminants incorporated into the yolk have opportunity to be absorbed by the

developing embryo. Because of the known adverse developmental effects of fipronil on vertebrates (Beggel et al. 2010, Stehr et al. 2006), maternal transfer of fipronil residues into yolks has potentially devastating effects on chicks. We have addressed this question through two experiments. We first examined the maternal transfer of both fipronil and its major metabolite (fipronil-sulfone) from exposed female zebra finches into eggs and hatchlings and its consequences on hatching success. Zebra finches are an ideal model as the species naturally co-occurs with locust-control operations in Australia and breed well in captivity over a large part of the year. In the second experiment, we injected known amounts of fipronil directly into the yolk of fertilised eggs to examine dose effects of this pesticide on chick development. Domestic chickens were chosen for these *in ovo* studies as there is a great deal known about normal embryonic and post-natal development of the chick, making it easier to assess any teratogenic effects of fipronil.

MATERIALS AND METHODS

Chemicals and Preparation of test substances

Fipronil (C₁₂H₄Cl₂F₆N₄O₂ or (±)-5-amino-1-(2,6-dichloro- α,α,α -trifluoro-p-tolyl)-4-trifluoromethylsulphonylpyrazole-3-carbonitrile), CAS No. 120068-37-3, of 97% purity was obtained from Sigma-Aldrich, Pty. Ltd., Australia (Fipronil PESTANAL®). Fresh test solutions were prepared on each day of dosing. Due to the low water solubility of technical grade fipronil (Tingle et al. 2003), the fipronil solution was prepared using a minimum amount of acetone solvent (approx. 60 μ l acetone per 20 mg fipronil). Acetone was chosen as the solvent due to the high solubility of fipronil in acetone (545.9 g.L⁻¹; BASF 2005). The solution was then diluted in canola oil to the required dose concentration. Canola oil is the vehicle used in common fipronil formulations for locust-control (e.g. Adonis®, BASF 2003). Control solutions consisted of acetone and canola oil only.

Maternal-transfer study

Adult zebra finches (*Taeniopygia gutatta*) were obtained from a breeding colony at the University of Wollongong (Wollongong, NSW, Australia). Four outdoor aviaries at the University of Wollongong were set up for the three treatment groups and one control group, with each housing 5 breeding pairs (n = 20 females). Commercial seed mix (Finch Mix, Avigrain, Berkeley Vale, NSW, Australia), tap water and grit were provided ad libitum. Breeding pair formation was induced by the introduction of covered nest baskets and nesting material. Nests were checked daily to monitor dates of egg-laying, and all eggs laid prior to dosing were removed. Once all four breeding groups had started laying eggs, females were captured and weighed (mean + SE, zebra finch weight of $12.6 \pm 0.4\text{g}$) and all previously laid eggs removed. Females within a given cage were administered either fipronil or control solution as a single oral dose via gavage. Fipronil treatment doses were 1, 5 and 10mg/kg body weight and were known to be sublethal to adult zebra finch (Kitulagodage unpublished data, Kitulagodage et al. 2008). Dosing volumes followed OECD Acute Oral Toxicity Testing guideline recommendations of limiting maximum liquid dose volumes to 2ml/100g body weight (OECD 2003). Females were immediately returned to their respective aviaries and closely monitored for the next three hours, then checked regularly over the duration of the study. Once females recommenced laying, alternate eggs were collected for analysis of fipronil and metabolite residue levels; the remaining eggs were allowed to hatch. Behavioural observations were made of chicks in control and treatment nests up to 10 days after hatching and the chicks were then euthanised to collect brain, liver and adipose tissue for residue analysis.

In ovo exposure study

Fertile domestic chicken eggs (*Gallus domesticus*) were obtained on the day laid from The University of Sydney's Poultry Research Unit (Camden, N.S.W., Australia). Eggs were placed in Octagon 40 self-rotating incubators (Brinsea Products Ltd, Somerset, UK); held at the

University of Wollongong animal housing facilities in a room with a light: dark photoperiod of 12h:12h, including 15min simulated dawn/dusk intervals using dimmed lighting. Incubators were set at 37.4°C and monitored daily to ensure humidity was maintained at levels that resulted in the optimal egg weight (water) loss of 14% over the 21-day incubation period (Rahn et al. 1974). In addition to being weighed, eggs were candled every third day to determine embryo viability; infertile eggs and early dead embryos were discarded once identified.

After nine days of incubation, 38 eggs containing viable embryos were split into five groups (three pesticide- treated groups, one vehicle-treated control group and one untreated control group). Fipronil treatment doses were 5.5, 17.5 and 37.5 mg/kg egg weight (based on pilot study doses of fipronil up to 1 mg/kg showing no effect); the vehicle consisted of acetone and canola oil only. We simulated maternal transfer of fipronil or vehicle-control solution by direct injection into the yolk on day 12 of incubation using a 50ul Hamilton glass syringe with a 26-gauge needle. The dosing volume of 0.36 ml solution/kg egg weight was adjusted accordingly for each egg. A hole at the injection site was made manually through the shell using a 1mm drill bit after candling to locate the position of the yolk sac and to avoid injecting into the developing embryo, while a polystyrene foam stopper was fitted onto the needle to enable injection at the same position in the yolk sac for each egg. Once injected, holes were sealed with melted paraffin wax and eggs then returned to the incubator. The vehicle-control group eggs were injected in the same manner with the acetone and canola oil control solution, whereas eggs in the control group were injection free. Eggs were then candled and weighed every second day and any eggs containing dead embryos were removed from the incubator once detected. Hatchlings were weighed as close to time of hatching as possible then placed in brooder boxes with heat-lamps. Commercial feed (chick starter feed, PETstock, Fairy Meadow, NSW, Australia) and tap water were provided *ad libitum*.

Behavioural and physical observations including ability to right itself, pecking response to food, and general gait and stance of each hatchling were observed within 24 h post-hatch. Chicks were euthanised at 48 h post-hatch and their brain and liver tissues collected for analysis of fipronil and metabolite residue levels.

All experiments were conducted in accordance with the National Health and Medical Research Council Guidelines for research involving native animals, as approved by the University of Wollongong Animal Ethics Committee (ethics approval number: AE03/26).

Assay Methods

Analysis for residue levels of fipronil and the sulfone metabolite (MB 46136) of fipronil was conducted at AgriSolutions Pty. Ltd. (Brisbane, Queensland, Australia). Zebra finch eggs, zebra finch hatchling tissue samples and domestic chicken hatchling tissue samples were homogenised, extracted in hexane and then filtered and liquid-liquid partitioned with acetonitrile. Residues were then isolated, and purified extracts brought up in acetonitrile, and passed through a 0.45 µm polytetrafluoroethylene filter prior to quantitative analysis via Gas Chromatography Mass Spectrophotometry (GC/MS). The level of parent fipronil and sulfone for each sample were expressed as mg fiproles per kg of tissue sample (Agrisolutions 2006).

Statistical Analysis

Comparative fipronil and fipronil-sulfone residue levels in brain, and liver tissue collected from *in ovo* treated chicks 48 hours after hatching for each treatment group were analysed using a t-test. The effect of time between, and within, treatment groups on measured chick weights were examined using one-way analysis of variance (1-way ANOVA) with Tukey's post-hoc tests. Analysis was performed using GraphPad Prism software (Version 5.02, GraphPad Software Inc., CA, USA).

RESULTS

Maternal Transfer of Fipronil

Fipronil and sulfone residues were detected in eggs from females in all three fipronil treatment groups, however only in eggs that were laid between 2 and 13 days post-treatment. The level of sulfone detected was consistently higher than that of fipronil in all eggs sampled and tended to persist longer (Table 5.1). At the lowest dose of 1 mg/kg, fipronil was only detected in eggs laid on day 2 post-treatment (0.01 mg/kg). Sulfone levels appeared to reach peak levels between 4 to 6 days post-dosing in all pesticide-treatment groups. No residue of either fipronil or sulfone was detected in eggs laid in the control group.

Table 5.1 Fipronil and Sulfone levels in eggs laid by female zebra finch administered a single oral dose of Fipronil at 1, 5 or 10 mg/kg body mass. Residue levels corrected for Limit of Quantification (LOQ) of 0.01 mg/kg egg mass. For all samples n=1 except for (a) where n=2. ND = no detectable residue level.

Maternal Dose of Fipronil	Days Post-Maternal Dose eggs laid	Fipronil in egg (mg/kg)	Sulfone in egg (mg/kg)
1 mg/kg	1 ^(a)	ND	ND
	2 ^(a)	0.01	0.13
	4	ND	0.19
	6	ND	0.10
	13	ND	ND
	17 ^(a)	ND	ND
5 mg/kg	1 ^(a)	0.10	0.09
	2 ^(a)	0.08	0.36
	4	0.11	1.28
	12	ND	0.01
	18	ND	ND
10 mg/kg	1	ND	ND
	4	0.22	2.16
	6	0.08	2.63
	9	ND	0.08
	12	0.01	0.19
	13	ND	0.03

Of the seven eggs of the pesticide-treatment group remaining in the nests, only one chick (14%) hatched, this was from the 1mg/kg treatment group, compared with 100% hatch rate of

control eggs (12 eggs). The one chick to hatch among the pesticide-treatment group appeared severely underdeveloped at 10 days of age. Its eyes were just opening and its legs were splayed with no demonstrated righting reflex. The hatchling was unable to remain erect and vocalisations were abnormal and muted. No feathers had emerged on its body and all primary feathers were still in sheaths approximately four mm long. Ventral feather tracks were visible, but not dorsal or head feather tracks. It weighed only 7.0 g when euthanased, but 0.83g of this mass was seed that was packed in its crop. In contrast, hatchlings from the control group weighed 8.4 ± 0.4 g at nine days old, had little or no seed stored in their crop, displayed strong begging behaviour, were very vocal, and able to right themselves. Furthermore, their body feathers had begun emerged both ventrally and dorsally, and primary feathers were unsheathed with one hatchlings measured to be approximately nine mm long. Tissue samples from the 10-day old pesticide-exposed chick contained fipronil in brain, liver, and fat tissues, while sulfone was detectable only in brain (Figure 5.1). No fiproles were detected in tissues of any control hatchlings (n=3).

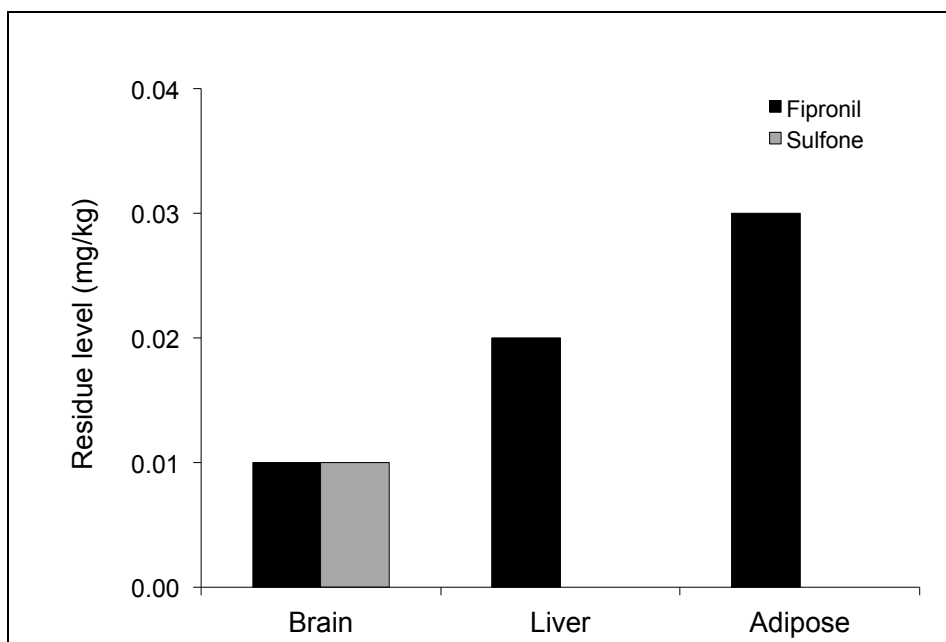


Figure 5.1 Fipronil and fipronil-sulfone residue levels in brain, liver and adipose tissue of the single zebra finch chick hatched (n=1) from eggs laid by females receiving a single oral dose of fipronil at 1mg/kg.body mass

In ovo exposure study

Of the 40 eggs incubated, two proved to be infertile prior to treatment (fertility rate of 95%). There was no difference in *in ovo* mortality post-treatment (Table 5.2); only one chick died during the hatching process after pipping and this was from the 5.5 mg/kg group.

Table 5.2 Survival rates of domestic chickens after *in ovo* exposure to fipronil at 0, 5.5, 17.5 or 37.5 mg/kg. egg weight.

Treatment group	<i>n</i>	<i>In ovo</i> mortality	Mortality during hatching process
Control	7	2	0
Vehicle-control	7	1	0
5.5 mg/kg	8	2	1
17.5 mg/kg	8	2	0
37.5 mg/kg	8	2	0

Of the 28 chicks that successfully hatched, behavioural and developmental abnormalities were only observed in the highest dose group (37.5 mg/kg). Observed signs included unsteadiness and resting on haunches. These chicks appeared to be uninterested in food provided in comparison to control chicks that ate considerable amounts of feed. This reduced feeding behaviour was reflected by loss in body mass over the 48-hour period post hatch (Figure 5.2); measured chick weights differed significantly across the four treatment groups at 48 hours after hatching, $F_{4, 15} = 4.258$; $p = 0.0168$. Tukeys post-hoc comparisons of the treatment groups indicate that body mass observed in the 37.5 mg/kg egg weight group chicks at 48 hours post hatch was significantly lower than those observed in control group chicks ($p < 0.05$). One chick from this highest treatment group was found dead 48 hours post hatch and weighed 29.3 grams compared to its weight of 36.2 grams immediately after hatching, necropsy revealed no abnormal findings however. Loss in body mass was also observed within the 5.5 mg/kg treatment group with measured chick weights differing significantly across the three time points, $F_{2, 11} = 8.409$; $p = 0.0061$. Tukeys post-hoc comparison of the time points

indicate chick weights measured both 24 hours and 48 hours after hatching were significantly lower than those measured immediately after hatching within the 5.5 mg/kg group ($p < 0.05$ and $p < 0.01$, respectively). There was no significant difference in chick weights within the 17.5 or 37.5 mg/kg groups, or between any of the treatment groups either immediately after hatching or at 24 hours post-hatch.

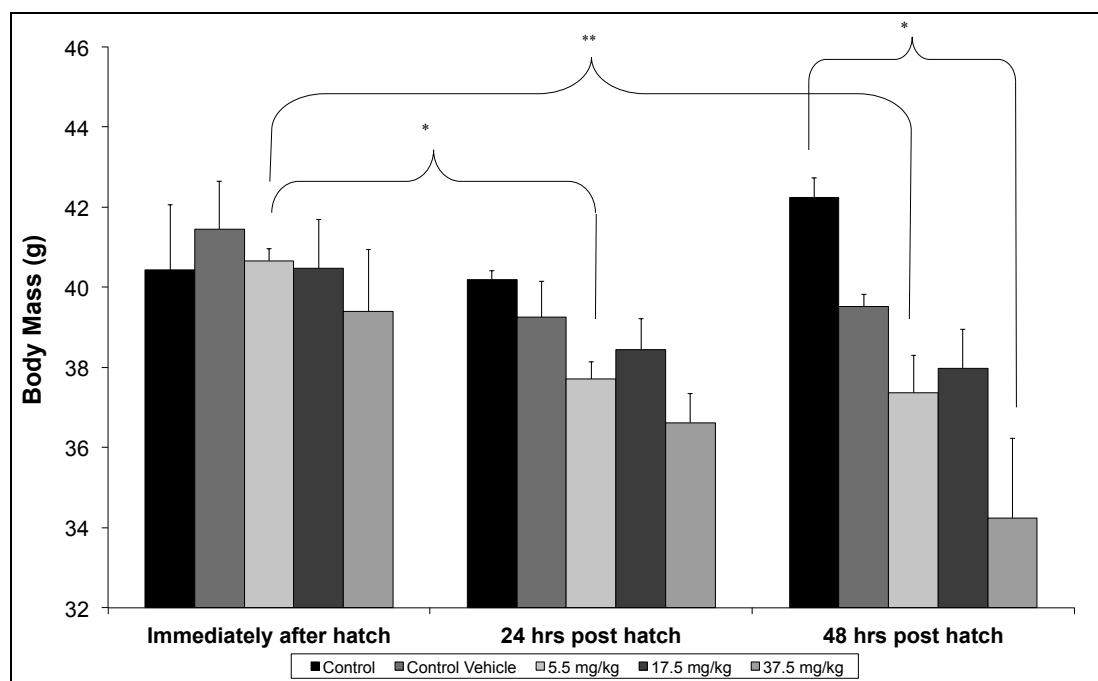


Figure 5.2 Body mass of domestic chicken hatchling (represented as Means \pm SE) after *in ovo* exposure to fipronil compared with control groups. Treatment group chicks were exposed to a single dose of fipronil via injection *in ovo* at 5.5, 17.5 or 37.5 mg/kg.egg weight. Measurements were taken immediately after hatching, at 24 hours after hatching and 48 hours after hatching. Asterisks indicate statistically significant differences; single asterisks indicate $p < 0.05$, two asterisks indicate $p < 0.01$

Fipronil and its sulfone metabolite were the only detectable residues in brain and liver tissue collected 48 hours after hatching from the 5.5, 17.5 and 37.5 mg/kg treatment group chicks (Table 5.3). No fiproles were detected in control hatchlings. Fipronil-sulfone concentrations were significantly greater than those of fipronil in both brain ($p = 0.0003$) and liver tissue ($p < 0.0001$) for all fipronil *in ovo* treated hatchlings. Comparing the two tissue types, concentrations detected in liver tissue were significantly higher than those in brain tissue for both fipronil ($p = 0.0061$) and fipronil-sulfone ($p < 0.001$).

Table 5.3 Fipronil and Sulfone residue levels in brain and liver (measured as means \pm SEM) of domestic chicken hatchlings exposed *in ovo* to fipronil at 5.5, 17.5 or 37.5 mg/kg.egg weight. Samples were collected at 48 hours post hatching. For all samples n=4 except for the 37.5 mg/kg groups where n=5.

Treatment Group	Residue in Brain (mg/kg)		Residue in Liver (mg/kg)	
	Fipronil	Sulfone	Fipronil	Sulfone
5.5 mg/kg	0.03 \pm 0.01	2.48 \pm 1.03	0.10 \pm 0.05	6.46 \pm 2.65
17.5 mg/kg	0.22 \pm 0.09	4.03 \pm 1.15	0.27 \pm 0.11	10.21 \pm 2.88
37.5 mg/kg	0.20 \pm 0.08	8.51 \pm 1.54	0.48 \pm 0.11	17.71 \pm 3.10

DISCUSSION

This is the first study to show unequivocally that both fipronil and its sulfone metabolite (fipronil-sulfone) are maternally transferred into eggs laid by fipronil-exposed females. This study also demonstrates that once fipronil is deposited in the avian eggs, it is absorbed by the developing embryo, and along with the fipronil-sulfone, accumulates in the tissue of the hatchlings. The higher levels of fipronil-sulfone detected in zebra finch eggs compared with fipronil residue levels is consistent with other studies examining post-exposure residue content in fipronil-exposed animals (JMPR 2001). Metabolic fate studies show that fipronil-sulfone formation occurs rapidly, being detectable within one hour after fipronil exposure in mice (Hainzl et al. 1998) and within the first eight hours in fipronil-treated avian species (Kitulagodage et al. unpublished data).

The lack of detectable fipronil or fipronil-sulfone residue in zebra finch eggs laid on the day immediately after dosing is likely due to those eggs being formed prior to dosing. The peak levels of sulfones found in eggs laid 4-6 days after dosing is consistent with the temporal pattern of yolk formation in ovulating birds, resulting in a higher proportion of yolk being exposed to maternal fipronil and sulfone in later laid eggs (Astheimer and Grau 1985).

The most striking result was the significant reduction in hatching success among female zebra finches exposed to fipronil (14% compared to 100% in controls). The doses that were administered were known to be sublethal from previous studies (Kitulagodage unpublished data) and none of the females dosed with fipronil showed any adverse effects or signs of toxicity.

The lowest fipronil dose used in this current study was 1 mg/kg, which for an adult zebra finch weighing 12g is equates to ingestion of 0.12 mg of fipronil. One sample of native grass seeds collected after locust control spray operations was found to contain 0.839mg/kg fipronil residue five days after application (Szabo 2005). Relating this field data to our study dose, in order to obtain a 1mg/kg dose of fipronil, a 12g zebra finch would have to eat 14g of contaminated seed. Zebra finch have been reported to consume their own body weight in seed over a 48-hour period, and can collect this amount daily during the breeding season when they are feeding their chicks (Zann 1996). The scenario of a zebra finch obtaining a 1mg/kg dose of fipronil in locust control areas is therefore feasible depending on whether the fipronil residue had accumulated in the seed or in the husk, as finches remove the husks from the seed prior to eating.

The standard rate of fipronil application for controlling locusts in Australia is currently 0.9g of active ingredient per 300ml per hectare (APLC, pers. comm.). Given the known exposure of zebra finches to locust-control pesticides in Australia (Fildes et al. 2006), further study of the dose sensitivity of reproductive output in zebra finches and sympatric species to fipronil is warranted.

In the domestic chicken *in ovo* study, the effect of drilling through the shell and injecting a solution had no effect on embryo survival rates. Although no clinical signs of toxicity were observed in any of the hatchlings, only the groups treated with fipronil showed significant

body mass declines after hatching, with one of the chicks that died 48 h post hatching losing approximately 19% of its body weight. Loss in body mass resulting from fipronil exposure has been demonstrated in studies on adult king quail (*Coturnix chinensis*) and bobwhite quail (Kitulagodage et al. unpublished data) and reduced feeding rates were observed in these species. No clear signs of reduced feeding behaviour were reportable in the chicken hatchlings as food consumption was not quantified.

The relative levels of fipronil and fipronil-sulfone residue detected in the brain and liver tissue of chicken hatchlings is consistent with the pattern seen in zebra finch eggs, with sulfone levels being significantly higher than fipronil levels across all treatment groups. These results do differ to the zebra finch hatchling tissue samples, however, as fipronil levels were higher than sulfone levels in the brain, and sulfone residue was not detected at all in the liver and adipose tissue. It is important to note though that this was based on measurements for one individual.

Comparing tissue types, both fipronil and fipronil-sulfone appear to accumulate at higher levels in liver as opposed to brain of the chicken hatchlings in all fipronil-treatment groups and in the surviving chick from the fipronil-dosed female zebra finches. The highest residue levels in the zebra finch chick however, were detected in the adipose tissue. Although pesticide residues were not determined in adipose from the chicken hatchlings, fipronil-dosing studies in rats, goats (JMPR 2001), adult zebra finches and bobwhite quail (Kitulagodage et al. unpublished data) have also documented greater amounts of fiproles in body lipids compared to either brain, or liver. Because adipose tissues are typically mobilized during periods of reduced food availability and, in females, when synthesizing egg yolk, there is great potential for fipronil to exert its toxic effects long after the time of initial exposure.

One conspicuous difference between the species we studied was the much greater sensitivity of zebra finch chicks to fipronil content in yolk compared to chickens. This may be due, in part, to differences in the stage of embryo development when first encountering fipronil. The zebra finch embryos of fipronil-exposed mothers were metabolizing yolk containing this pesticide and its metabolites from very early embryogenesis. By contrast, the chicken embryos were nearly 60% developed when their yolk was injected on day 12 with fipronil. Another consideration is that injected pesticide would be located predominantly in a discrete location of the yolk, whereas maternal transfer would result in an even mix of the pesticide throughout each yolk layer deposited in accord with circulating levels of fiproles at the time of yolk synthesis. Resolution of ontogenetic and phylogenetic effects on fipronil sensitivity of developing avian embryos is thus of paramount importance when considering risk.

CHAPTER 6. GENERAL CONCLUSIONS

AVIAN SENSITIVITY TO FIPRONIL

This research has demonstrated that variability in fipronil sensitivity across avian species appears to reflect a pattern at the ordinal level. The Passeriformes (zebra finch, house finch and house sparrow) and budgerigar, a psittaciform species, appear to be moderately sensitive to fipronil. The Galliformes, however, appear to be highly sensitive to fipronil (king quail, bobwhite quail, ring-necked pheasant and red-legged partridge).

Fipronil acts as a gamma-aminobutyric acid (GABA) receptor antagonist and the expected signs of fipronil intoxication resulting from this mode of action were observed in all species. Clear differences, however, were observed in times of onset and duration of these signs between the different orders of avian species. The Passeriformes displayed rapid onset of signs of intoxication, with complete remission within 24 h of exposure. In contrast, signs of intoxication were not observed in the Galliformes until at least day 2, then lasting up to 3 days after exposure. Additional responses to fipronil exposure in the Galliformes were cessation of feeding, inactivity and lack of response to disturbances. These symptoms were, not observed in any of the other species. For the budgerigars, the only psittaciform species tested, their rapid onset of signs of intoxication were similar to those observed in passeriforms, but differed in persisting up to 2 days after exposure.

While there have been varying reports indicating that the sulfone metabolite of fipronil (fipronil-sulfone) is just as toxic or more toxic than its parent fipronil compound in vertebrates (USEPA 1996; Hainzl *et al.* 1998), this is the first study demonstrating fipronil-sulfone to be less toxic than fipronil *in vivo* in a vertebrate species; fipronil-sulfone was at least 2.5-fold less toxic than fipronil in zebra finch. In king quail however, fipronil and its fipronil-sulfone metabolite, were relatively equitoxic. One major observation from this study was that identical

signs of intoxication observed in fipronil-treated king quail including anorexia and weight loss were also observed in fipronil-sulfone treated king quail, which raised questions about the influence of metabolism of fipronil to fipronil-sulfone on the overall toxicity of fipronil.

Fipronil exposure in the highly sensitive bobwhite quail resulted in reduced feeding behaviour and progressive loss in body mass. Residue analysis of brain, liver and adipose tissue from fipronil-treated bobwhite quail revealed a significant positive correlation between weight loss and fipronil-sulfone residue levels, the most notable being the correlation against brain tissue, a body tissue with a high concentration of GABA receptors.

As the fipronil-sulfone metabolite is just as toxic as the parent compound in Galliformes, the significant losses in body mass may exacerbate the toxic effects of pesticides. As these birds begin to rely on their fat stores where lipophilic fipronil residues are known to accumulate, subsequent mobilisation of these energy stores will redistribute fipronil-sulfone metabolites to other tissues, as demonstrated by the correlation between weight loss and tissue residue levels.

The rapid conversion of fipronil to fipronil-sulfone may be a determining factor in the differing sensitivity to fipronil between the galliformes and passeriforms. As fipronil-sulfone is over 2.5-fold less toxic than fipronil in zebra finch, metabolism of fipronil in this species would result in conversion to a less toxic metabolite. In king quail however, fipronil-sulfone is just as toxic as fipronil, therefore metabolism of fipronil in this species results in conversion to a metabolite of equivalent toxicity to the parent fipronil. This may contribute to the greater fipronil sensitivity in Galliformes. Further research is needed however to understand the impact of the sulfone metabolite on the toxicity of fipronil across the different avian orders.

The prolonged periods without feeding and the significant weight loss observed in the galliform species exposed to fipronil would additionally have a negative impact on energy

reserves for thermoregulation and general immune system requirements. Inactivity and lack of response to disturbances, both displayed in the Galliformes after exposure to fipronil, would make these birds considerably more vulnerable to predation in the wild. These factors combined pose a significant risk to these non-target organisms, especially when considering the relatively small amount of fipronil they would need to be exposed to in order to elicit these effects.

A LESSON LEARNED ABOUT EVALUATING FORMULATED PRODUCTS

The formulation comparison study revealed the most unexpected finding of this research in demonstrating that the solvent ingredient (diacetone alcohol) contributes significantly to the toxicity of the commercial fipronil formulation (Adonis 3UL) used for locust control spraying in Australia. There is no available information on the ecotoxicity of diacetone alcohol for terrestrial organisms. While narcosis, decreased hemoglobin and erythrocytes numbers and hepatic lesions have been reported in rats after oral administration of sublethal doses of diacetone alcohol (OECD 2000), further information on this study, such as test methods used, is not publicly available. Assessment of the active ingredient toxicity alone, in this case fipronil, is inadequate in evaluating the toxicity of pesticide formulations. These findings clearly highlight the importance of identifying and differentiating between the toxicity of active ingredients and that of its formulation in order to understand the realistic risks of exposure of these pesticides to non-target organisms.

POTENTIAL POPULATION-LEVEL IMPACTS FROM THE AGRICULTURAL USE OF FIPRONIL

This is the first study to demonstrate that both fipronil and its fipronil-sulfone metabolite are maternally transferred into eggs laid by fipronil-exposed birds. This resulted in significantly reduced hatching success among the clutches laid by the female zebra finches exposed to

fipronil. All of the females dosed with fipronil, however, were asymptomatic of signs of fipronil toxicity. This study also demonstrated that once fipronil is deposited in the avian eggs, it is absorbed by the developing embryo, and along with the fipronil-sulfone, accumulates in the tissue of the hatchlings and has adverse effects on development and behaviour.

The lowest fipronil dose administered having reproductive effects on breeding female zebra finch in this study was 1 mg/kg. Based on fipronil residue levels detected in grass seeds collected after locust control spray operations, and on the feeding rates of zebra finch, it is feasible for a zebra finch to ingest enough fipronil-exposed seeds in the wild during a breeding event to equate to a 1mg/kg dose of fipronil. Further examination of fipronil exposure in seed-eating birds requires assessment of whether fipronil residue accumulates in the seed or in the husk, as birds remove the husks from seed prior to ingesting it.

Given the pronounced effects that low doses of fipronil have on developing embryos and the coincidence of breeding by many species when locust outbreaks occur, more attention must be paid to the potential of this pesticide to affect reproductive success. Such research should recognise that any fipronil accumulating in fat stores will likely be transferred to the egg yolk of birds and reptiles but also to milk of lactating mammals. Thus, predictions of fipronil effects on particular vertebrates are likely to vastly underestimate the impact of low-level exposure on free-living populations if such modelling relies on adult pesticide sensitivity alone.

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APPENDIX 1. REPORTED EFFECTS OF FIPRONIL ON NON-TARGET ORGANISMS

Organism	Impact	Reference
<u>Non-target Insects:</u> Ground beetles <i>(Carabidae)</i> and darkling beetles <i>(Tenebrionidae)</i> Bees <i>(Scelionidae)</i> and Wasps <i>(Sphecidae)</i>	Locust-control spraying reduced populations by 90% within two days of treatment regardless of dose Almost 100% decline was observed in both populations due to locust-control spraying.	(Balanca and deVisscher 1997) (Balanca and deVisscher 1997)
<u>Aquatic Organisms:</u> Black-fly larvae <i>(Simulium vittatum)</i> Water flea <i>(Daphnia)</i> Japanese carp Rainbow trout European carp	An acute toxicity study of fipronil resulted in a median lethal concentration (LC50) value range of 0.31 – 0.18 µg/L after 48 hours The median lethal dose of fipronil (LD50) was determined to be 0.19mg/L after 48 hours LC50 of 0.34mg/L LC50 of 0.248 mg/L LC50 of 0.430 mg/L	(Overmyer <i>et al.</i> 2005) (Colliot <i>et al.</i> 1992) (Colliot <i>et al.</i> 1992) (Aventis 2001) (Aventis 2001)
<u>Vertebrates:</u> Fringe-toed lizard <i>(Acanthodactylus dumerili)</i> Rats	LD50 of 30mg/kg Acute oral LD50 of 97 mg/kg.	(Peveling and Demba 2003) (USEPA 1996)

Organism	Impact	Reference
Vertebrates (cont'd):		
Rats	<p>In an acute toxicity study on rats, the clinical signs of toxicity were observed within 24hrs of treatment and included tremors and convulsions of various types, hunched posture, and hind-leg splay. Motor activity had decreased by 40% 8hrs after treatment.</p>	(JMPR 2001)
Rats	<p>In a biotransformation study three treatments were assessed using labelled fipronil; a 4mg/kg and a 150mg/kg single oral dose, and a daily dose of 4mg/kg over 14 days. The majority of fipronil TRR (Total Radioactive Residues) i.e. fipronil and its residues, was excreted via faeces (45-75%). Of the fipronil absorbed, the highest radioactive concentrations were found in the fat; the main residue detected was fipronil sulfone (MB 46136).</p>	(JMPR 2001)
Goats	<p>Three lactating goats were orally dosed daily for 7 days with either 0.05, 2 or 10ppm labeled fipronil. The majority of the radioactivity was excreted via faeces. The 2ppm dosed goat proportionally absorbed the greatest fipronil and also had the highest percentage of residue detectable in the collected milk. Residues were not detected in milk at the 0.05 dose, however for both the 2 and 10ppm doses, residues levels were detected and rose over the 7 days.</p>	(JMPR 2001)

Organism	Impact	Reference
<u>Vertebrates (cont'd):</u>		
Hens	<p>In a study on laying hens, doses were same as for goats (0.05ppm, 2pp, and 10ppm) however over a 28 day daily treatment schedule. The highest recoveries were from faeces (52-58%) and egg yolks (13-16%) and whites (~2%). The residue levels detected in the egg yolk were higher than those detected in the tissue of the hens. At all doses the main residue detected in both eggs and tissues was the sulfone (MB 46136).</p>	(JMPR 2001)
Cows	<p>In a feeding study on lactating cows given daily doses of either 0.04, 0.13 or 0.43ppm fipronil for 35 days, almost all the residue detected in milk was fipronil-sulfone (MB 46136). Of the tissues collected, the highest TRR levels were detected in the fat, also mainly sulfone (MB 46136).</p>	(JMPR 2001)
New Zealand white rabbits	<p>Dermal LD50 for rabbits of 445mg/kg for males and 354mg/kg for females. Clinical signs of toxicity for rabbits described in this study included convulsions, sluggishness, salivation, spasms, tremors, hyperactivity and diarrhea. It is interesting to note that convulsions were not observed until 3 to 9 days after treatments and some deaths did not occur until 11 to 14 days after treatments.</p>	(JMPR 2001)

Organism	Impact	Reference
<p data-bbox="284 293 536 322"><u>Vertebrates (cont'd):</u></p> <p data-bbox="284 342 459 371">Dogs (Beagle)</p>	<p data-bbox="639 342 1145 824">In three chronic dosing studies on beagles, the main observations reported were decreases in mean body weight and food consumption. Clinical signs of toxicity were observed at doses low as 1mg/kg; these included hunched posture, hypothermia, excessive salivation, irregular heart rate, convulsions, tremors, abnormal reflexes and apparent lack of vision.</p> <p data-bbox="639 846 1145 981">Some of the dogs deteriorated to such an extent due to effects of treatment that they had to be euthanased during the study.</p> <p data-bbox="639 1003 1145 1182">Serum concentrations of the fipronil-sulfone (MB 46136) were higher than of that of the parent fipronil in treatment doses above 0.075mg/kg.</p>	<p data-bbox="1169 1149 1334 1178">(JMPR 1997)</p>